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ANTI APOPTOTIC PROTEINS AS TARGETS OF CHEMOTHERAPY

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Programmed cell death or apoptosis has currently become one of the hottest areas of research. Apoptotic cell death is found throughout the animal kingdom, and it culminates in the execution, packaging and disposal of the dying cells. It allows the organism to tightly control cell numbers and tissue size and to protect itself from damaged cells that threaten homeostasis. Apoptosis is characterized by distinct morphological and biochemical changes mediated by a family of cysteine proteases called caspases, which are expressed as inactive zymogens and are proteolytically processed to an active state following an apoptotic stimulus.

Two distinct pathways (extrinsic and intrinsic) leading to caspase activation have been characterized (Wang, 2001).The extrinsic pathway is initiated by ligation of transmembrane death receptors (CD95, TNF receptor and TRAIL receptor) to activate membrane-proximal (activator) caspases (caspase-8 and –10), which in turn cleave and activate effector caspases such as caspase-3 and caspase-7. The intrinsic pathway requires disruption of the mitochondrial membrane and the release of mitochondrial proteins including Smac/ DIABLO, HtrA2 (Omi) and cytochrome C. Cytochrome C functions with Apaf-1 to induce activation of caspase-9, there by initiating the apoptotic caspase cascade while Smac/ DIABLO and HtrA2 bind to the antagonists IAPs (Wang, 2001; Suzuki et al, 2001).

Tumorigenesis is a multistep process in which mutations in key cellular genes produce a series of acquired capabilities that allow cancer cells to grow overcoming growth- inhibitory signals and host immune responses. Moreover, the ability of cells to evade apoptosis is also an essential "hallmark of cancer" (Hanahan and Weinberg, 2000). The increased understanding of the mechanism of apoptosis has led to novel therapeutic approaches of enhancing it in tumor cells.

Chemotherapy for the treatment of cancer was introduced into the clinic more than 50 years ago. In reality the effectiveness of chemotherapy has suffered from a range of confounding factors including systemic toxicity due to a lack of specificity, rapid drug metabolism and both intrinsic and acquired drug resistance. Why tumors are often inherently resistant to chemotherapeutic drugs or become resistant after an initial round of treatment? Defects in the apoptosis inducing pathways as well as resistance to apoptosis can eventually lead to expansion of a population of neoplastic cells

Disruption of the intrinsic apoptotic pathway is extremely common in several cancers. Besides over-expression of antiapoptotic genes, tumors can acquire apoptosis resistance by down regulating or mutating proapoptotic molecules. This review will focus predominantly on different antiapoptotic molecules over-expressed by various

tumors and their role in regulating the effectiveness of chemotherapy.

NF-KB

An important factor influencing apoptosis of tumor cells is the transcription factor, nuclear factor of kappa B (NF-KB). It is a transcriptional regulator that is made up of different protein dimers that bind a common sequence motif known as the KB site. Normally, NF-KB remains sequestered in an inactive state by the cytoplasmic inhibitor of NF-KB (IKB) proteins. However a variety of external stimuli including cytokines, pathogens, stress and chemotherapeutic agents can lead to activation of NF-KB by phosphorylation, ubiquitilation and subsequent degradation of IKB (Karin and Ben, 2000). The DNA binding subunits of NF-KB migrate into the nucleus and activate target genes. Depending on the stimulus and the cellular context NF-KB can activate anti apoptotic genes such as those encoding IAP's and BCL-X_L (Karin and Lin, 2002). Genes encoding NF-KB or IKB proteins are amplified or translocated in human cancers (Rayet and Jelinas, 1999). NF-KB may be activated by cytokines, by alterations in epidermal growth factor or receptor expression, Ras activation or through cell damage and changes in oxidation-reduction state (Karin et al, 2002).

The target genes of NF-KB include anti-apoptotic genes, genes that positively regulate cell proliferation, genes that encode negative regulators of NF-KB and immunoregulatory and inflammatory genes. Genes of all these categories can contribute to tumorigenesis. NF-KB has been shown to be constitutively activated in certain types of cancers (Wood et al, I998). Several viral oncoproteins activate NF-KB by means of different mechanisms (Mosialos, 1997). Oncogenic activation of upstream signaling molecules, chronic infections and proinflammatory cytokines have also been shown to stimulate IKK activity, which leads to constitutive NF-KB activation. Constitutively activated NF-KB promotes tumorigenesis by stimulating cancer cell proliferation, preventing apoptosis, and increasing tumors angiogenic and metastatic potential. Inactivation of NF-KB by over-expression of IKB can sensitize cancer cells to chemotherapy (Biswas et al, 2003). In our laboratory, it has been observed that inactivation of NF-KB by over-expression of IKB can sensitize A431 cells to EGF induced apoptosis (Anto et al, 2003) and ectopic over-expression of NF-KB confers resistance to curcumin-induced apoptosis (Anto et al, 2000).

Bcl2

The members of the Bcl2 family, which regulate apoptosis at the mitochondrial level, are an important class of regulatory proteins. They can be divided into antiapoptotic (Bcl2, BCLX_L, BCLw, MCL1, etc.) and proapoptotic (BAX, BAK, BID, BAD, BOK/MTD, etc) proteins according to their function. Although the impact of Bcl2 family members on apoptosis is well known, the biochemical mechanism of their function is not entirely clear. It is believed that the mitochondrial membrane permeabilization is regulated by the opposing action of pro-and anti apoptotic Bcl2 family members.

Many oncogenic mutations probably impair apoptosis indirectly, by affecting signal transduction pathways that promote or repress expression of Bcl2 family members. For example, Bcl2 is over expressed in a variety of cancers (Reed, 1999; Tyagi et al, 2003) and its over expression can accelerate tumorigenesis in

transgenic mice (Adams et al, 1999). Over expression of Bcl2 occurs in 70% of breast cancer, 30-60% of prostate cancer, 80% of B-cell lymphomas, 90% of colorectal adenocarcinomas, and many other forms of cancer (Liu et al, 2003). Over-expression of Bcl2 in patients receiving synchronous chemoradiotherapy has been reported as an independent indicator of poor survival (Hussain et al, 2003).

We have observed that over expression of Bcl2 and $BCLX_L$ induces resistance to apoptosis in HL-60 cells (Anto et al, 2002). Mutations or altered expression of Bcl2 related proteins could drastically alter drug sensitivity in experimental models (Reed, 1999; Schmitt et al, 2000). Infusion of a Bcl2 antisense oligonucleotide into tumor bearing mice has been shown to reduce tumor growth and the preliminary results of clinical studies seem promising (Jansen et al, 1998; Waters et al, 2000). In addition, mutations of altered expression of upstream regulators of Bcl2 proteins are associated with cancer. For example, the Bad kinase Akt is positively regulated by various oncoproteins and negatively regulated by the PTEN tumor suppresser (Datta et al, 1999).

Akt

The main biological consequences of Akt activation that are relevant to cancer –cell growth can be catalogued loosely into three categories- survival, proliferation (increased cell number), and growth (increased cell size). Several components of the PI3K-Akt pathway are deregulated in a wide spectrum of human cancers. Therapeutic strategies that target the PI3 kinase pathway are now in development. Akt functions in an anti apoptotic pathway, because constitutively active Akt rescues PTEN mediated apoptosis (Li et al, 1997).

The mechanism by which Akt protect cells from death is likely to be multifactorial because Akt directly phosphorylates several components of the cell-death machinery. For example, BAD, a pro-apoptotic member of the Bcl-2 family promotes cell death by forming a non-functional heterodimer with the survival factor $BCL\text{-}X_L$. Phosphorylation of BAD by Akt prevents this interaction (Datta et al, 1997) restoring $BCL\text{-}X_L$'s antiapoptotic function. Similarly, Akt inhibits the catalytic activity of a pro-death protease caspase-9, through phosphorylation (Cardone et al, 1998). Finally, phosphorylation of FKHR- a member of the Forkhead family of transcription factors- by Akt prevents its nuclear translocation and activation of FKHR gene targets, which include several proapoptotic proteins such as BIM and Fas ligand.

Akt can also influence cell survival by means of indirect effects on two central regulators of cell death- NF-KB (Romashkova, and Makarov, 1999; Anto et al, 2003) and p53(Mayo and Donner, 2001). Akt can exert a positive effect on NF-KB function by phosphorylation and activation of IKB kinase (IKK), a kinase that induces degradation of the NF-KB inhibitor, IKB (Romashkova,.and Makarov, 1999). Gong et al (2003) have reported that inactivation of NF-KB by genistein is mediated via Akt signaling pathway in breast cancer cells. Akt influences the activity of p53 through phosphorylation of p53 binding protein, MDM2 which is a negative regulator of p53 function (Mayo and Donner, 2001). The PTEN gene, located on chromosome 10 inhibits the activation of Akt. In vivo gene therapy with PTEN suppresses tumor growth, downregulates phosphorylated Akt, and increases sensitivity of human bladder cancer to doxorubicin (Tanaka and Grossman, 2003). Amplified Akt and mutated PTEN have been found with high frequency in a

variety of solid cancers, indicating the importance of this pathway in regulating tumorigenesis.

IAPs

The 'inhibitor of apoptosis' (IAP) gene family, which was discovered less than a decade ago, encodes a group of structurally related proteins that, in addition to their ability to suppress apoptotic cell death, are involved in an increase in number of seemingly unrelated cellular functions. As their name implies, the IAP proteins confer protection from death- inducing stimuli (Deveraux and Reed, 1999).

So far eight human IAPs have been identified, namely IAP-1, IAP-2, XIAP(X-linked IAP), BIR (baculovirus IAP repeat), CARD (caspase recruitment domain), ILP (IAP-like protein), MIHA (mammalian IAP homologue A), NAIP (neuronal apoptosis inhibitory factor and survivin. Only five of these contain a predicted RING domain. C-IAP1 and c-IAP2 are the only IAPs to have been identified biochemically (Rothe et al, 1995). However, the best characterized IAP is XIAP as it has the most observable biological properties (Holcik et al, 2001).XIAP is an extremely potent suppressor of apoptosis and these effects are mediated, at least in part by its ability to suppress caspases directly(Deveraux et al, 1997, 1998).

Expression levels of certain IAPs are subject to tight transcriptional control. For example, expression of survivin is regulated in a cell cycle dependant manner and seems to be induced in normal cells at the G2-M boundary (Ambrosini et al, 1997). C-IAP2 (Chu et al, 1997) and XIAP (Tang et al, 2001) are regulated by the stress responsive transcription factor, NF-KB (Ghosh et al, 1998). It remains to be tested whether the induction of IAPs such as X-IAP and c-IAP2 by NF-KB directly correlates with the situations in which NF-KB activation exerts an anti-apoptotic effect.

HSPs

The Hsps are a large family of highly conserved proteins broadly categorized according to their size. Some are constitutively expressed and associated with specific intracellular organelles, and others are rapidly induced in response to cellular stress. Hsps functions collectively to protect cells from the potentially fatal consequences of adverse environmental, physical or chemical stresses by their ability to prevent protein aggregation and to promote the refolding of denatured proteins (Parsell and Lindquist, 1993). The protective function of the Hsps may be extended to include an antiapoptotic role for several members of the Hsp family, including Hsp90, Hsp70 and Hsp27 (Beere and Green, 2001). Kanasawa et al, (2003) have reported that Hsp 70 and Hsp 40 are overexpressed in cancer tissue samples compared with normal tissues. From our laboratory, Rashmi et al (2003) have observed a protective role for Hsps against curcumin induced apoptosis in colon cancer cells.

Many of the stresses that cause the elevation of JNK activity also increase the expression of HSPs. In fact, several studies have shown that Hsp70 can inhibit JNK activation induced by a number of stresses, including heat shock (Mosser et al, 1997). Hsp90 was recently reported to sustain Akt activity by directly associating with Akt to prevent its dephosphorylation by protein phosphatase 2A (PP2A) (Sato et al, 2000), a mechanism proposed for the modulation of JNK activity by Hsp70 (Meriin et al, 1999). However, like targets of most chemotherapeutics, Hsp is not a cancer-specific protein.

Elevated levels of Hsp27 are observed in many tumor types and are often correlated with a drug- resistant phenotype. When over-expressed in vitro, Hsps27 confers enhanced cellular survival in response to a variety of proapoptotic stimuli. The protective capacity of Hsp27 is likely due to its unique ability to modulate the dynamics of microfilament reorganization so as to maintain stability of the cytoskeleton under conditions of cellular stress (Guay et al, 1997).

Other promising therapeutic targets include components of the prosurvival signals and transduction pathways involving RAS and mitogen activated protein kinases (MAPKs). Ras proteins control signaling pathways that are key regulators of several aspects of normal cell growth and malignant transformation. They are abberant in most human tumors due to activating mutations in the Ras gene or to alteration in upstream or downstream signalling. The extra cellular signal-regulated kinase/mitogen activated protein (ERK/MAP) kinase family of proline-directed serine/threonine kinases is important in a range of signalling pathways. In mammals, they comprise a cascade of three kinases, namely ERK, JNK and p38. The wide spread involvement of these kinase cascades in death and survival signalling makes them potentially useful candidates for therapeutic modulation.

Tumor cells can acquire resistance to apoptosis by various mechanisms one of which is the over expression of antiapoptotic genes which will interfere at different levels of apoptosis signaling. Down regulation of survival signalling pathway to enhance the proapoptotic ability of conventional chemotherapeutic agents, as described above is a powerful strategy towards overcoming drug resistance. However; further studies must be conducted to exploit the true therapeutic benefit of apoptosis modulation.

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CHEMOPREVENTIVE ACTIVITY OF AQUEOUS EXTRACT OF A MACROFUNGUS *PHELLINUS- RIMOSUS* AGAINST *N*-NITROSODIETHYLAMINE INDUCED HEPATOCELLULARCARCINOMA

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ABSTRACT

Chemoprevention is an important alternative approach to control cancer. Chemical substances with multiple inhibitory properties would be a welcome addition to the class of chemopreventive drugs. In this study, we examined the antioxidant, anti-inflammatory, antimutagenic and cancer preventive activities of aqueous extract of a macrofungus *Phellinus rimosus* (Berk) Pilat. The extract exhibited significant superoxide anion (O_2^-), hydroxyl radical (OH), nitric oxide (NO) scavenging and lipid peroxidation inhibiting activities. The inhibitory concentrations required by the extract to scavenge 50% (IC_{50}) of the superoxide anion, hydroxyl radical and nitric oxide generated were 126 ± 5.1, 71 ± 4.7 and 31 ± 4.5 μg/ml respectively. The concentration required to inhibit 50% of Fe^{2+} induced lipid peroxidation in rat liver was 318 ± 2.4 μg/ml. The extract showed significant anti-inflammatory activity in a dose dependent manner. Extract at a concentration of 100 mg/kg body wt inhibited 44.5%, 45.4 % and 47 % carrageenen, dextran and formalin induced inflammations respectively. The antimutagenic activity was determined by the Ames mutagenicity assay using histidine mutant *Salmonella typhimurium* strains. TA 98 and TA 100. The extract at concentration of 5 mg/plate showed profound antimutagenic activity against benzo[*a*]pyrene (B[*a*]P) and 4-nitro-*o*-pheneylenediamine (NPDA) induced mutations of TA98 and TA100 respectively. Anticarcinogenic activity was evaluated using *N*-nitrosodiethylamine (NDEA) induced hepatocellular carcinoma in rats. Serum gamma glutamyl transpeptidase (GGT), glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) and alkaline phosphatase (ALP) activities and lipid peroxidation level (MDA) were elevated significantly ($P<0.01$) in the NDEA alone treated group of animals. Treatment of the extract (25 and 50 mg/kg body wt, p.o.) prior to the NDEA administration decreased the serum GGT, GOT, GPT and ALP activities and MDA level in a dose dependent manner. The NDEA treated animals also showed altered serum albumin/globulin ratio (A:G ratio), hyperfibrinogenaemia, increased hepatic glutathione S-transferase (GST) activity, glutathioneperoxidsae (GPx) activity and reduced glutathione (GSH) level compared to the extract plus NDEA treated animal group. The extract also inhibited *invitro* aniline hydroxylase (AH) activity of rat liver induced by phenobarbital in a dose dependent manner. The results, thus suggest the significant chemopreventive properties of the aqueous extract of the *Phellinus rimosus* against NDEA induced hepatocellular carcinoma.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies world wide, with limited effective therapeutic options available. Chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection and dietary aflatoxin B1 (AFB_1)

contamination are considered as important etiological factors (1). The absence of effective systemic chemotherapeutic agents and the mortality associated with intrahepatic, rather than metastatic growth of HCC have led many investigators to focus on developing better methods of local tumor control. Among the several group of chemical carcinogens implicated in human hepatocellular carcinogenesis, nitrosocompounds form a largest group (2). The exposure to *N*-nitrosamines can be either from environment or from the diet. Majority of human cancers are currently thought to be caused by environmental factors (3) with food being one of the most important modifying agent (4). Cooking methods like roasting, grilling, baking and deep-frying in open furnaces, of foods, seem to increase the formation of *N*-nitrosamines. Major tumorigenic agents contributed to the initiation and promotion of tumor in the gas phase of cigarette smoke has also been identified as nitrosamines such as dimethylnitrosamine, diethylnitrosamine and nitropyrrolidine (5).

Numerous perspective and case control studies have firmly established that reactive electrophilic intermediates of carcinogens are formed during the metabolism by the host enzymes mainly cytochrome P450 system. The intermediates can interact with cellular macromolecules such as DNA, RNA and protein to form adducts. This can produce mutation that activate oncogene or inactivate tumor suppressor gene resulting in uncontrolled growth. In HCC, a specific hot spot mutation at codon 249 of the P^{53} gene with G to T transversion was found in nearly one-half of the tumor samples from South Africa, Taiwan and China, where HBV and AFB_1 are risk factors (1). Many of these genetic changes are acquired from the action of any one or a combination of chemical, physical, biological and genetic insults to the cell. Evidences indicated that activated neutrophils in inflammation have been shown to stimulate mutagenesis *in vitro* and oxidative stress from acute or chronic inflammation favors cancer development in many organs (6). Free radicals generated from the exogenous or endogenous pathways have been shown to involve in all the three stages of carcinogenesis. Heterocyclic amines in food and cigarette smoke are important exogenous source of free radical. There are many potential strategies for chemical protection against the multiple stages of carcinogenesis. An alternative approach to control cancer is chemoprevention which refers to the administration of chemical agents to prevent the initiation (mutational) and promotional events that occur during the process of neoplastic development (7). Hence a chemopreventive agent exhibiting activities such as antiinflammation, inhibition of carcinogen induced mutagenesis, inhibition of phase I enzyme activity and scavenging of free radical could play a decisive role in the inhibition of chemical carcinogenesis either at the initiation or promotion stage.

Mushrooms are macrofungi and they have a notable place in folk medicine throughout the world, since ancient times. Attempts have been made in many parts of the world to explore the use of mushrooms and their metabolites for the treatment of a variety of human sufferings (8). *Phellinus* species are polypore macrofungi mostly tropical and 18 species are known from Kerala. One of the species, *P. lintus* is reported to be extensively used in Chinese medicine (9). *Phellinus rimosus* (Berk) Pilat (Family Hymenochaetaceae) is often found growing on jackfruit tree trunks in Kerala (India). The basidiocarps of this fungus have been used by some local tribes for the treatment of mumps (10). Our earlier investigations showed that ethyl acetate and methanol extracts of *P. rimosus* possessed antioxidant, anti-inflammatory, hepatoprotective and

nephroprotective activities (11-13). In this communication we report the antioxidant, anti-inflammatory antimutagenic and the protective effect of aqueous extract of *P. rimosus* against NDEA induced hepatocellular carcinoma in rat.

Materials and Methods

Animals

Male Swiss albino mice of 6-8 weeks old (25 ± 2 g) and male Wistar rats of 10 weeks old (160-180 g) purchased from the Small Animal Breeding Center, Kerala Agriculture University, Mannuthy, Kerala, India were used for the studies. They were maintained in environmentally controlled conditions with free access to standard food (Lipton, India) and water. The experiments were conducted according to the prescribed guidelines of the Animal Ethic Committee.

Chemicals

N-nitrosodiethylamine (NDEA), Benzo[*a*]pyrene (B[*a*]P), 4-nitro-*o*-pheneylene diamine (NPDA) and carrageenan were purchased from Sigma, St. Loius, USA. Nicotinamide adenine dinucleotide phosphate (disodium) (NADP), glucose-6-phosphate, D-biotin, L-histidine and agar-agar were purchased from Sisco Research Laboratories, Mumbai and dimethylsulphoxide (DMSO) and formaldehyde from Merck India Ltd, Mumbai. All other chemicals and reagents used were of analytical grade.

Bacterial strains

Salmonella typhimurium strains TA98 and TA100 were originally obtained from Prof. B. N. Ames, University of California, Berkely, USA. The strains were subcultured in nutrient broth for 12 h and stored at –70°C as frozen permanents in the presence of dimethyl sulphoxide (9%). Thawed frozen permanent (40 µl) was used to inoculate for the fresh overnight culture of each strain in nutrient broth (5 ml). The inoculated nutrient broth was incubated overnight at 37°C and used for the antimutagenic assay.

Preparation of the extract

Sporocarps of *P. rimosus* growing on the jackfruit tree trunks were collected from the out skirts of Thrissur, Kerala. The specimen was identified and voucher specimen was deposited in the Herbarium of Centre for Advanced Studies in Botany, University of Madras, Chennai, India (HERB MUBL 3171).

The sporocarps were cut into small pieces, dried at 40-50°C for 48 h and powdered. Five hundred gram of the powdered material was boiled in 5 litre distilled water for 1h at 90-95°C. The supernatant removed and the extraction was repeated once again. The supernatants thus obtained were combined and filtered through Whatmann No. 1 filter paper, the filtrate was concentrated at low temperature and finally lyophilized. The residue (35 g) was designated as aqueous extract. The extract dissolved in distilled water was employed for the *in vitro* and *in vivo* assays.

Assay of in vitro antioxidant activity

a) Superoxide anion scavenging activity

Superoxide anion scavenging activity was determined according to the method of Mc Cord and Fridovich (14). The reaction mixture contained, EDTA (6 mM) contained 3 µg NaCN; riboflavin (2 µg); NBT (50 µg); KH_2PO_4-Na_2HPO_4 buffer (67 mM, pH 7.8) and various concentrations of the aqueous extract in a final volume of 3 ml. The tubes were illuminated under incandescent lamp for 15 min. The optical density at 560 nm was measured before and after illumination. The inhibition of the superoxide radical

generation was determined by comparing the absorbance values of the control with that of treatments.

b)Inhibition of lipid peroxidation

Lipid peroxidation induced in rat liver homogenate (15) and its inhibition by the extract was determined by the method of Ohkawa *et al.* (16). The reaction mixture contained 0.1 ml of rat liver homogenate (25%, w/v) in Tris-HCl buffer (20 mM, pH 7); KCl (30 mM); $FeSO_4$ $(NH_4)_2SO_4$. $6H_2O$ (0.16 mM); ascorbate (0.06 mM); and various concentrations of the extract of *P. rimosus* in a final volume of 0.5 ml. The reaction mixture was incubated for 1h at 37°C. After the incubation period, 0.4 ml was removed and treated with 0.2 ml SDS (8.1%); 1.5 ml thiobarbituric acid (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4 ml by distilled water and then kept in a water bath at 95-100°C for 1 h. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1, v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4,000 rpm for 10 min. The organic layer was removed and its absorbance at 532 nm was measured. Inhibition of lipid peroxidation was determined by comparing the optical density of the treatments with that of control.

c) Assay for hydroxyl radical scavenging activity

The reaction mixture contained deoxyribose (2.8 mM); $FeCl_3$ (0.1 mM); K_2HPO_4-KOH buffer (20 mM, pH 7.4); EDTA (0.1 mM); H_2O_2 (1.0 mM); ascorbic acid (0.1 mM) and various concentrations of the aqueous extract of *P. rimosus* in a final volume of 1 ml. The reaction mixture was incubated at 30°C for 60 min. The thiobarbituric acid reacting substance (TBARS) formed was estimated by the method of Ohkawa *et al.* (16). The hydroxyl radical scavenging activity was determined by comparing absorbance of control with that of treatments.

d)Assay for nitric oxide scavenging activity

Nitric oxide scavenging activity was determined by the method of Sreejayan and Rao (17). Immediately before the experiment, 10 mM stock solution of sodium nitroprusside was prepared in PBS (pH 7.4). Various concentrations of the aqueous extract of *P. rimosus* and sodium nitroprusside (1 mM) in a final volume of 3 ml were incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction solution was removed and diluted with 0.5 ml of Griess reagent (1% sulphanilamide, 2% orthophosphoric acid and 0.1% naphthyle thylenediamene dihydrochloride). The absorbance of the chromophore was read immediately at 546 nm. Production of nitrite from of 1 mM sodium nitroprusside solution incubated in the presence and absence of aqueous extract of *P. rimosus* at various time intervals (50, 100 and 150 min) was determined and compared with the absorbance of standard solutions of sodium nitrite (0.1µM-5 µM) treated similarly with Griess reagent.

Determination of anti-inflammatory activity

Anti-inflammatory activity of aqueous extract of *P. rimosus* was determined by carrageenan and dextran induced acute and formalin induced chronic mouse paw edema.

a) Carrageenan induced paw edema

Animals were divided into four groups of six animals each. In all groups the inflammation was induced by single sub-plantar injection of 0.02 ml of freshly prepared 1% carrageenan in normal saline (11). Group treated with carrageenan alone was served as control. Two groups received aqueous extract of *P. rimosus* at concentration of 50 and 100

mg/kg body wt orally 1 h before the carrageenan injection. The paw thickness was measured using vernier calipers before and 3 h after carrageenan injection. Increase in paw thickness was calculated using the formula Pt-Po, where, Po initial paw thickness at time to and Pt is the thickness at time t (3 h). Percent inhibition was calculated by the formula (1-PT/PC) X100, where PT is the increase in paw thickness of the treated and PC is that of control. Diclofenac (10 mg/kg body wt., i.p) was used as the reference drug.

b) Dextran induced paw edema

Experiment was same as described above except that single dose of 0.02 ml of dextran (1%) was used as the inducer of inflammation (11).

c) Formalin induced paw edema

Experiment was same as described before except that single dose of 0.02 ml of formalin (2%) was used as the inflammation inducer (11). The extract was administered once daily for 6 consecutive days.

Antimutagenic assay

Antimutagenic activity was determined by the method of Ames (18) using *Salmonella typhimurium* strains TA 98 and TA 100.

a) Antimutagenic assay using direct acting mutagens

NPDA was used as the direct acting mutagens. Freshly grown overnight cultures of Salmonella tester strains of TA 100 in nutrient broth (0.1 ml, approximately 10^9 bacterial cells/ml) were mixed with 0.1 ml of various concentrations of aqueous extract of *P. rimosus* (5 mg, 2.5 mg and 1 mg /plate), 0.2 ml of 0.5 mM histidine/biotin solution and 0.01 ml of mutagen (20 ¼g NPDA in DMSO) in 2 ml of molten agar at 40°C. The mixture was poured onto minimal glucose agar plate and incubated for 48 h at 37°C. After the incubation period, number of revertants per plate were counted using a colony counter. To evaluate the toxicity of the extract, plates without mutagens but with extract (5 mg/plate) treated in the similar manner were employed.

All the experiments were repeated twice in triplicate. The percent inhibition was calculated using the formula $\{(1-(R_2\text{-}SR)/ (R_1\text{-}SR)\}$ X100, where R_1 is the average number of revertants in the presence of mutagen alone, R_2 the average number of revertants in the presence of mutagen plus extract and SR is average number of revertants in the plate without extract or mutagen (spontaneous revertants).

b) Antimutagenic assay using indirect acting mutagens

For this assay B[*a*]P (5 ¼g in DMSO) was employed as mutagen. Activation was accomplished by treatment with the rat liver microsomal fraction before plating onto the minimal agar plate.

Preparation of rat liver microsomal fraction (S9)

Male Spraque Dawly rat (200 g) was treated with sodium phenobarbitone (0.1 %) in drinking water for 4 days (19). After an overnight fasting, animal was killed by decapitation, liver removed and homogenate was prepared aseptically. The homogenate was centrifuged in a cooling centrifuge at 8,600 rpm for 10 min at 4°C. The supernatant was used as the S9 fraction.

The assay was carried out by the Ames plate incorporation method. The reaction mixture was prepared by adding 0.5 ml of S9 mix [containing 0.2 ml of 0.2 M sodium phosphate buffer, pH 7.4, 0.025 ml of 0.1 M NADP, 0.025 ml of 1 M glucose-6-phosphate, 0.01 ml of $MgCl_2$-KCl solution (1.65 M KCl +0.4 M $MgCl_2$) and sterile distilled water], 0.01 ml of 0.5 mg/ml mutagen B [*a*] P (in DMSO), 0.1 ml

of various concentration of the extract (5 mg, 2.5 mg and 1 mg), 0.1 ml of the freshly grown overnight *S. typhimurium* culture strains (TA100) and 0.2 ml of histidine/biotin solution. The mixture was poured onto the minimal agar plate. The plates were incubated at 37°C for 48 h. Number of revertance and percentage of inhibition were calculated as described above.

Effect of P. rimosus extract on invitro aniline hydroxylase activity

Male Spraque Dawly rat (200 g) was treated with sodium phenobarbitone (80 mg/ kg body wt, p.o) for 5 days. After an overnight fasting, animal was killed by decapitation and the microsomal fraction (S9) was prepared as described above. Aniline hydroxylase was determined (20) in 1 ml of the microsomal fraction in the presence and absence of various concentrations of the *P. rimosus* extract (1, 2.5 and 5 mg). The activity was compared to the reaction mixture devoid of the extract.

Effect of P. rimosus extract on NDEA induced hepatocellularcarcinoma

Male wistar rats (140 ± 20g) were used for the experiment. HCC was induced according to the method of Jose et al. (21) with slight modifications. Animals were divided into 4 groups of 6 animals in each group. Group 1 treated with NDEA (4 mg/kg body wt. p.o.) for 5 days/week for 20 weeks was kept as control. Group 2 and 3 were administered orally with 25 and 50 mg/kg body wt respectively with aqueous extract of *P. rimosus* 1 h prior to each NDEA administration. The group 4 treated with vehicle (distilled water) was maintained as normal. 1 week after the last dose of NDEA administration, animals were kept fasting overnight and then sacrificed. Coagulated and noncoagulated (heparin) blood were collected from the heart for plasma and serum respectively. Serum was used for the determination of gammaglutamyl transpeptidase (GGT) (22), glutamate pyruvate transaminase (GPT) (23), glutamate oxaloacetate transaminase (GOT) (23), total protein (24), albumin (25), alkaline phosphatase (ALP) (26) and lipid peroxidation (malondialdehyde) (27). Plasma was analyzed for fibrinogen (28).

Liver was removed and washed thoroughly in ice-cold saline and homogenate (10 %) was prepared in PBS (50 mM, pH 7). A part of the homogenate was used for the estimation of reduced glutathione (GSH) (29). The remaining homogenate was centrifuged at 10,000 rpm for 10 min in a cooling centrifuge, after removal of the cell debris; supernatant was used for the assay of glutathione S-transferase (GST) (30) and glutathione peroxidase (GPx) (31). Protein was determined by the method of Lowry *et al.* (32).

Histopathological examination

A portion of the liver was fixed in 10 % formalin and then embedded in paraffin. 6 μm microtome sections were prepared from each liver and stained with hematoxilin-eosin.

Statistical analysis

Experimental data were statistically analysed using ANOVA. If found significant ($P<0.05$) the extract treated group was compared with the control group using Dunnett's t-test. P values less than 0.05 were considered significant. All data were represented as mean ± SD.

Result

Antioxidant activity

Aqueous extract of *P. rimosus* showed significant free radical scavenging activities. The activity of the extract was in a dose dependent

manner (Table I). Inhibitory concentration of the extract required for scavenging 50% (IC_{50}) of the super oxide anion radical generated from the photoreduction of riboflavin was found to be 126 ± 5.1 μg/ml. The extract was effective in scavenging the OH radical generated from the Fenton reaction. The IC_{50} was observed 71 ± 4.7 μg/ml. Incubation of whole liver homogenate with Fe^{2+} ion showed the induction of lipid peroxidation, where as the extract significantly inhibited the Fe^{2+} induced lipid peroxidation. The IC_{50} was found at 318 ± 2.4 μg/ml. The extract exhibited scavenging of nitric oxide generated from the sodium nitroprusside. The IC_{50} was found 31 ± 4.5 μg/ml. The incubation of sodium nitroprusside with various concentration of aqueous extract of *P. rimosus* at different time intervals resulted in linear time-dependant reduction of nitrite production (Fig 1).

Anti-inflammatory activity

Extract was effective in reducing the inflammatory edema induced by all the three edematogenic agents. Anti-inflammatory activity of the extract was in a dose dependent manner (Table 2, 3 and 4). The extract at concentration of 100 mg/kg body wt showed significant ($P<0.01$) inhibition of edema induced by acute inflammatory agent carrageenan (44.5 %) and dextran (45.4 %). The extract at the same concentration was highly effective in reducing the edema induced by the chronic inflammatory agent formalin (47 %). In both models the effect was comparable to the standard reference drug diclofenac.

Antimutagenic activity

Effect of aqueous extract of *P. rimosus* against B[*a*]P and NPDA induced mutation is presented in fig. 2& 3. The extract inhibited NPDA induced mutation of TA100 in a dose dependent manner. The extract at 5 mg/plate inhibited (42.6 %) mutation of TA 100 induced by NPDA (Fig. 2). The extract at 5 mg/plate also showed a significant inhibition of mutagenicity induced by the S9 activated B[*a*]P to the *S. typhimurium* strain TA 98 (86.5%) (Fig. 3) reversion of bacterial colonies in the extract alone treated plate was comparable to that of the spontaneous reversion of the strains (30 ± 2 for TA 98 and 130 ± 13 for TA 100).

Effect of P. rimosus extract on invitro aniline hydroxylase activity

Extract of *P. rimosus* inhibited the activity of *invitro* aniline hydroxylase in a dose dependent manner (Table 5). The activity of aniline hydroxylase induced by phenobarbitone was inhibited 9.4, 29.1 and 53.7 % by 1, 2.5 and 5 mg of the extract respectively.

Effect of extract on NDEA induced hepatocellularcarcinoma

Aqueous extract of *P. rimosus* inhibited the NDEA induced hepatocellular carcinoma in a dose dependent manner. Treatment of NDEA 5 days/week for 20 weeks induced hepatocellularcarcinoma in all the control group animals. The number of tumors and percent of incidence was reduced significantly in animals administered with the 50 mg/kg body wt extract. The activity of the SGOT, SGPT and ALP was elevated significantly ($P<0.01$) in the NDEA treated animals compared to the normal group of animals. Marked decline ($P<0.01$) was observed in the activities of these enzymes in group of animals treated with the extract plus NDEA (Table 6). The NDEA alone treated animal group also showed hyperfibrinogenemia compared to the extract plus NDEA treated animals (Table 7). The control animal group showed elevation of total protein (Table 7), hence the albumin/globulin ratio (Table 8) was

altered ($P<0.01$) compared to the normal animal group. Treatment of the extract prevented the alteration of the A:G ratio. The activity of the serum GGT was found reduced significantly ($P<0.01$) in the extract plus NDEA treated animal group (50 mg/ kg body wt) (Table 9). The index of lipid peroxidation, MDA was elevated ($P<0.01$) in the serum of the NDEA alone treated animal group compared to the normal and *P. rimosus* treated groups (Table 9). The activities of GST (Fig. 4), GPx and GSH (Table 10) level in the liver homogenate of the extract (50 mg/ kg) plus NDEA treated animals showed a significant decrease ($P<0.01$) compared to the NDEA treated group.

Histopathological analysis indicated that the NDEA alone treated liver cells were arranged mostly in solid and trabecular pattern, with cellular polymorphism, fatty infiltration, varying mitotic figures and focal necrotic changes. All these changes clearly indicated the hepatocellularcarcinoma. These pathological manifestations were decreased high to moderate level respectively in the 50 mg/ kg and 25 mg/kg body wt extract treated group of animals.

Discussion

Results of the present investigations indicate that the aqueous extract of *P. rimosus* is an effective chemopreventive agent against the NDEA induced hepato cellular carcinogenesis. This conclusion is supported by various biological properties of the extract. Treatment of the extract prior to the NDEA administration significantly reduced the tumor incidence compared to the control group of animals. The serum GGT activity was significantly elevated in the NDEA alone treated group of animals indicating the induction of hepatocellular carcinoma. However, treatment of the extract prior to NDEA showed a significant reduction of the tumor marker in a dose dependent manner. This is in agreement with elevated hepatic GST activity in the NDEA treated animal. Various hepatomas exhibited high levels of GST-P protein, as usually observed in pre-neoplastic and neoplastic lesions after chemical hepatocarcinogenesis (33). The low level of the hepatic GST in the extract plus NDEA treated animal supports its ability to inhibit tumor progression. Further, the hepatocellularcarcinoma is associated with hyperfibrinogenemia (34). This was due to synthesis of this protein by the carcinoma cells. The plasma fibrinogen level decreases in the extract plus NDEA treated group than the NDEA alone treated animals. The elevated serum GOT, GPT, ALP and altered A:G ratios are indicative of poor hepatic function of the NDEA treated animals compared to animals administered with extract prior to NDEA treatment. The elevated hepatic GGT activity is responsible for the increased GSH level in the control group, which is found decreased in the extract treated group. In addition to elevated GST, increased expressions of both G/GT and GPx have been implicated in drug resistance (35). Hence the decreased hepatic GPx, GST and serum G/GT activity in the extract plus NDEA treated animal group compared to those treated only with NDEA also support the efficacy of the treatment.

NDEA has been shown to metabolized by the microsomal mixed function oxidase (MFO) system to its active ethyl radical metabolites $CH_3CH_2^+$. This reactive radical interact with DNA producing mutation and oncogenesis. Studies in the hepatoma indicate disequilibria of the delicate oxidant versus antioxidant balance, which is tilted towards an oxidant side (36). This oxidative stress might be the reason for the elevated MDA level in the serum of NDEA treated animals. Lipid peroxidation can result in the formation of several toxic bye-products such as 4-hydroxynonenal and malondialdehyde that can attack the cellular targets

including DNA, inducing mutagenicity and carcinogenesity (37,38). The treatment of the extract prior to the NDEA administration significantly reduces the level of lipid peroxidation. The histopathological observations support the above findings. The liver of animals treated with the extract and NDEA shows a significant reduction of mitotic level and hyperplasia compared to the liver of NDEA alone treated animals.

Reactive oxygen species such as O_2^-, $^{\cdot}OH$, H_2O_2 and NO participate in the initiation or promotion of cancer through their ability to cause point mutations, DNA cross-links and DNA strand breaks (37, 39). Oxidants have the capacity to induce the transcription of growth competence related protooncogene C-*fos* and C-*jun* in several systems (40). Induction of these immediate genes represents a prerequisite for the stimulation of the cell proliferation. A marked increase in the expression of cellular oncogenes such as C-*ras*, C-*fos*, C-*myc* and N-*myc*, involved in neoplastic transformation, has been detected in the rat hepatomas as early as the first month after diethylnitrosamine treatment (36).

The *in vitro* radical scavenging activity of the extract partially explains its mechanism in the prevention of hepatocarcinogenesis. The extract shows significant superoxide anion, hydroxyl radical and nitric oxide scavenging and lipid peroxidation inhibiting activity in a dose dependent manner. The Fe^{2+} ion induced lipid peroxidation in the rat liver homogenate is also inhibited by the extract in a dose dependent manner, which might be either by direct scavenging of the hydroperoxyl radical generated from the self perputating chain reaction or by transferring reducing equivalent to the unstable lipid peroxide intermediates of the pathway. The activity is in agreement with the low serum MDA level in the extract plus NDEA treated animals. The inhibition of nitric oxide could possible be the result of direct scavenging of nitric oxide by *P. rimosus* extracts or might be due to a consequence of the reaction of the extracts with other oxides of nitrogen i.e. NO_2, N_2O_3, N_2O_4 and $OONO^-$, the possible intermediates in the oxidation of NO to nitrite. Nitric oxide or reactive nitrogen species (RNS) formed during its reaction with oxygen are mutagenic agents with the potential to produce nitration, nitrosation and deamination reactions on DNA bases. Simultaneous generation of NO and O_2^- favors the production of a toxic reaction product, peroxynitrite ($ONOO^-$) (41). The scavenging of the superoxide anion generated from the photoreduction of the riboflavin and nitric oxide from sodiumnitroprusside indicate the possibility of preventing the peroxynitrite formation in the cell. Reducing the nitric oxide generation in the digestive tract was found to be effective in preventing the reactions of nitrate with amines and amides to form carcinogenic nitrosamines and nitrosamides (42). Hence, the NO scavenging activity of *P. rimosus* extract could also support the preventive role against NDEA induced hepatocellularcarcinoma.

Chemical carcinogens and cocarcinogens are considered to be responsible for many of the cancers of humans. It is widely accepted that the reaction of carcinogens with cellular macromolecules (especially DNA) is an important event in carcinogenesis, consequent to mutational events. The *P. rimosus* extract inhibited mutagenic activity elicited by carcinogen B[*a*]P against TA 98 and a non-carcinogen NPD against TA100 in a dose dependent manner. This indicates the antimutagenic activity of the extract against frame shift and base substitution mutations. The mechanism of antimutagenicity of the extract against direct acting mutagens is probably due to the inactivation of the mutagens. However, the extract alone (5 mg/plate)

did not produce any toxicity, which was evident from the background lawn. Moreover, the number of revertant colonies was similar to those of the spontaneous reversion. Hence, the antimutagenic activity is not the consequence of the toxic effect of the extract on bacterial colony. The procarcinogen, B[*a*]P is metabolized by mixed function oxidase (MFO) of the rat liver to an active intermediate benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE) (43). Incubation of phenobarbitone-induced rat liver S9 in the presence of *P. rimosus* extract inhibited the *in virto* aniline hydroxylase activity, a component of the MFO system. Hence, the incubation of extract the B[*a*]P in the presence of S9 may be inhibited its activation or probably due to the interaction with the active intermediate BPDE. The effect can also be attributed to direct scavenging of the free radicals, which are generated during the activation of B[*a*]P by MFO (44).

Inflammatory cells produce a wide range of reactive oxygen species, and several lines of evidence indicate the association of inflammation with cancers in various tissues including liver, stomach, colon/rectum, esophagus, lung, skin, bladder, mouth and pancreas. Recently, neutrophil-mediated nitrosamine formation has been showed to be a possible endogenous carcinogen, which may promote neoplasia (45). The results of the present study reveal that aqueous extract of *P. rimosus* inhibits both acute and chronic inflammations in a dose dependent manner. Arachidonic acid metabolites are surmised to be involved as inflammatory agents that cause paw edema. The arachidonic acid metabolism and prostaglandins have long been postulated to be involved in many aspects of carcinogenesis (46). In addition to the COX mediated arachidonic acid metabolites, kinins, histamine and serotonin are also accounted for the edema formation caused by formalin, dextran or carrageenan (47). Inhibition of these mediators or their release might be the mechanism of the antiinflammatory activity of the extract.

All the doses used in the experiments were selected based on the previous studies conducted by us. The extract showed no acute toxicity or sub-acute toxicity (unpublished data). Phytochemical analyses of the extract show the presence of polysaccharides and polyphenols. A number of polysaccharides and protein bound polysaccharides isolated from mushrooms are clinically used for the treatment of cancer. Krestin (PS-K) isolated from *Coriolus versicolor,* lentinan from *Lentinus edodes* and Schizophyllan from *Schizophyllum commune* are sold in Japan as anticancer drugs and extensively used in treatment (48). High molecular weight polysaccharide especially glucan are found to stimulate both nonspecific host resistance and specific immunological activity against tumors (49). A large number of the polyphenols are found to possess antioxidant, anti-inflammatory and antimutagenic activities (50). Hence, the cancer chemopreventive properties possessed by the aqueous extract of *P. rimosus* might be mediated through these active ingredients.

Acknowledgement

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Table I- *Invitro* antioxidant activity of aqueous extract of *P. rimosus* IC 50 (μg/ml)

Activities	IC 50 (μg/ml)
Superoxide radical scavenging	126.0 ± 5.1
Hydroxyl radical scavenging	71.00 ± 4.7
Lipid peroxidation inhibiting	318.0 ± 2.4
Nitric oxide radical scavenging	31.00 ± 4.5

Values are mean ± SD, n=3.

Table 2-Effect of aqueous (AQ) extracts of *P. rimosus* on carrageenan induced paw edema in mice. Reference drug diclofenac (DF).

Groups/ treatment (mg/kg)	Initial paw thickness (cm)	Paw thickness after 3 h (cm)	Increase in paw thickness (cm)	Percent inhibition
Control	0.157 ± 0.004	0.323 ± 0.013	0.166 ± 0.015	---
AQ				
50	0.161 ± 0.002	0.270 ± 0.007	0.109 ± 0.006[a]	34.3
100	0.166 ± 0.003	0.258 ± 0.005	0.092 ± 0.004[a]	44.5
DF				
25	0.166 ± 0.004	0.243 ± 0.004	0.077 ± 0.005[a]	53.6

Values are mean ± SD, n= 6 animals

[a]$P<0.01$(Dunnett's *t*- test) significantly differ from control group.

Table 3-Effect of aqueous (AQ) extracts of *P. rimosus* on dextran induced paw edema in mice. Reference drug diclofenac (DF).

Groups/ treatment (mg/kg)	Initial paw thickness (cm)	Paw thickness after 3 h (cm)	Increase in paw thickness (cm)	Percent inhibition
Control	0.171 ± 0.003	0.314 ± 0.009	0.143 ± 0.007	—
AQ				
50	0.167 ± 0.007	0.273 ± 0.005	0.106 ± 0.010[a]	25.8
100	0.158 ± 0.006	0.237 ± 0.008	0.078 ± 0.010[a]	45.4
DF				
25	0.168 ± 0.005	0.240 ± 0.008	0.072 ± 0.010[a]	49.6

Values are mean ± SD, n= 6 animals

[a] $P < 0.01$(Dunnett's *t*- test) significantly different from control group

Table 4-Effect of aqueous (AQ) extracts of *P. rimosus* on formalin induced paw edema in mice. Reference drug diclofenac (DF).

Groups/ treatment (mg/kg)	Initial paw thickness (cm)	Paw thickness after 6 day (cm)	Increase in thickness (cm)	Percent inhibition
Control	0.168 ± 0.006	0.406 ± 0.014	0.238 ± 0.012	—
AQ				
50	0.155 ± 0.005	0.328 ± 0.012	0.173 ± 0.014[a]	27.3
100	0.165 ± 0.007	0.291 ± 0.006	0.126 ± 0.010[a]	47.0
DF				
25	0.164 ± 0.008	0.305 ± 0.009	0.141 ± 0.010[a]	40.7

Values are mean ± SD, n= 6 animals

[a] $P<0.01$(Dunnett's *t*- test) significant with respect to control group

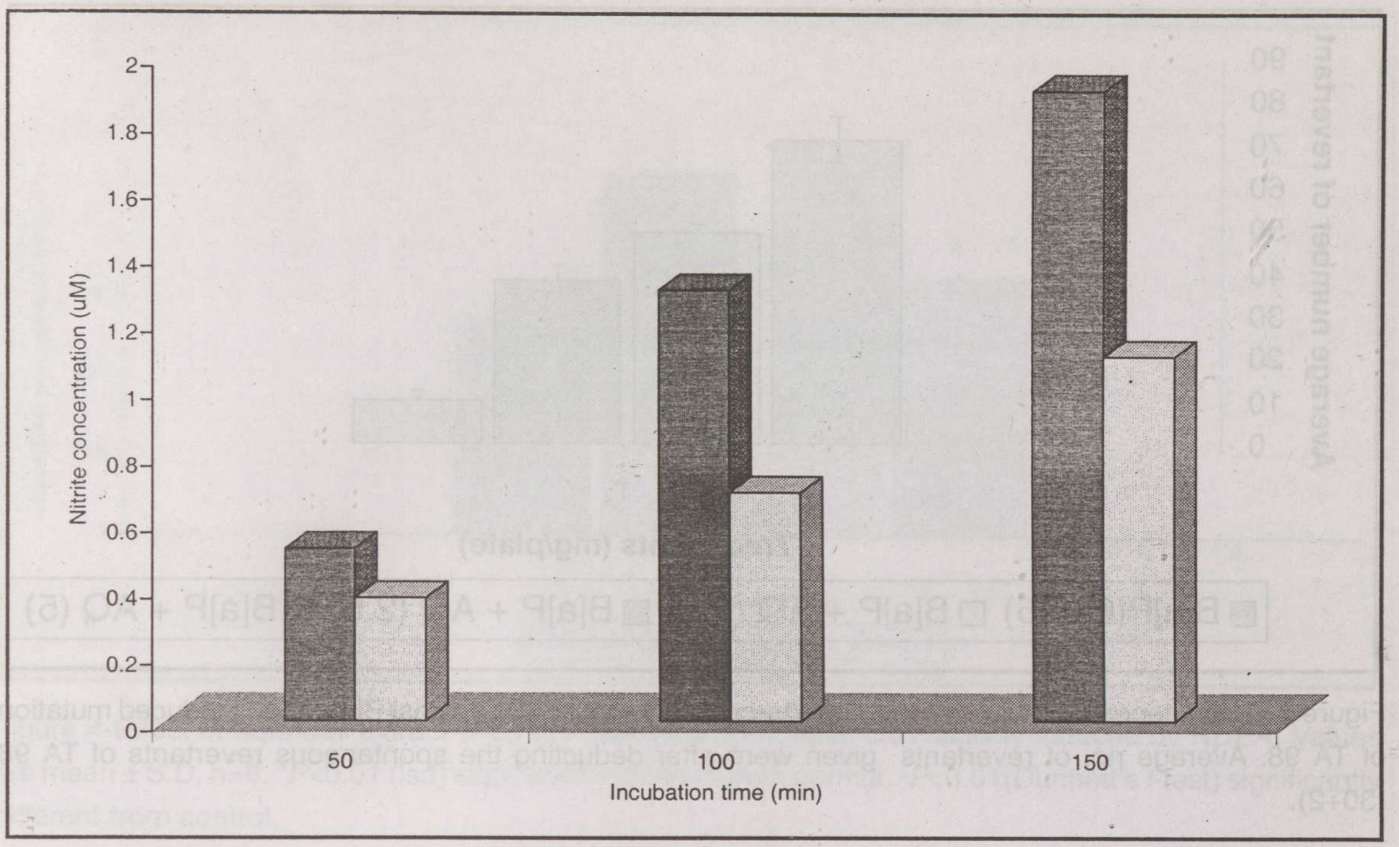

Figure I- Nitric oxide scavenging activity of aqueous extract of *P. rimosus* (AQ). Extract 31 μg/ml (IC_{50}) incubated with sodiumnitroprusside at various time intervals and optical density obtained by Griess reaction was compared with the sodium nitrite standard curve.

■ Sodiumnitroprusside, □ Sodiumnitroprusside + AQ.

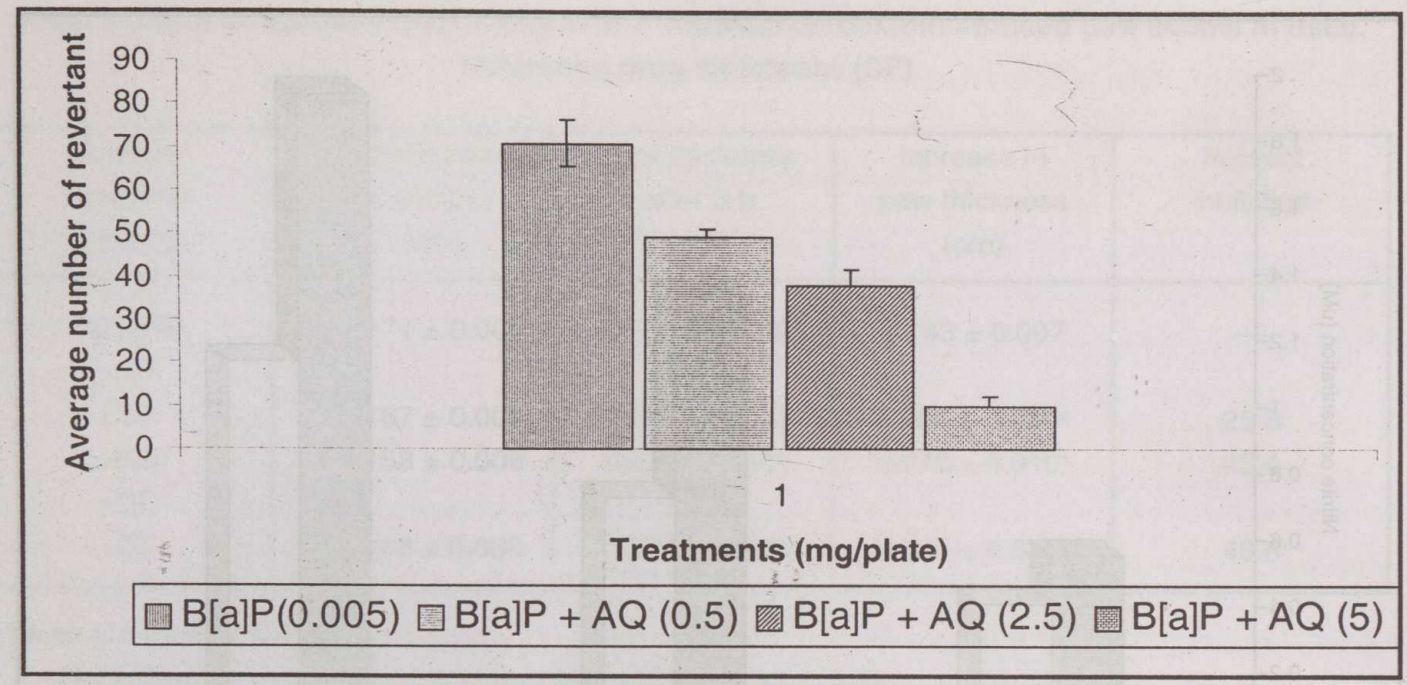

Figure 2- Antimutagenic activity of aqueous extract of *P. rimosus* (AQ) against B[*a*]P (5μg) induced mutation of TA 98. Average no. of revertants given were after deducting the spontaneous revertants of TA 98 (30+2).

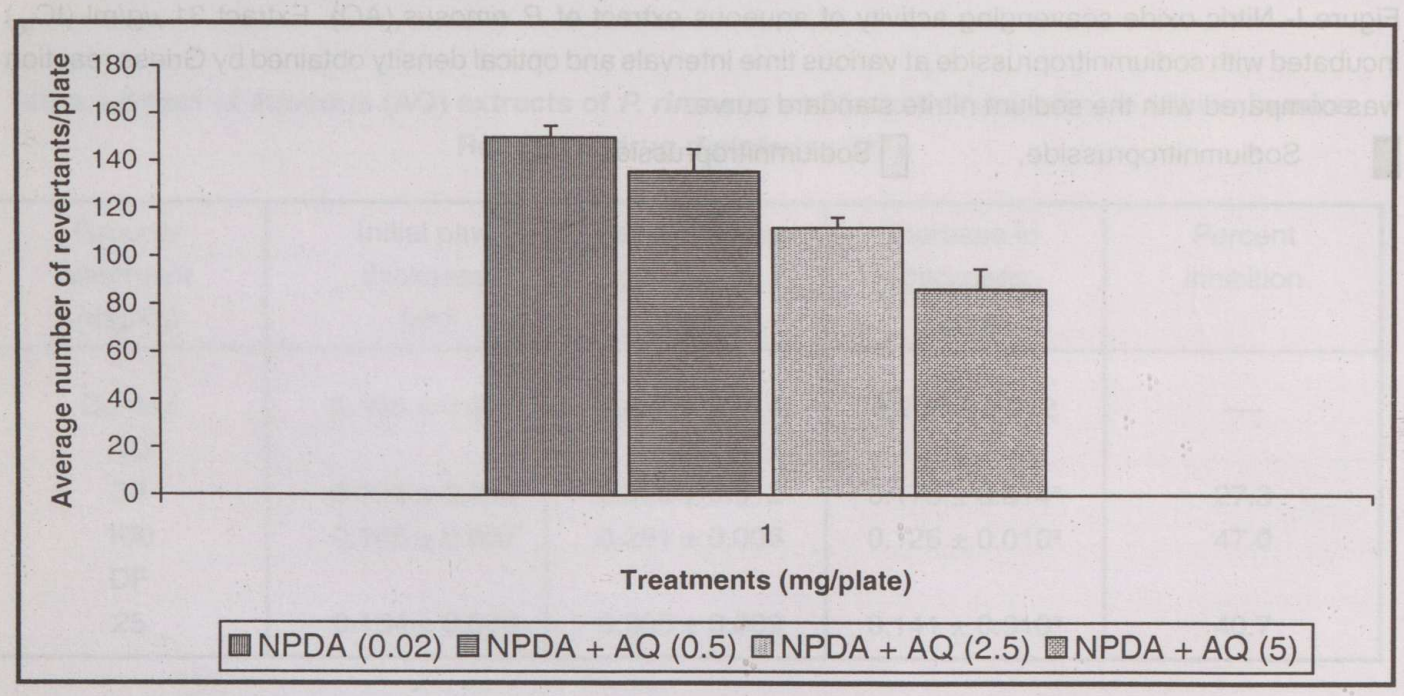

Figure 3 -Antimutagenic activity of aqueous (AQ) extracts of *P. rimosus* against 4-Nitro-*o*-phenylenediamine (NPDA, 20 μg) induced mutations of TA 100. Values are mean ± S.D, n=3, Average number of revertants/ plate expressed after deducting the spontaneous revertants of TA 100 (130 ± 13).

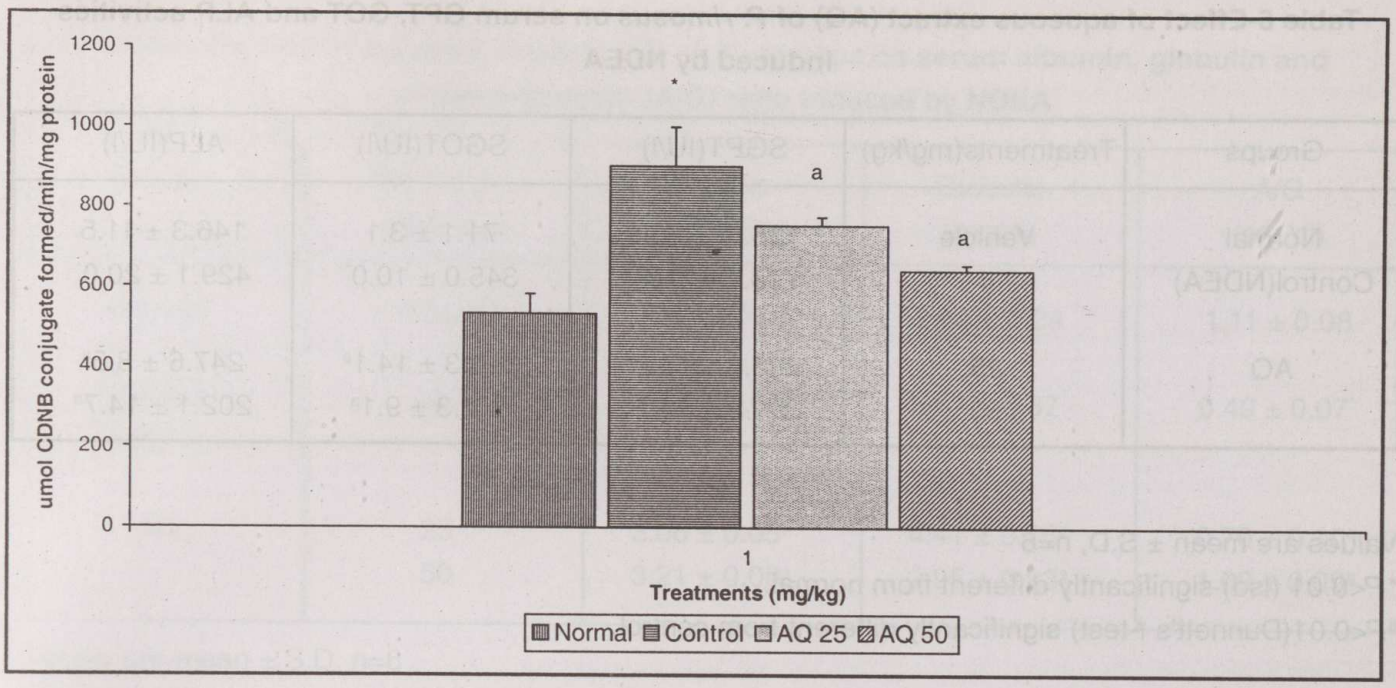

Figure 4-Effect of aqueous extract (AQ) of *P. rimosus* on hepatic GST activity induced by NDEA. Values are mean ± S.D, n=6. *P<0.01 (lsd) significantly different from normal. [a]P<0.01 (Dunnett's *t*-test) significantly different from control.

Table 5– *In vitro* aniline hydroxylase inhibiting activity of aqueous (AQ) extracts of *P. rimosus*.

Groups	Treatments (mg/ml)	Aniline hydroxylase (nmol p-aniline formed/min/mg protein)	Percent inhibition
Control	Vehicle	1.58 ± 0.02	—
AQ	1.0	1.43 ± 0.02	9.40
	2.5	1.12 ± 0.01	29.1
	5.0	0.73 ± 0.03	53.7

Values are mean ± S.D, n=2.

Table 6-Effect of aqueous extract (AQ) of *P. rimosus* on serum GPT, GOT and ALP activities induced by NDEA

Groups	Treatments(mg/kg)	SGPT(IU/l)	SGOT(IU/l)	ALP(IU/l)
Normal	Vehicle	125.8 ± 10.6	71.1 ± 3.1	146.3 ± 11.5
Control(NDEA)	4	798.1 ± 25.2*	345.0 ± 10.0*	429.1 ± 20.0*
AQ	25	442.8 ± 16.2[a]	210.3 ± 14.1[a]	247.6 ± 8.5[a]
	50	311.8 ± 14.7[a]	142.3 ± 9.1[a]	202.1 ± 14.7[a]

Values are mean ± S.D, n=6

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$(Dunnett's *t*-test) significantly different from control.

Table 7-Effect of aqueous extract (AQ) of *P. rimosus* on serum fibrinogen and total protein levels induced by NDEA

Groups	Treatments(mg/kg)	Fibrinogen(mg/dl)	Total protein(mg/dl)
Normal	Vehicle	96.6 ± 5.6	5.93 ± 0.08
Control (NDEA)	4	167.2 ± 14.5*	8.46 ± 0.92*
AQ	25	123.0 ± 6.8[a]	7.19 ± 0.20[a]
	50	110.6 ±1.9[a]	6.17 ± 0.22[a]

Values are mean ± S.D, n=6

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$(Dunnett's *t*-test) significantly different from control.

Table 8-Effect of aqueous extract (AQ) of *P. rimosus* on serum albumin, globulin and albumin/globulin (A/G) ratio induced by NDEA

Groups	Treatments (mg/kg)	Albumin (mg/dl)	Globulin (mg/dl)	A/G (mg/dl)
Normal	Vehicle	3.16 ± 0.07	2.84 ± 0.24	1.11 ± 0.08
Control (NDEA)	4	2.78 ± 0.05*	5.67 ± 0.87*	0.49 ± 0.07*
AQ	25	3.06 ± 0.05[a]	4.41 ± 0.76[a]	0.70 ± 0.10[a]
	50	3.21 ± 0.08[a]	2.95 ± 0.23[a]	1.09 ± 0.09[a]

Values are mean ± S.D, n=6

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$(Dunnett's *t*-test) significantly different from control.

Table 9-Effect of aqueous extract (AQ) of *P. rimosus* on serum GGT and MDA activity induced by NDEA

Groups	Treatments(mg/kg)	GGT(U/l) at 25°C	MDA(nmol/ml)
Normal	Vehicle	21.5 ± 6.09	1.46 ± 0.08
Control (NDEA)	4	70.5 ± 13.1*	3.40 ± 0.21*
AQ	25	42.5 ± 3.67[a]	2.75 ± 0.18[a]
	50	31.1 ± 3.25[a]	1.90 ± 0.05[a]

Values are mean ± S.D, n=6

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$(Dunnett's *t*-test) significantly different from control.

Table 10-Effect of aqueous extract (AQ) of *P. rimosus* on hepatic GPx and GSH activity induced by NDEA

Groups	Treatments(mg/kg)	GPx(U/l)	GSH(nmol/mg protein)
Normal	Vehicle	22.5 ± 1.8	8.40 ± 0.32
Control (NDEA)	4	36.8 ± 4.0*	11.10 ± 1.00*
AQ	25	32.8 ± 2.6[b]	9.30 ± 0.47[a]
	50	24.7 ± 0.8[a]	8.55 ± 0.20[a]

Values are mean ± S.D, n=6

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$ and [b] $P<0.05$ (Dunnett's *t*-test) significantly different from control.

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SCREENING OF *ALANGIUM SALVIFOLIUM* L. FOR ITS HELICOBACTERICIDAL ACTIVITY

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ABSTRACT

Stem and root bark of *Alangium salvifolium* belonging to the family Alangiaceae was screened for its probable helicobactericidal activity. Aqueous extracts (hot and cold) and solvent extracts (acetone, chloroform and methanol) were screened. Among those, chloroform extract was observed to recover bioactive principles from the plant materials effectively. The MIC and MLC was also observed to be significantly low for human *Helicobacter pylori* (Hp) isolates. Samples drawn during flowering seasons were better than that of vegetative period. So, the plant need to be pursued as promising candidate in *H. pylori* eradication.

KEYWORDS

Alangium, Alangiaceae, Helicobactericidal, Hp, PUD.

INTRODUCTION

The pathogenic involvement of *Helicobacter pylori* (Hp) in peptic ulcer diseases (PUD) is recognised only in the recent past by Drumm, *et al.* (1990) and Sonnenberg (1995). In the mid 1990's Suzuki and Ishii (1996) declared *H. pylori* as the causal organism of various PUD. Due to its persistent and malignant consequences, more attention is diverted towards successful therapeutic intervention for comprehensive eradication of the pathogen (Jain and Santani, 1994). Since the available drugs are expensive with side effects, herbal drugs possessing anti-ulcer effects in traditional systems were tried. *Alangium salvifolium* L. is one such plant, which is being used (Anonymous, 1985). Even though antibacterial, antifungal and antiyeast activities of the plant was reported by Avirutnant and Pongpan (1983) and Pongpan *et al.* (1982), the helicobactericidal activity of the plant has not been evaluated. Hence the present work was carried.

MATERIALS AND METHODS

Alangium salvifolium L. belonging to the family Alangiaceae, selected for this study was collected from Shenkottai, Tirunelveli district, Tamilnadu, India and identified according to Gamble (1967). A voucher specimen is being deposited at the department herbarium for future reference. Two seasonal samples were collected ie., flowering (April – May) and vegetative season (September – October) with a gap of six months between the period of sample collection of the plant. The plant samples were shade dried and pulverised in a stone mortar and filtered in a sieve (40 mm) and stored in an air tight container. This was used as a basic sample in this study. Aqueous extracts (hot and cold) and solvent extracts (acetone, chloroform and methanol) are prepared according to Indian Pharmacopoeia (Anonymous, 1980).

The human isolates of *H. pylori* were

collected from the gastric antral biopsy specimens at the site of active lesions with the help of sterile (2% gluteraldehyde) endoscopic forceps. It was transported to the laboratory in Brain Heart Infusion (BHI) soft agar tubes (Anonymous, 1998). The biopsy specimen was transferd to modified BHI agar plates (Calf brain infusion – 200 g/l; Beef heart infusion – 250 g/l; Proteose peptone – 10 g/l; Dextrose – 2 g/l; NaCl – 5 g/l; Disodium phosphate – 2.5 g/l; Agar – 15 g/l; Triphenyl tetrazolium chloride – 40 mg/l; Cefatoxime – 10 mg/ml; Defibrinated sheep blood – 50 ml; Final pH – 7.4 ± 0.2) and incubated at 37°C under microaerophilic conditions for 72 – 96 h. The bacterial outgrowth from the biopsy specimen was characterised on the basis of culture, microscopic characteristics, biochemical and physiological properties. *H. pylori* human isolated were stored in modified BHI agar slants at 4°C and used for this study.

Standard, pre-sterilised filter discs were obtained from Hi-media, Mumbai (Anonymous, 1998) and various extracts of the medicinal plant were incorporated aseptically in them at a concentration of 5 µl/disc. Simultaneously, broth culture of human *H. pylori* isolates was seeded on air-dried sterile Muller-Hinton agar plates obtained from Hi-media, Mumbai using a sterile cotton swab.

The crude plant extract impregnated filter discs (5 µl/disc) were placed on the *H. pylori* inoculated plates with the help of flame sterilised forceps and pressed gently. These plates were incubated at 37°C under micro-aerophilic condition for 48 – 72 h and the zone of inhibition was recorded according to Bauer *et al.* (1966).

Minimal inhibitory concentration (MIC) and Minimal lethal concentration (MLC) of the crude plant drug preparations was determined as per the methodology of Presscot *et al.* (1996). For comparison, sterile filter discs with known concentration of antibiotics were obtained from Hi-media, Mumbai, namely Amikacin, kanamycin, vancomycin, methicillin, ceftazidime, netillin, tobramycin, chloromphenicol and tetracycline at 30 µg concentration; streptomycin, gentamycin, ampicillin, amoxycillin and norfloxacin at 10 µg concentration; cotrimoxazole at 25 µg concentration; nitrofurantoin at 300 µg concentration and ciprofloxacin at 5 µg concentration were used as standard antibacterials against *H. pylori.*

RESULTS

Among the extracts tested chloroform extract was found to exhibit notable anti *H. pylori* properties (Table I). No significant difference was observed in anti *H. pylori* activity among the seasonal samples. The anti Hp activity of the extracts was comparable with many conventional antibiotics (Table II).

Since chloroform extract possessed a wide range of zone of inhibition, the extract was further subjected for MIC and MLC against *H. pylori. A salvifolium* was observed to possess bioactive principles (in chloroform extract) with relatively higher MIC and MLC for *H. pylori.* Stem bark extract demonstrated MIC at 75 µg in both seasonal samples but MLC was 100 µg for that collected during vegetative period and 75 µg for that collected during flowering period. For root bark extract, MIC was 50 µg for vegetative period and 75 µg during flowering period, whereas MLC was 75 during vegetative period and 75 µg for that collected during flowering period.

Table II categorically demonstrates the emergence of multiple drug resistance in *H. pylori* resistance to a wide class of bacterials, which includes pencillins, aminoglycosides with relatively low sensitivity to cephalsporins, such as ceftazimide.

DISCUSSION

Micorbial etiology of peptic ulcer was elucive for a very long time. Only in the recent past, the etiological correlation of *H. pylori* (earlier known as *Camphylobacter pylori*) was categorically authenticated which has brought a diabolic change in PUD management. In view of the inherent cost, patient noncomplaince, side effect profiles and the risk of drug resistance, a search for therapeutic alternative for *H. pylori* eradication is on. In this back ground medicinal plants, which is traditionally used to treat PUD, was screened for anti *H. pylori* molecule.

Owing to its therapeutic versatility, *A. salvifolium* has been one of the most extensively used medicinal plant. Its medicinal property viz., antidiabetic activity has been well documented (Anonymous, 1985). The abundance in chemical molecules such as alkaloids has made many researchers to analyse Alangium for its potential to treat various ailments (Anonymous, 1985). In these lines, *A. salvifolium* both stem and root barks were analysed for anti *H. pylori* activity and discussed. In tune with its versatality, *A. salvifolium* had demonstrable anti *H. pylori* activity in chloroform extract with a comparable zone of inhibition with that of many conventional antibiotics. They also had moderate MIC and MLC for *H. pylori* with root collected during vegetative period having slightly lower MIC and MLC. Dhar *et al.* (1968) has demonstrated the anti-amoebic activity of ethanol extracts of *A. salvifolium*. Pongpan *et al.* (1982) have demonstrated the antifungal activities of petroleum ether fraction of *A. salvifolium* dried fruit against *Candida albicans* and other antimicrobial activity of ethanol extracts. Similar observations on the antimicrobial activity of *A. salvifolium* were reported by Avirutnant and Pongpan (1983).

Even though, *A. salvifolium* is a promising antimicrobial with documented activity against various fungi, yeast and bacterial and protozoan pathogens, its helicobactericidal activity is not being reported as far. As the Pharmacological profile of this plant is relatively well known, it provides an opportunity to expand the medicinal application of *A. salvifolium* to treat PUD caused by *H. pylori*.

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Table I : Helicobactericidal activity of *Alangium salvifolium* L. stem and root bark extracts

Extracts tested	Zone of inhibition (mm)			
	Vegetative period		Flowering period	
	Stem bark	Root bark	Stem bark	Root bark
Cold aqueous extract	-	-	-	-
Hot aqueous extract	-	-	-	-
Acetone extract	1	2	1	1
Chloroform extract	12	14	13	13
Methanol extract	-	-	-	-

- - No zone of inhibition

Table II : Antibiotic susceptibility pattern of human isolates of *Helicobacter pylori*

Antibiotics tested	Symbol	Concentration (µg/disc)	Zone of inhibition (mm)	R/S pattern
Amikacin	Ak	30	18	S
Kanamycin	K	30	-	R
Vancomycin	Va	30	-	R
Methicillin	M	30	-	R
Ceftazidime	Ca	30	14	R
Netillin	Nt	30	14	R
Tobramycin	Tb	30	23	S
Chloromphenicol	C	30	-	R
Tetracycline	T	30	12	R
Streptomycin	S	10	12	I
Gentamycin	G	10	16	S
Ampicillin	A	10	-	R
Amoxycillin	Am	10	1	R
Norfloxacin	Nx	10	31	S
Cotrimoxazole	Co	25	13	I
Nitrofurantoin	Nf	300	-	R
Ciprofloxacin	Cf	5	38	S

- - No zone
S - Sensitive
R - Resistant
I - Intermediate

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METHANOLIC EXTRACT OF A MEDICINAL MUSHROOM *GANODERMA LUCIDUM* PROTECTS RADIATION INDUCED OXIDATION DAMAGE IN MICE

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ABSTRACT

Ionizing radiation damages cellular molecules directly by transferring energy or indirectly by generation of oxygen derived free radicals. Development of effective radioprotective agents with least side effects is a compelling urgency because radiotherapy is increasingly found useful in cancer treatment. Antioxidant enzymes and other scavengers of reactive oxygen intermediates are involved in numerous defense systems in cells. Investigations were carried out on the antioxidant activity and radioprotective effect of methanolic extract of fruiting body of a medicinal mushroom, *Ganoderma lucidum* . Antioxidant capacity of the extract of this mushroom was assayed by FRAP assay, free radical scavenging activity by DPPH assay and pulse radiolysis method, inhibiting effect of free radical generation by ferrylmyoglobulin – ABTS assay and oxygen radical absorbance capacity by ORAC assay. Radioprotective effect of the extract was determined by whole body irradiation (4.5Gy) after treatment with extract for five consecutive days. Significant radiation induced metabolic changes in the liver of the irradiated animals were observed. *G.lucidum* extract was found to delimit these changes to a great extent. The effect of the extract was evident from the restoration of the activities of GSH, GPx, SOD, CAT, and inhibition of lipid peroxidation. The results also indicated that the extract possessed significant antioxidant activity as evident from its ORAC coupled with free radical scavenging and inhibition of radical generation properties. The findings thus suggest the potential therapeutic use of *G.lucidum* extract in complementary cancer therapy.

INTRODUCTION

In recent years people have become more health conscious and the use of nutrition in the control of degenerative diseases is thus, gaining importance. Antioxidants, that can keep the toxic and volatile free radicals in check, are becoming an important aspect in this regard. A biochemical paradox that has been observed for many years is now becoming understood. Though oxygen is essential for aerobic life, it can be toxic to an organism if inappropriately metabolized. It can accept less than four electrons to form a reactive metabolite, which may be toxic to cells. The production of toxic oxygen-derived free radicals and related reactive species is associated with various forms of disease, injury or trauma to an organism (Armstrong, 1998). Excess reactive oxygen species (ROS) generation can result from exposure to various physical and chemical agents, including ionizing radiation, air pollutants, chemical toxicants, photosensitization etc. besides various pathophysiological states. Ionizing radiation damages cellular molecules directly by transferring

energy or indirectly by generation of oxygen derived free radicals, excited states and other reactive species collectively known as ROS (Von Sonntag 1987). The effects of low-LET radiation are mainly mediated by generation of ROS. Most of the radiation induced damage to biomolecules in aqueous media; such as those prevailing in living systems is caused by the formation of free radicals resulting from radiolysis of water and macromolecules. ROS such as superoxide anion radical ($O_2^{\cdot}$) hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) are considered to be important in the etiology of several pathological conditions such as cardiovascular diseases, neurological disorders, arthritis, diabetes, inflammation, cancer etc. (Hennmani and Parihar, 1998)

Radiation therapy evolved as a treatment of cancer because it permitted tumor eradication with preservation of normal tissue functions. Mammalian cells are most sensitive to radiation-induced damage in the late G_2 and M phase of the cell cycle. Cellular damage produced by the radiation therapy is an indirect result of ionizing chemicals in the cell to very reactive compounds. Cytotoxicity is primarily caused by oxygen-derived free radicals such as hydrogen peroxide (H_2O_2), Superoxide (O) and hydroxyl radical (OH) (Parker,1990). In modern clinical practice, radiotherapy is frequently combined with surgery and chemotherapy to provide the most effective tumor control with least damage to normal tissue. Synthesis of radiosensitizors, which augment the amount of injury induced by radiation to hypoxic cells that are relatively resistant, has increased the effectiveness of radiation therapy (Dische, 1985).

One of the major draw back of radiotherapy is that it produced severe side effects generated due to the damage of normal tissues during irradiation (Maunch et al, 1995) The sub lethal whole body gamma irradiation also inflicts drastic changes in the haematopoietic system as it is very sensitive to the ionizing effects of irradiation (Anderson, 1990). Radiation can induce the formation of free radicals and this radiation induced free radicals produce peroxidation of lipids, leading to structural and functional damage to cellular membranes (Rayleigh, 1987). The natural antioxidant system of the body, consisting of reduced glutathione (GSH) and the related enzymes as well as superoxide dismutase (SOD) are believed to be the major cellular constituents involved in the defense against lipid peroxidation. Reduced glutathione (GSH), which amounts to about 90% of the nonprotein thiols in the cell is involved in a number of reductive reactions in the cell and acts as a substrate or co-factor for the antioxidant enzymes, GSH peroxidase, GSH transferase and reductases which are involved in the termination of peroxidation GSH have been shown to protect cells against oxidative stress by reacting with peroxides and hydroperoxides. Increase in GSH was found to be correlated with thiol induced radioprotection and chemoprotection in mouse. Superoxide dismutase (SOD) reacts with Superoxide radical and converts them to H_2O_2, which is catalyzed by catalase or GSH peroxidase (Uma Devi and Ganasoundari, 1999).

Antioxidants are a group of substances which when present, significantly inhibit or delay oxidative process, while often being oxidized themselves. (Yoshikawa et al., 2000). Antioxidant enzymes and other scavengers of reactive oxygen intermediates are involved in numerous defense systems in cells. Antioxidants may exert their effects on biological systems by different mechanisms including electron donation (as reducing agent) metal ion chelation (there by

eliminating potential free radicals) sparing by antioxidants (co-antioxidants) or by gene expression regulation (Yoshikawa et al., 2000)

Mushrooms are fleshy fungi and they have attracted the attention of man since ancient times. References to their existence are found in classical Indian, Greek and Roman writings (Butler and Bisbay, 1960). For centuries mushroom lovers have picked up wild mushrooms for their aroma and palatability. Mushrooms are a valuable source of food, being rich in protein with a high digestibility coefficient of about 87%. The protein contains many of the essential amino acids, which in some species equals the muscle protein in nutritive value. Its biological value is intermediate that of vegetable and animal protein. The mushrooms are also an excellent source of vitamins and minerals. (Munjal and Seti, 1980). Mushrooms are claimed to possess antiviral, antibiotic, anti-inflammatory hypoglycemic, hypocholestrolemic and hypotensieve activities (Andrae, 1999).

Herbalists consider *Ganoderma* an adaptogen or natural regulator of suppressing the immune system if it is over active and boosting if it is under active. Many health claims are made on the effect of *Ganoderma* on the immune system. These claims are based primarily on the presence of high molecular weight polysaccharides and free radical scavenging or antioxidant activity of *Ganoderma* extracts.

Development of effective radioprotective agents with least side effects is a compelling urgency because radiotherapy is increasingly found useful for cancer treatment. Although a large number of biological properties and clinical use of *Ganoderma* species have been reported, no attempt has been made to exploit the radioprotective effects of this medicinal mushroom. Investigations were carried out on the antioxidant activity and radioprotective effect of the methanolic extract of the fruiting body of *Ganoderma lucidum* occurring in South India and the findings are reported in this communication.

MATERIALS AND METHODS

MATERIALS

Ascorbic acid, 2,2-azobis-3-ethylbenzthiazoline–6-sulphonic acid diammonium salt (ABTS), butylated hydroxytoluene (BHT), 1,1 –diphenyl 2- picryl hydrazyl (DPPH), ethylene diamine tetra acetic acid (EDTA), ferric chloride, hydrogen peroxide, methanol (HPLC grade), myoglobin, potassium ferricyanide, sodium acetate, 2,4,6-tripyridyl- s-triazine (TPTZ), 2-thiobarbituric acid, triphenyl phospene (TPP), trichloro acetic acid and xylenol orange were purchased from Sigma Chemical Co. USA. Hydrogen peroxide (H_2O_2) was purchased from Merck, India Ltd, Mumbai. Sodium azide (NaN_3), Reduced glutathione (GSH), 5,5-dithiobis- (2-nitrobenzoic acid) (DTNB), Nitroblue tetrazolium (NBT), 1-chloro-2,4-dinitrobenzene (CDNB), riboflavin from Sisco Research Laboratories Pvt.LTD. Mumbai.

All other chemicals and reagents used were analytical reagent grade.

Animals

Swiss albino female mice weighing 20-25gm were purchased from Veterinary College Mannuthy. Animals were fed with standard mouse feed and water.

Irradiation

Animals were exposed to Cobalt-60 gamma rays (4.5 Gy) at Radiation Therapy Unit, Amala Cancer Hospital.

METHODS

Preparation of the extract

Sporocarps of *G.lucidum* were collected from the out skirts of Thrissur, Kerala. The sporocarps were cut into small pieces, dried at 40-50°C for 48 h and powdered. Two hundred gram of the powdered materials was extracted with petroleum ether. The defatted material was extracted with 70% methanol for 8-10 h twice (Suffness and Douros, 1979). The extracts combined and the solvents was completely evaporated at 40°C using a rotary vacuum evaporator. The residue was designated as methanol extract. The methanol extract was dissolved in distilled water to form a uniform suspension, and used in the experiments.

Determination of antioxidant activity

Antioxidant capacity of the extract was assayed by FRAP assay, free radical scavenging activity by DPPH assay and pulse radiolysis methods, inhibitory effect on free radical generation by ferrylmyoglobulin/ABTS assay and Oxygen radical absorbance capacity by ORAC assay.

For the antioxidant activity assays *G. lucidum* 0.1%, 0.5%, 1% concentrations of the extracts were used. The results were expressed as Ascorbic acid Equivalent Antioxidant Capacity (AEAC). AEAC is the concentration of Ascorbic acid (%) required to give the same antioxidant capacity as test substance (Gil et al, 2000).

Ferric reducing antioxidant power (FRAP) assay:

The ferric reducing ability was measured at low pH (Benzie and Strain, 1996; Pulido et al 2000). The stock solution of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ and 0.3 M acetate buffer (pH3.6) were prepared. The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution and 25 ml acetate buffer. It was prepared freshly and warmed to 37°C. Then, 900 µl of distilled water and 30 µl test sample/methanol/distilled water were added to standard solutions. The reaction mixture was then incubated at 37°C for 30 min and absorbance was recorded at 595 nm. An intense blue colour complex was formed when ferric tripyridyl triazine (Fe^{3+}TPTZ) complex was reduced to the ferrous (Fe^{2+}) form and the absorption at 595 nm was recorded. The calibration curve was plotted with absorbance at 595 nm versus concentration of $FeSO_4$ in the range of 0-1 mM . The concentrations of $FeSO_4$ were in turn plotted against concentrations of standard antioxidants (L-ascorbic acid).

DPPH radical scavenging assay

In this method a commercially available and stable free radical ($DPPH^*$, 2,2-diphenyl-1-picrylhydrazil), which was soluble in methanol, was used (Aquino et al, 2001). DPPH in its radical form has an absorption peak at 515nm, which disappeared on reduction by an antioxidant compound. An aliquot (37.5 µl) of the extract was added to 1.5 ml of freshly prepared DPPH solution (0.25 g/l in methanol). Absorbance was measured at 515 nm 20 min after the reaction was started. The DPPH concentration in the reaction medium was calculated from the calibration curve of % DPPH scavenged versus concentration of the standard antioxidant (L-ascorbic acid).

ABTS radical scavenging assay by spectrophotometry

In this assay, the radical scavenging activity of extract was determined by using ferryl

myoglobin/ABTS protocol (Alzoreky and Nakahara, 2001). The stock solutions of 500 μM ABTS diammonium salt, 400 μM myoglobin, 740 μM potassium fericyanide and 450 μM H_2O_2 were prepared in phosphate buffered saline (PBS) (pH7.4). Metmyoglobin (MbIII) was prepared by mixing equal amounts of myoglobin and potassium ferricyanide solutions. The reaction mixture (total volume 2 ml) contained the following substances (final concentrations in the reaction mixture): ABTS (150 μM), MbIII (2.5 μM), 16.8 μl of the sample and 978 μl PBS. The reaction was initiated by adding 75 μM H_2O_2 (330 μl) and the lag time in seconds was recorded before absorbance of $ABTS^{\cdot+}$ at 734 nm began to increase . The calibration curve was plotted with lag time in seconds versus concentration of the standard antioxidant (L-ascorbic acid).

ABTS radical scavenging by Pulse radiolysis:

Antioxidant activity of the extracts was also assayed by ABTS radical scavenging by pulse radiolysis technique using a linear accelerator. For pulse radiolysis measurements, the absorbed dose was kept to a minimum to avoid decomposition of the test compound. Typical maximum doses with 50 pulses were 15 Gy. The standard pattern of decay with ascorbic acid having four different concentrations of 2.5, 5, 7.5 and 10 μg per ml showed typical concentration dependent curves (Scotl et al, 1993). The ascorbic acid equivalent was computed by extrapolating the results with the standard graph.

Oxygen Radical Absorbance Capacity (ORAC) assay

In ORAC assay oxygen radical absorbance capacity was measured on detection of chemical damage to β-phycoerythrin through the decrease in the fluorescence emission. The fluorescence was recorded every 5 min till the last reading is less than 5% of the zero minute reading. ORAC values were calculated in terms of μmoles/g of fresh weight.

Determination of Radioprotection

Experimental design:– One hundred twenty Swiss albino mice were used in the experiment. They were divided into four groups of 30 animals per group. Each group was again divided into five groups of 6 animals per group.

Group I (Normal): Distilled water was given for five consecutive days orally.

Group II (Control): Distilled water was given for five consecutive days orally and whole body irradiated with 4.5 Gy of gamma rays.

Group III: 70% methanolic extracts of *G.lucidum* (*1000* mg/kg) was given orally for five consecutive days and then whole body irradiated with 4.5 Gy of gamma rays.

Group IV: 70% methanolic extracts of *G.lucidum* (*500* mg/kg) was given orally for five consecutive days and then whole body irradiated with 4.5 Gy of gamma rays.

Preparation of liver homogenate

Animals from each of the four groups were sacrificed at 30min, 2hr, 4hr, 8hr and 24hr after the irradiation and the liver was excised, was washed immediately with 0.9% saline, the liver was blotted dry and weighed quickly. It was then homogenized in Tris buffer to yield a 10% homogenate. An aliquot of this was used to assay GSH. The remaining sample was centrifuged at 10,000rpm for 10minutes. The supernatant was collected and used for the assay of lipid peroxidation, protein, catalase, GPx, GST, and SOD. Reduced glutathione in the tissue was

determined according to the method of Moron et al., (1979). Superoxide dismutase activity was determined according to the method of Mc Cord and Fridovich, (1969). Tissue Catalase activity was determined according to the method of Beer and Siezer, (1952). Glutathione peroxidase activity was determined according to the method of Hafemann et al., (1974). Glutathione-s-transferase activity was determined according to the method of Habig et al., (1974). The level of lipid peroxidation was measured as malondialdehyde (MDA) according to the method of Ohkawa et al, (1979). Protein content in the tissue was determined according to the method of Lowry et al., (1951).

RESULTS

Antioxidant capacity of extract – FRAP assay

The ferric reducing antioxidant power of the *G. lucidum* was found to increase in a concentration dependent manner. The extract at a concentration of 0.1%, 0.5%, and 1% showed AEAC values of 0.0678, 0.0378, 0.0199 respectively (Fig.1).

Radical scavenging activity of the extracts – DPPH assay

The methanolic extract of *G. lucidum* at concentration of 0.1%, 0.5%,1% showed AEAC values of 0.557,0.524,0.470respectively (Fig.2).

Inhibitory effect of the extract on free radical generation – Ferrylmyoglobin/ABTS assay

There was a concentration dependent increase in radical scavenging activities concentration of 0.1%, 0.5%,1% showed AEAC values of 0.13,0.15 (Fig.3).

Radical scavenging activity of the extract – Pulse radiolysis assay of ABTS

Pulse radiolysis study of ABTS involves scavenging of primary radicals. *G.lucidum* showed scavenging activity of 4.2µg ascorbic acid equivalent per ml (Fig 4)

Oxygen Radical Absorbance Capacity (ORAC) assay

The ORAC values for the mushroom extracts were determined by spectrofluorimetric assay. The methanolic extract of *G.lucidum* showed oxygen radical absorbance capacity of 38.94±9.0

Radioprotective effects

Radiation caused several deleterious changes at cellular level. Significant radiation induced metabolic changes in the liver of irradiated animal were observed. Radiation caused severe oxidative stress and damage to the antioxidant defense. The *Ganoderma* mushroom extract was found to delimit these changes to a great extent. The effect of the extract was evident from the restoration of the activities of GSH, GPx, GST, SOD, CAT and inhibition of lipid peroxidation

Reduced Glutathione (GSH)

G.lucidum extract administration before irradiation resulted in significant increase in GSH level of liver at 2hr, with a further increase to a maximum at 24hr. Irradiaton produced a significant decrease in GSH from 2hr to 4hr followed by recovery by 24hr. *G.lucidum* extract treatment before irradiation prevented the radiation induced depletion of liver GSH and maintained the values to normal or above normal levels at all observation time. (Fig.5)

Glutathione peroxidase (GPx)

GPx activity increased to a peak value at 24hr in *G.lucidum* extract treated group of animals before irradiation. Radiation caused significant decrease in GPx activity at 30 minutes and at 2hr after which it rose. The activity although below normal, was maintained till 24hr. (Fig.6)

Glutathione- s- transferase

Irradiation caused a significant decrease in GST activity at 2hr but the activity recovered to normal range by 24hr. As in case of other enzymes, *G.lucidum* extract treatment before radiation therapy prevented the radiation induced decrease in GST (Fig.7).

Superoxide dismutase

Irradiation caused a significant decrease in SOD activity, which was evident at 2hr, and dropped to lowest value at 4hr with recovery at later intervals. *G.lucidum* extract pretreatment checked the initial fall of SOD activity and maintained it at normal level thereafter(Fig.8).

Catalase

Irradiation induced a significant decrease in catalase activity at 4hr. *G.lucidum* extract checked the fall of catalase activity and significant increase was maintained at 24 hr. (Fig.9).

Lipid peroxidation

Irradiation resulted in a highly significant increase of lipid peroxidation after 4hr, after which the level dropped significantly . In animals treated with *G.lucidum* extract (500mg/kg) before irradiation also showed a higher activity of lipid peroxidation than normal. However lipid peroxidation was significantly inhibited in group of animals with *G.lucidum* extract at a dose of 1000mg/kg administered before irradiation. (Fig.10).

DISCUSSION

Antioxidants show activities at different levels of protection (Cadenas and Packer, 1996). Although organisms are bestowed with antioxidant and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to prevent the damage totally (Hemnani and Parihar, 1998). Hence antioxidants in diet are of importance as possible protective agents to help human body to reduce oxidative damage. Recently a large number of natural antioxidants have been isolated from different plant materials (Packer and Ong, 1998; Jovanovic and Simic, 2000; John et al., 2002). Mushrooms are functional foods and are traditionally used in folk medicine of several systems of medicine. Medicinal mushrooms possessing antioxidant properties in human diet would be potentially useful to help human body to reduce oxidative damage.

In Chinese medicine fruiting bodies of *Ganoderma lucidum* is considered as a panacea because of its proven ability to treat a number of diseases. The broad-spectrum medicinal property of *G. lucidum* might be due its significant antioxidant activity. Our earlier reports also confirm the significant antioxidant activity of this medicinal mushroom (Jones and Janardhanan, 2000; Sheena *et al* 2003). Non-enzymatic antioxidants react with pro-oxidants and inactivate them. In this redox reaction, antioxidant acts as a reductant. In this context, the antioxidant power can be referred to as reducing ability. In FRAP assay, an easily reducible oxidant Fe III is used in excess. Thus on reduction of Fe III- TPTZ complex by antioxidant, Fe II –TPTZ is formed which can be measured spectrophotometrically at 595 nm

(Benzie and Strain, 1996). The first line of defense is the preventive antioxidants, which suppress the formation of free radicals. In ferryl myoglobin-ABTS assay, on addition of antioxidant, formation of ABTS radical by reaction of ferryl myoglobin and ABTS is delayed and inhibition of formation of the radical is measured as the lag time in seconds (Alzoreky and Nakahara, 2001). In the DPPH assay the ability of antioxidant to scavenge stable purple colored primary radical DPPH is tested by its depolarization spectrophotometrically at 515nm (Aquino et al, 2001). In the pulse radiolysis study, ABTS radicals are generated as 'primary radicals' due to electron bombardment in a linear accelerator. The ORAC values are used as 'standard' measures for comparing antioxidant activity of food materials. Results of various methods of antioxidant activity indicate that *G.lucidum* extract possesses significant antioxidant capacity coupled with free radical scavenging and inhibition of radical generation.

Ionizing radiation is toxic to organisms since it induces deleterious structural changes in essential macromolecules (Navaro et al, 1997). The interaction of ionizing radiation with biological system results in generations of free radicals, H and OH radicals, H_2 and H_2O_2. Radiations induced free radicals in turn impair the antioxidant defense mechanism leading to increased membrane lipid peroxidation, which results in the damage of membrane, bound enzymes (Halliwell et al, 1989). The increased lipid peroxidation is due to the low concentration of GSH. Antioxidant enzymes are among the endogenous system that are available for the removal or detoxification of these free radicals and their products formed by ionizing radiation. The GSH/GST detoxification system is an important part of cellular defense against a large array of endogenously or exogenously formed injurious agents. GSH offers protection against oxygen-derived free radicals and cellular lethality following exposure to ionizing radiation. GST enzymes also possess peroxidase activity and can directly attack the peroxides that may be generated via oxidative reduction recycling, resulting in decreased cytotoxicity.

The present study demonstrates that a significant reduction in GSH and the activities of all antioxidant enzymes in radiation treated group. This could be due to the enhanced utilization of antioxidant defense system in an attempt to detoxify the radicals generated by radiation. In the intact and healthy cells the enzymes are restored immediately after each interaction and GSH is also restored by synthesis (Meister and Anderson, 1983). But in the irradiated animals, the normal synthesis/repair will be disrupted due to damage to DNA and membranes. As a result, restoration will be delayed till the cells are recovered. This could explain the slow recovery in the levels of GSH and antioxidant enzymes after radiation treatment.

The result of present study indicates that methanolic extract of *G.lucidum* inhibits lipid peroxidation in a dose dependent manner.

Radiation causes increase in the levels of superoxide radicals (after 4 hour of radiation). The increased S0D activity after *G.lucidum* treatment was therefore due to the elimination of the superoxide radicals. As a result of this dismutation reaction, highly reactive H_2O_2 are formed. Catalase and GPx are enzymes responsible for degradation of H_2O_2, *G. lucidum* treatment increased the activity of catalase in liver.

GPx is an important scavenger enzyme and has an incorporated selenium molecule to

which it owes its activity (Ursini et al 1995). The treatment of *G.lucidum* also increased the activity of GPx significantly.

The level of GSH was decreased maximally after 2 hours of radiation exposure when compared to normal group of animals. This may be due to the electrophilic burden generated as a result of irradiation. The data clearly indicate that treatment of *G.lucidum* extract increase the level of liver GSH. The increased activity of GPx in the group of animals that were treated with the extract prior to irradiation might be due to the increased level of its substrate GSH.

Since, *G.lucidum* extract showed profound antioxidant activity, radiation-protecting abilities of extract could be attributed to its direct antioxidant or free radical scavenging properties. The radioprotective effect also might be due to the enhanced activity of the hepatic antioxidant defense system. However the present investigation reveal that *G.lucidum* extract possesses significant ability to protect radiation induced oxidative damage. Phytochemical analysis indicates that the predominant constituents of the extract are polysaccharides and triterpenes. Further work on the characterization of the active principle contributing to the radioprotective effect of the mushroom would be of significant use for exploiting its potential in complementary cancer therapy.

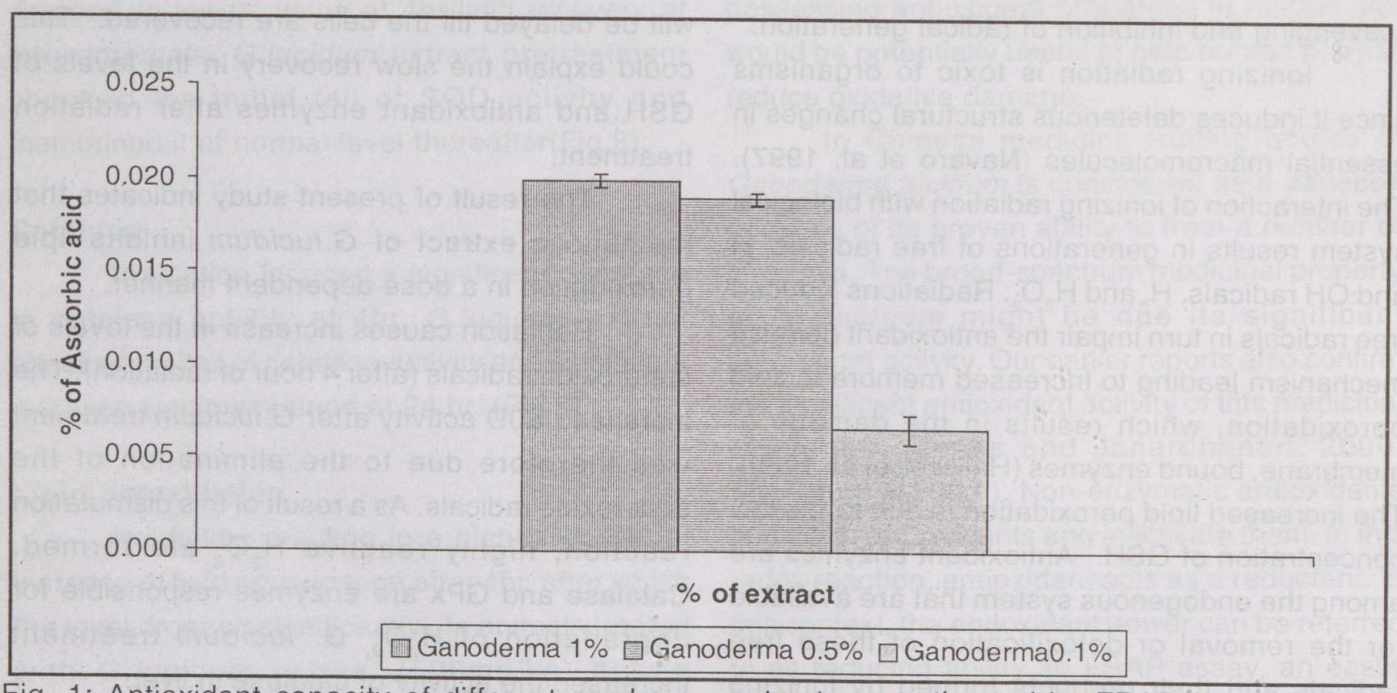

Fig. 1: Antioxidant capacity of different mushroom extracts as estimated by FRAP assay. Figure represents the results for aqueous extracts of, *G. lucidum*. The antioxidant capacities of the aqueous extracts are equivalent to indicated concentrations of ascorbic acid (%). Values are mean ± S.E. from four estimations .

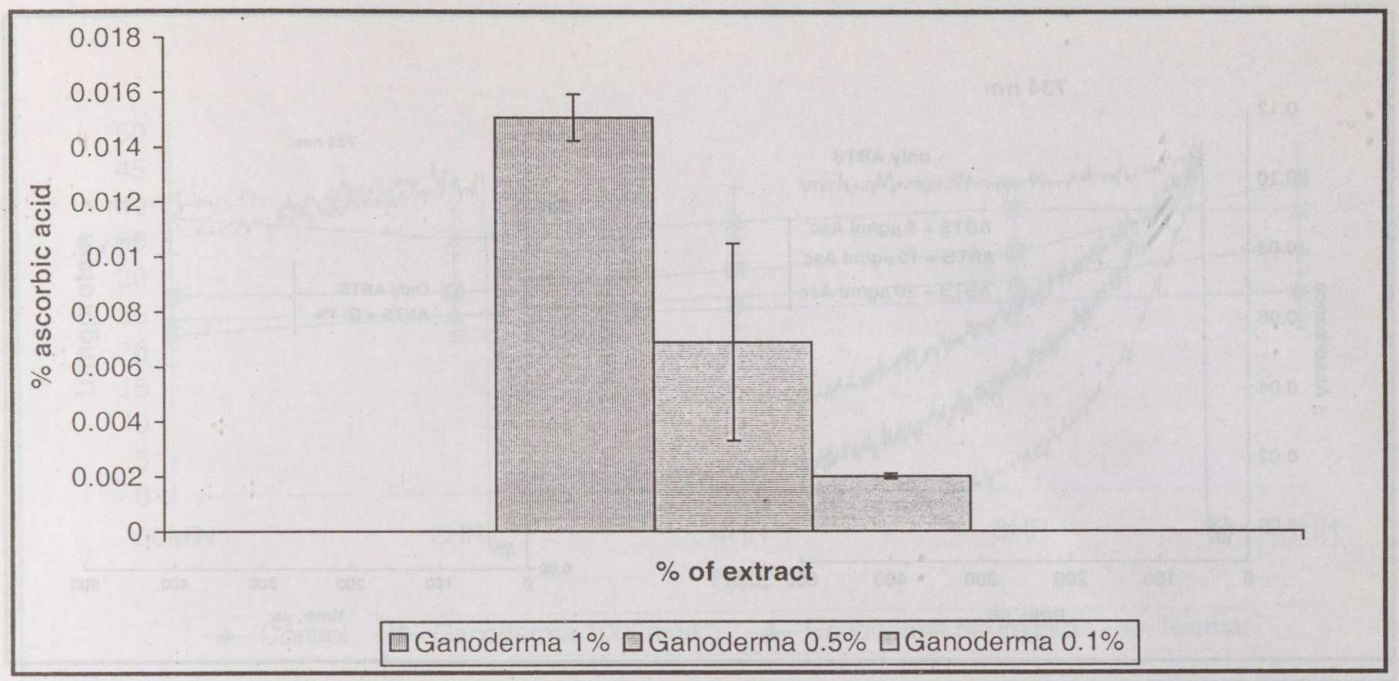

Fig. 2: Antioxidant capacity of different mushroom extracts as estimated by DPPH assay. Fig. represents the results for aqueous extracts of *G. lucidum*. The antioxidant capacities of the aqueous extracts are equivalent to indicated concentrations of water-soluble standard antioxidant ascorbic acid (%). Values are mean ± S.E. from six estimations.

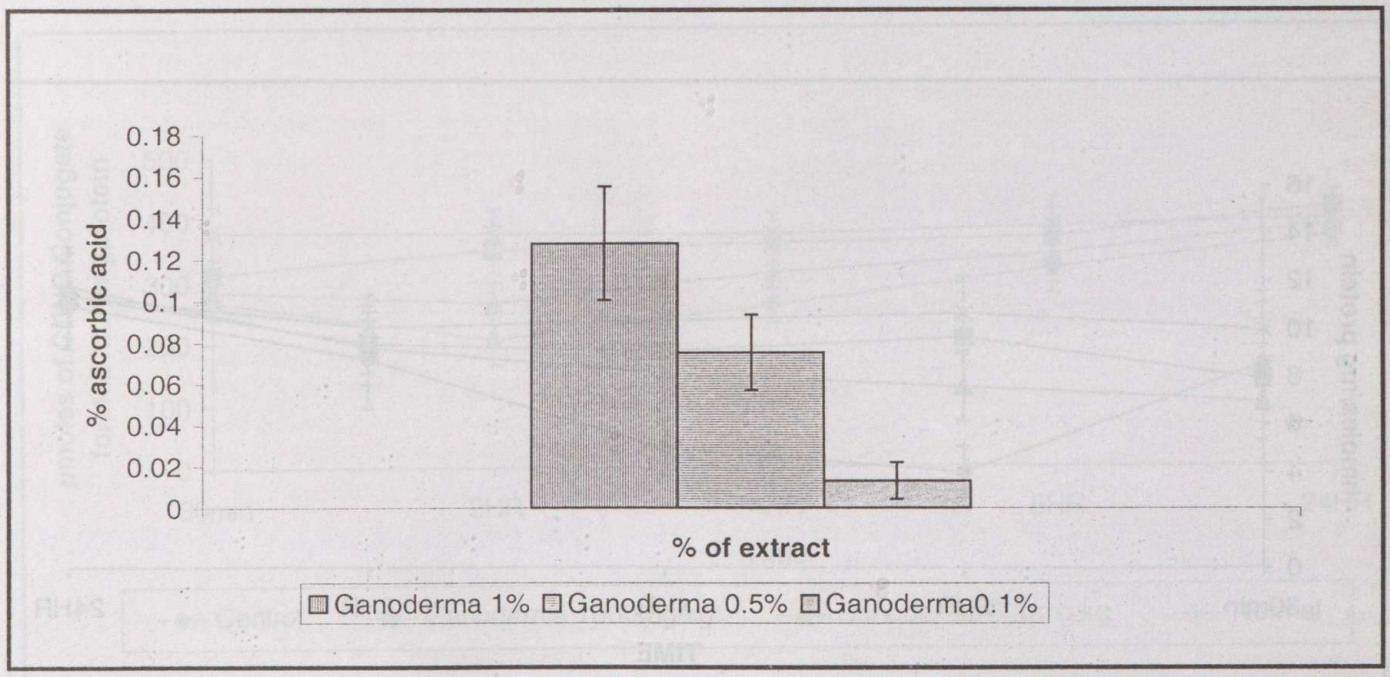

Fig 3: Antioxidant capacity of different mushroom extracts as estimated by using spectrophotometric ferrylmyoglobin/ABTS^{+} method. Figure represents the results for aqueous extracts of *G. lucidum*. equivalent to indicated concentrations of water-soluble standard antioxidant ascorbic acid (%). Values are mean ± S.E. from four estimations each.

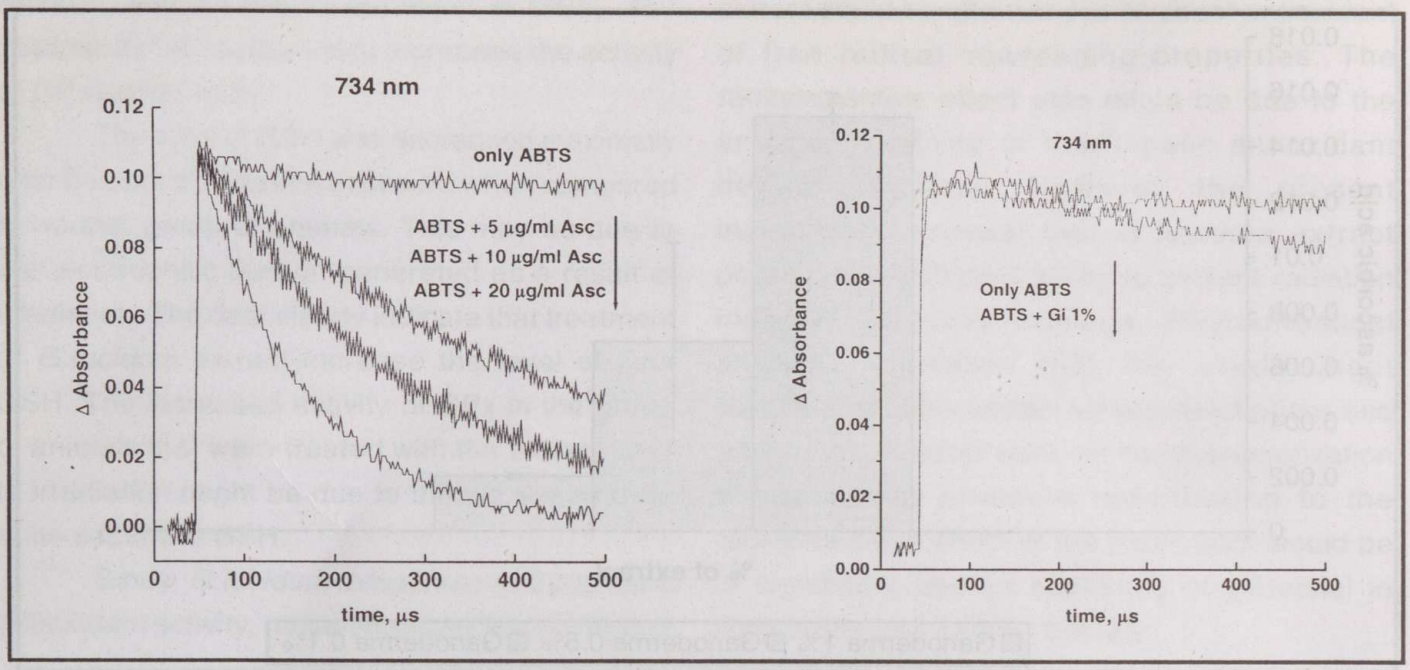

Fig. 4: The antioxidant capacities of the mushroom extracts as determined by pulse radiolysis, by measuring the decay of ABTS·⁻. Fig. 4a represents the decay of in absence (a) and presence of standard antioxidant, ascorbic acid 5 µg/ml (b); 10 µg/ml (c); 20 µg/ml (d). Fig. 4b, shows ABTS radical scavenging patterns with aqueous extracts *G. lucidum* respectively.

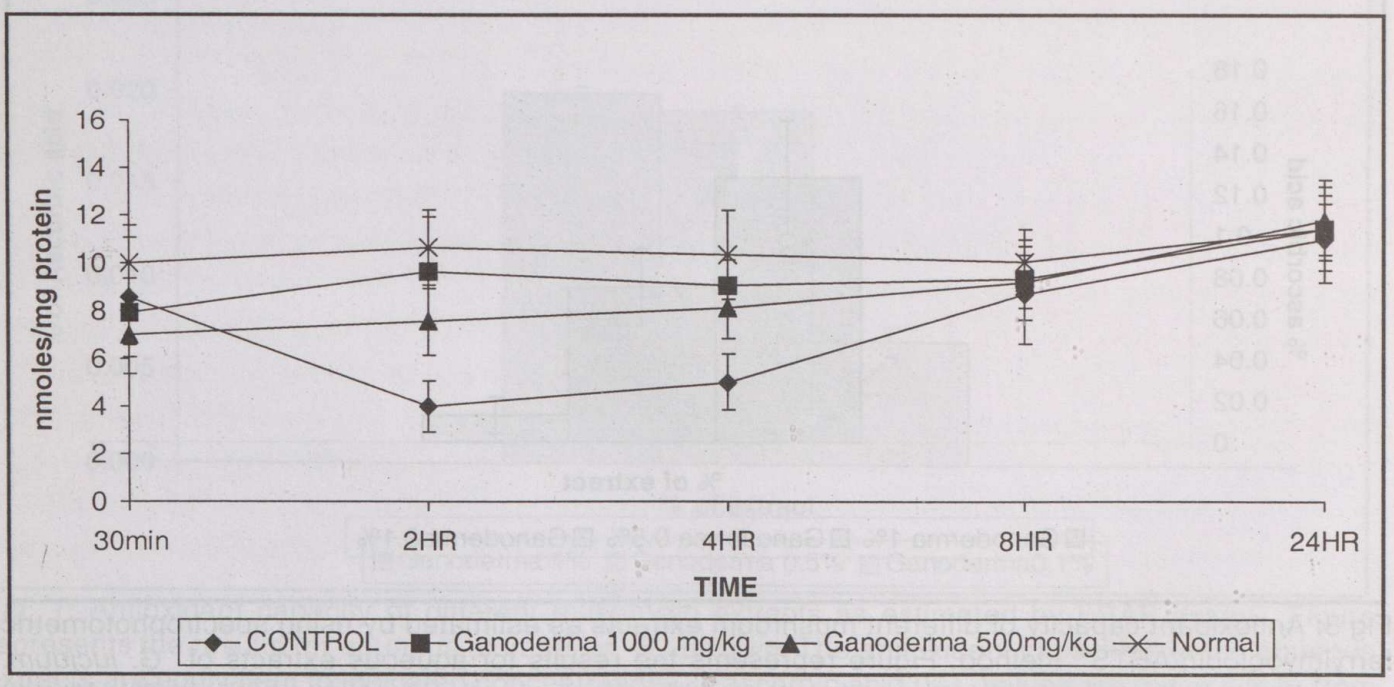

Fig. 5: Effect of *G.lucidum* on tissue glutathione of radiation treated mice.
Values are mean ± SD, n = 6 animals

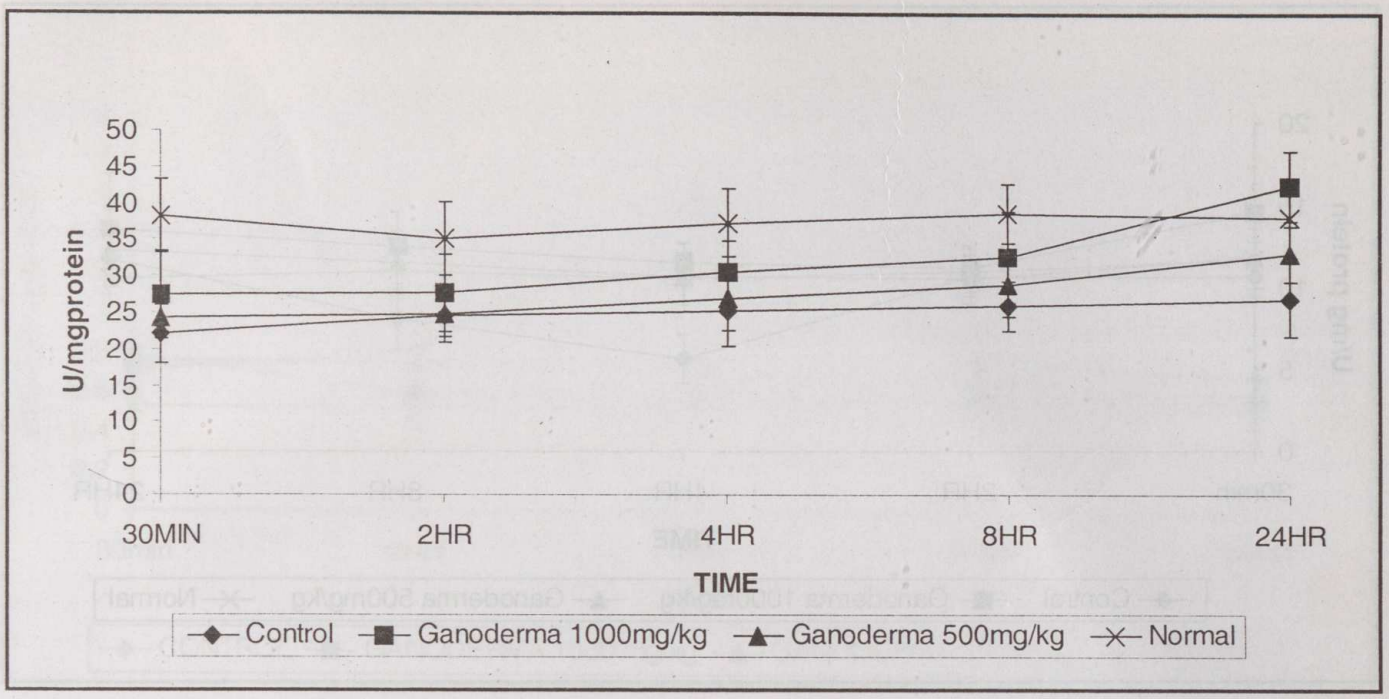

Fig. 6: Effect of *G.lucidum* on tissue GPx of radiation treated mice.
Values are mean ± SD, n=6 animals

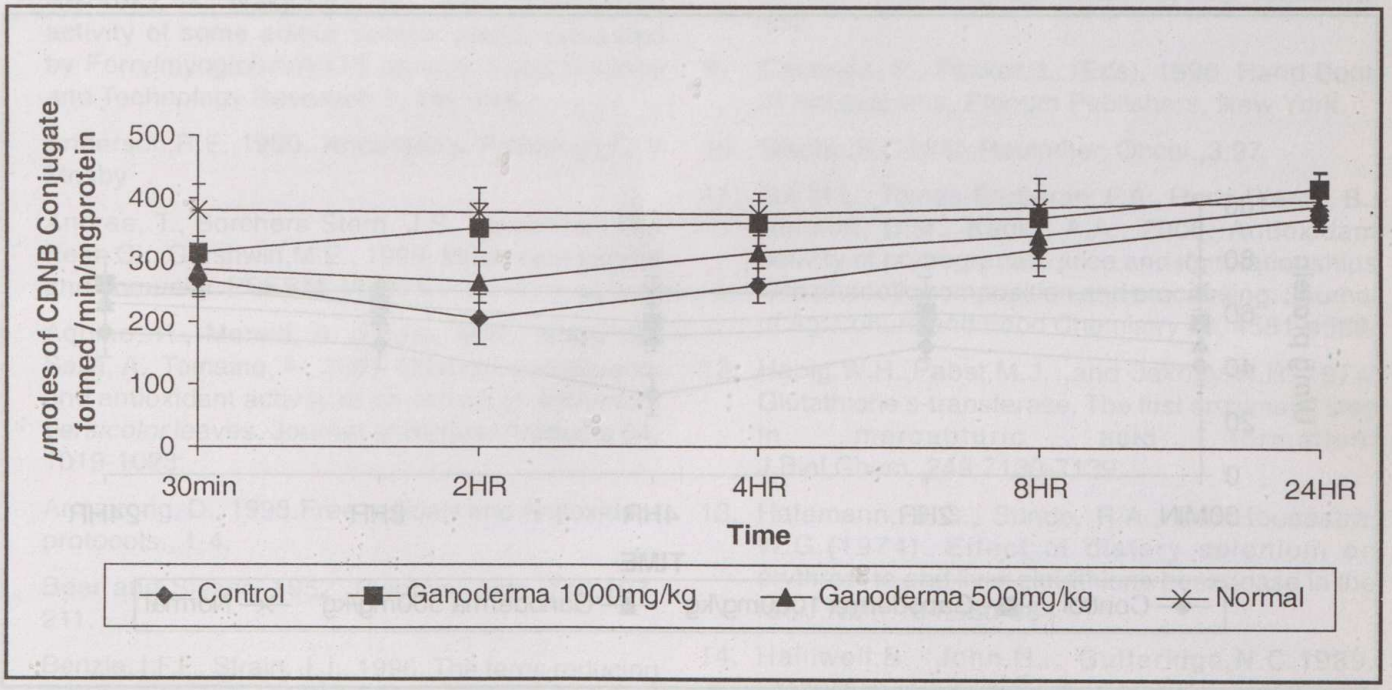

Fig. 7: Effect of *G.lucidum* on tissue GST of radiation treated mice.
Values are mean ± SD, n=6 animals

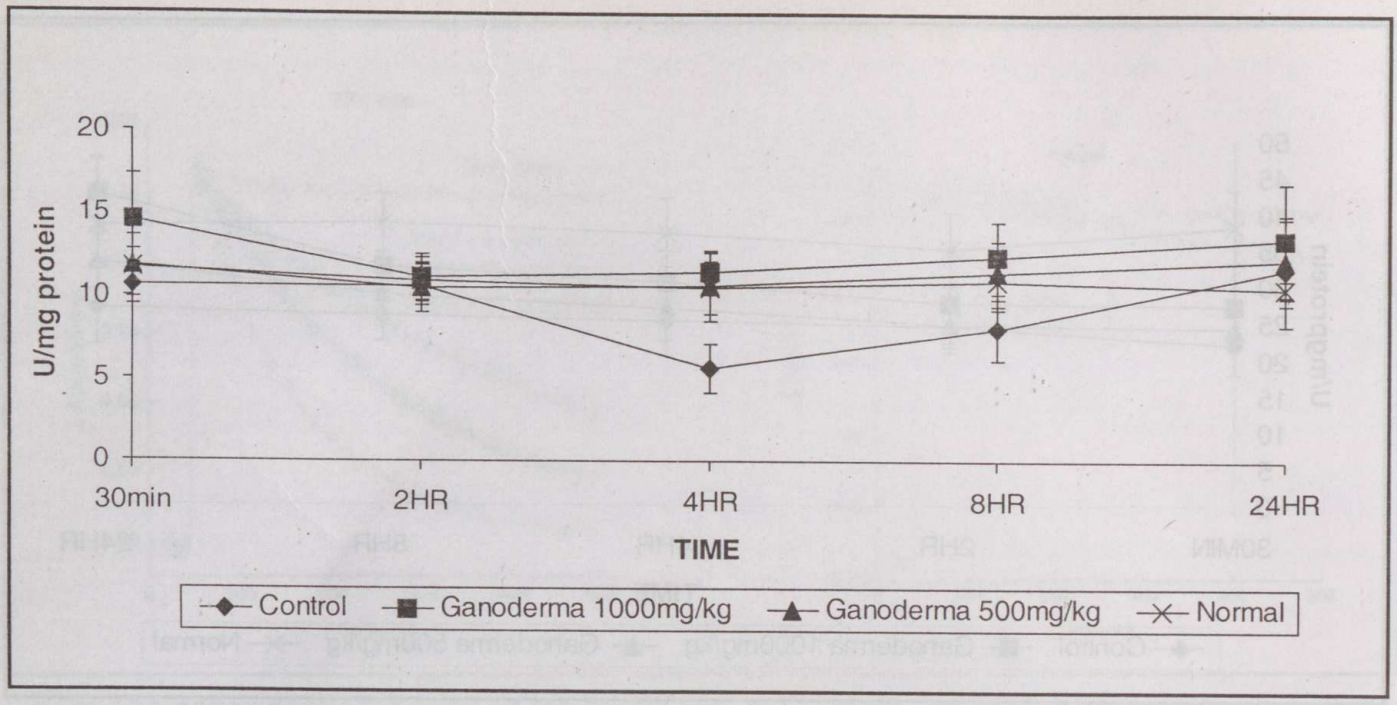

Fig. 8: Effect of *G.lucidum* on tissue SOD of radiation treated mice.
Values are mean ± SD, n=6 animals

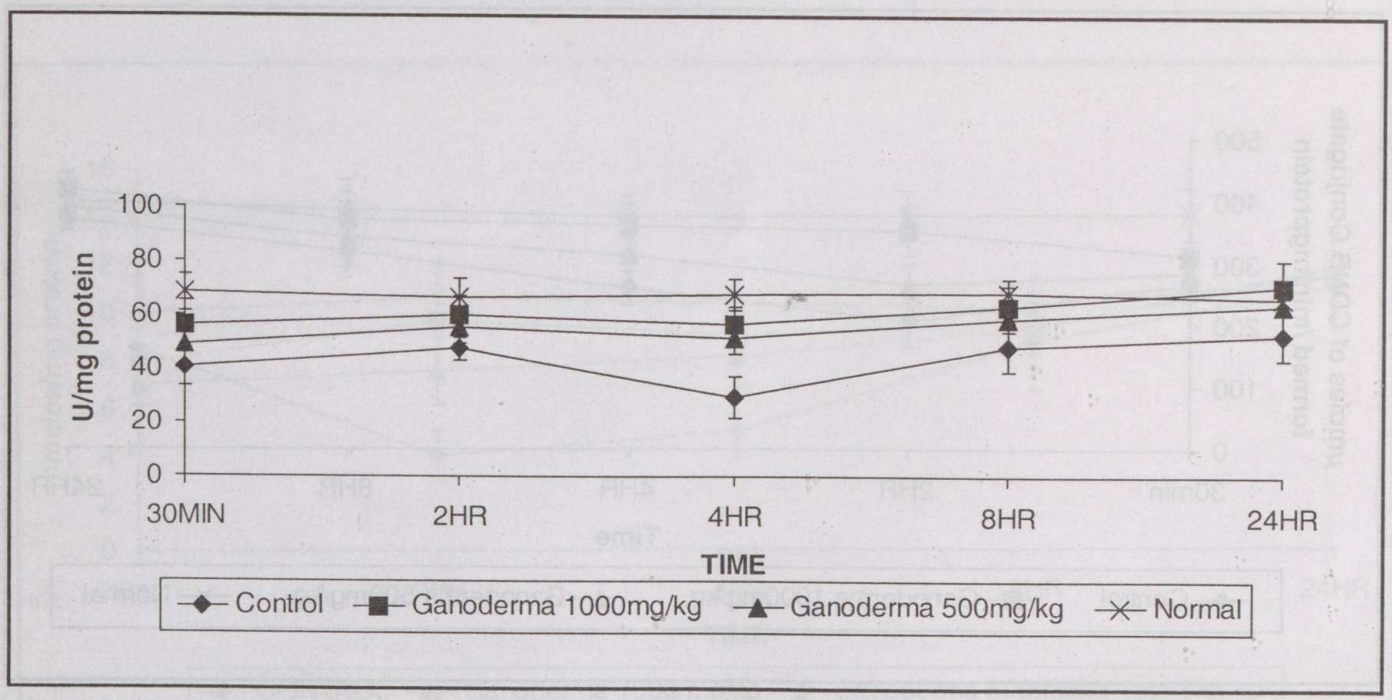

Fig. 9: Effect of *G.lucidum* on tissue CAT of radiation treated mice.
Values are mean ± SD, n=6 animals

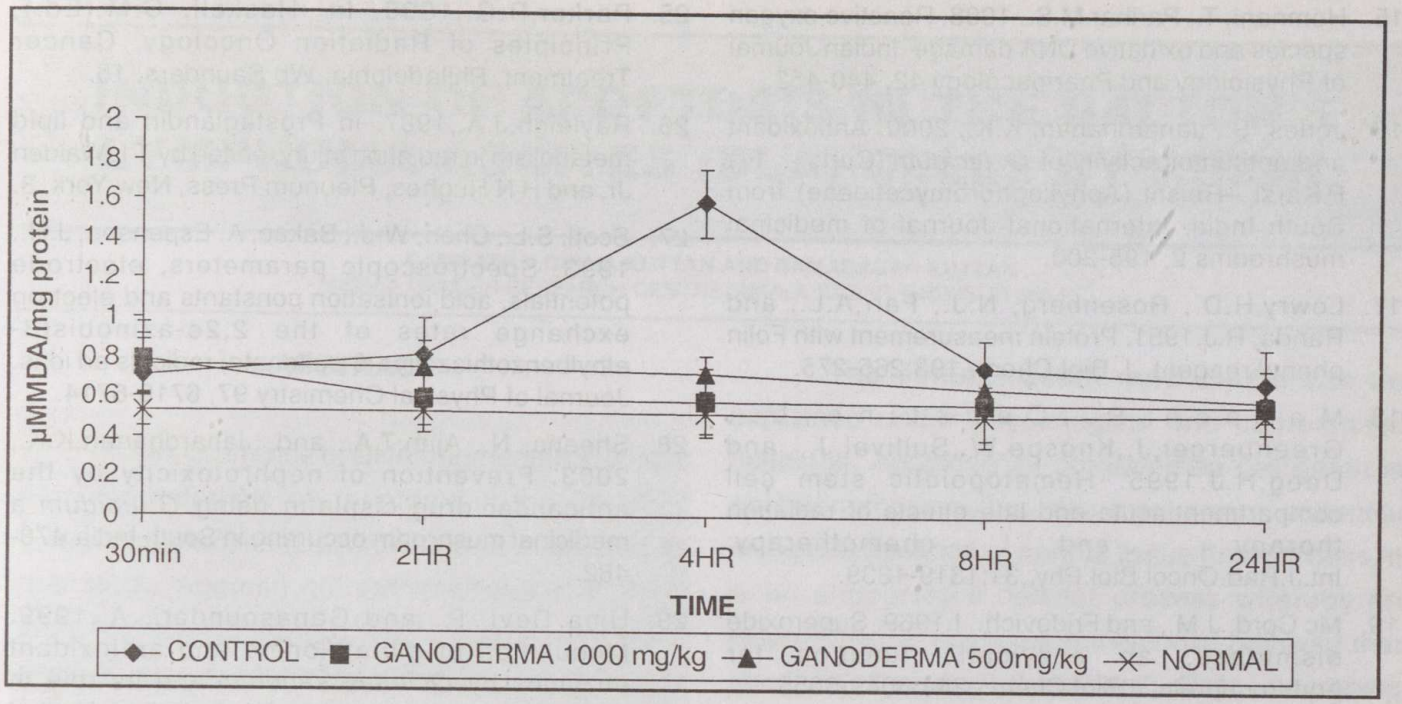

Fig. 10: Effect of *G.lucidum* on tissue lipidperoxidation of radiation treated mice. Values are mean ± SD, n=6 animals

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INDUCTION OF APOPTOSIS IN THE DALTON'S LYMPHOMA ASCITES (DLA) CELLS BY ABRIN

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ABSTRACT

The occurrence of DNA fragmentation which is a characteristic of apoptosis in the Dalton's Lymphoma Ascites (DLA) cells were evaluated in this study. Agarose gel electrophoresis of DNA samples isolated from DLA cells treated with abrin showed fragmentation. Morphological changes associated with apoptosis were assessed by light microscopy in the treated cells. Results suggested that, abrin effectively induced apoptotic changes in the DLA cells that lead to cellular death.

INTRODUCTION

Abrus precatorius Linn which is commonly known as Rosary pea / Jequirity bean belonging to family Leguminoseae and subfamily Papilionoideae has a toxic glycoprotein lectin known as abrin in its seed. By using single step affinity column chromatography this lectin can be isolated from seeds (1). Like ricin, abrin is also a type II ribosome inactivating protein (2). Some of the other documented actions of abrin being galactose specific (3), mitogenic (4), haemagglutinating (5), tumouricidal (6,7) And immunopotentiating (8).

We have earlier reported the *in vivo* tumouricidal property of abrin against DLA and EAC (Ehrlich's Ascites Carcinoma) cells induced solid tumours. It was also found that DLA cells were more sensitive to sub lethal doses of abrin than EAC cell line (9).

The cellular death mechanism can be explained under physiological and pathological headings. Apoptosis or programmed cell death is an autonomous cell suicide mechanism, essential in the maintenance of normal tissue homeostasis. It is an endogenous cellular process whereby an external signal activates a metabolic pathway that results in physiological cell death. Cells undergoing apoptosis in normal and neoplastic cells denote a distinctive sequence of morphological and biochemical changes. Several investigators have proposed that apoptosis inducers are ideal for cancer therapy (10).

The present study was undertaken to evaluate weather the *in vitro* cytotoxic effects of abrin in the Dalton's Lymphoma Ascites cell line of murine origin are mediated via apoptotic or by necrotic pathways.

MATERIALS AND METHODS

Drug preparation

Abrin was isolated from seeds of red variety of Abrus *precatorius* using Sepharose 4B affinity column chromatography and purified (11). The stock solution so obtained, was diluted with Phosphate Buffered Saline (PBS, pH 7.2) to get a concentration of 250ng/ml.

Cell culture

DLA cell line was obtained from Adayar Cancer Institute (Chennai,India)and was propagated

as transplantable ascites tumours in female Swiss albino mice. For this, tumour cells were aspirated from the peritoneal cavity of mouse and one million cells were inoculated to fresh animals every 2 weeks.

DLA cells were aspirated freshly from peritoneum, washed with PBS and concentration of cells was determined. Viability of cells was checked by the dye exclusion method using tryphan blue. Five million live cells were added to 15ml of Dulbecco's minimum essential medium (DMEM) supplemented with 10% foetal calf serum in culture vials. Concentrations of 10 and 25ng of abrin per million DLA cells were utilised for culture in triplicate. A control culture of DLA cells in triplicate was also maintained. After the addition of the drug, 48 hours incubation was allowed. Following these treatments, cells were washed thrice with PBS and tested for viability by using tryphan blue (1%) exclusion method. Cell pellet was separated following centrifugation. To study morphological changes on tumour cells, a small portion of the pellet was resuspended in PBS and cell smear was prepared and stained with haematoxylin and eosin.

Analysis of DNA fragmentation

DNA isolation from freshly collected DLA cells as well as from control and abrin treated DLA cells were performed (12). In brief, washed the cells with Tris borate saline (TBS) and centrifuged to discard the supernatant. Resuspended in Saline EDTA buffer and vortexed. Added 20μl of proteinase K (20mg/ml) and 50μl of 20% SDS and incubated at 50^{0}C for 3 h. To the cooled digested sample added 100μl of 5 M Nacl and mixed. Saturated phenol (pH 7.8) was added in equal volume, mixed thoroughly by inversion. Centrifuged and collected the aqueous phase.

A mixture of saturated phenol: chloroform: isoamyl alcohol (24: 25: 1) was added in equal volume, mixed thoroughly and centrifuged at 4000 rpm for 15 min and the aqueous phase was collected in fresh tube. To this added chloroform: isoamyl alcohol mixture (24:1) and the aqueous phase were reextracted, same as above. To the aqueous phase added 1/10th volume of 3M sodium acetate (pH 5.5) and mixed well. Equal volume of isopropanol was added and centrifuged. Precipitated DNA was recovered, air dried, resuspended in Tris EDTA buffer (pH 8.0) and stored.

Purity and quantification of the DNA was ascertained by looking into the optical densities at 260 and 280 nm. One μl (50ng) of each of samples were resolved at 90V for 2 hours on a 1.8 % agarose gel containing 0.5μg/ml ethidium bromide. A 100 bp ladder DNA sample was used as marker in the lane 1. The resulting DNA fragmentation was noted by U V visualisation.

RESULTS

Cytotoxicity

Viable cells were counted in haemocytometer by using tryphan blue (1%) exclusion method. Abrin treated DLA cells revealed clear evidence of cytotoxic injury, which was dose dependent (table 1) when compared to control cells.

Morphology

Microscopic examination of abrin treated and control DLA cells revealed that the cell death could be with necrotic and apoptotic characteristics. On studying the morphology of cultured DLA cells, necrotic cells were identified as swollen / disintegrating with more eosinophilic cytoplasm and pale but conserved chromatin (fig 1). While apoptotic cells were distinguished as highly condensed / shrunken cell with nuclear elongation, margination, fragmentation and sacculation, cell blebbing and presence of apoptotic bodies (fig 2). Active normal

DLA cells were charectrised by less eosinophilic cytoplasm and nucleus with uniform distribution of chromatin material. The proportions of DLA cells with apoptotic changes were very high and accounted for 570 ± 37 per 10^3 cells counted in the culture containing 10 ng abrin per million DLA cells. However, in the culture having 25 ng abrin, DLA cells with apoptotic changes were 393 ± 23 per 10^3 cells. Control culture cells exhibited 33 ± 4 per 10^3 cells, with apoptotic changes. Since freshly isolated DLA cells were used for primary culture, number of necrotic cells after 48 h incubation was on higher side even in control culture. The proportions of necrotic and apoptotic DLA cells after 48 h incubation vary widely in different culture (table 2).

Assessment of DNA fragmentation

Results of electrophoretic run of DNA samples showed that abrin treated cells exhibited extensive DNA double strand breaks; there by yielding a ladder appearance, while the DNA of control DLA cells exhibited a minimum breakage (lane 3). The DNA isolated from fresh DLA cells did not show any double strand breaks (lane 2). The DNA isolated from DLA cells treated with 10 ng abrin showed more fragmentation (lane 4) than those treated with 25 ng abrin (lane 5) (fig 3). This kind of DNA cleavage is one of the characteristics of apoptosis.

DISCUSSION

Results of the present study suggested that abrin could induce tumour cell death both by physiological and pathological means. Most of the cytotoxic anticancer drugs in current use have been shown to induce apoptosis in susceptible cells (13). Apoptotic cells display DNA fragmentation at internucleosomal sites followed by morphological changes and loss of membrane integrity (14).

This study revealed that the potency of abrin to bring about apoptotic changes decreases with dose, as more cell death through necrotic pathway dominated when 25 ng of abrin was present in the culture in place of 10 ng. Ricin,a type II ribosome inactivating protein is reported to induce apoptosis in target cells by inhibiting protein synthesis (15). It is shown that D-galactose specific lectin from *Viscum album* L, namely ML I brings about apoptosis in peripheral lymphocytes (16).

It could be established that nuclear changes as a part of the apoptosis precedes the loss of membrane integrity there by making tryphan blue impermeable. More than 50% of DLA cells incubated with abrin showed intact cell membrane responding as live cells in the dye exclusion test, but DNA fragmentation could be detected at that time. This finding closely agreed with earlier reports (17,18) suggesting that DNA cleavege of treated cells occur before loss of membrane integrity.

To summarise, the induction of apoptosis in tumour cells that make them more render for host phagocytic clearance without initiating inflammation, could be attributed for abrin's tumouricidal activity.

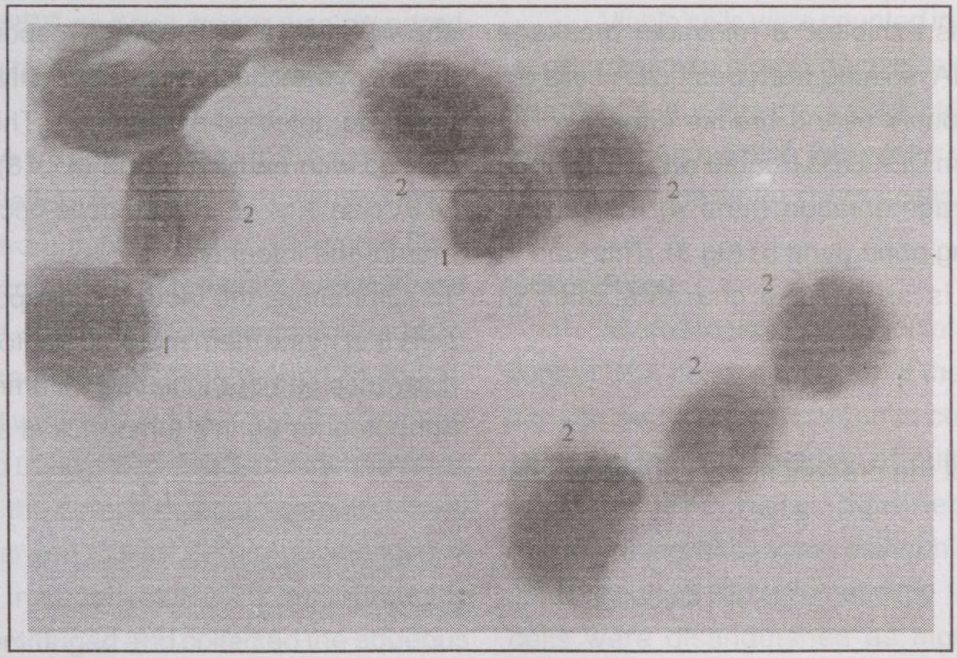
Table 1. Effect of abrin on DLA cell cytotoxicity

Concentration of abrin / 10^6 DLA cells	% Of dead cells after 48 h
0	22 ± 4
10ng	40 ± 5
25ng	53 ± 5

Table 2. Effect of abrin on morphological changes of DLA cells

Concentration of abrin per 10^6 DLA cells	Number of necrotic cells per 10^3 DLA cells.	Number of apoptotic cells per 10^3 DLA cells.
0	234 ± 27	33 ± 4
10ng	280 ± 14	570 ± 37
25ng	331 ± 31	393 ± 23

Fig 1. Morphology of metabolically active normal and apoptotic DLA cells.



Metabolically active normal DLA cells (1) show uniform distribution of chromatin material. Apoptotic DLA cells (2) show vacuolation, elongation, vertical cleavage, margination, and chromatin condensation of nucleus and cell shrinkage.

Fig 2. Morphology of necrotic and apoptotic DLA cells.

Necrotic DLA cell (3) shows enlargement of cell, eosinophilic cytoplasm, intact cell and nuclear membranes. Apoptotic bodies (curved arrows) and blebbing (straight arrow) are other features of DLA cells undergoing apoptosis (2).

Fig 3. DNA fragmentation of DLA cells.

Lane 1- marker DNA (100 bp ladder).
Lane 2- DNA isolated from fresh DLA cells.
Lane 3- DNA isolated from DLA cells of control culture.
Lane 4- DNA isolated from DLA cells cultured with 10 ng abrin.
Lane 5- DNA isolated from DLA cells cultured with 25 ng abrin.

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AMELIORATION OF CYCLOPHOSPHAMIDE INDUCED TOXICITY IN MICE BY AN EXTRACT OF *PHYLLANTHUS AMARUS*

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ABSTRACT

The effect of 75% methanolic extract of *Phyllanthus amarus (P.amarus)* was studied against cyclophosphamide (CTX) induced toxicity in mice. Administration of CTX (25mg/kg b.wt, i.p) for fourteen days produced significant myelosuppression as seen from the decreased WBC count and bone marrow cellularity. Administration of *P.amarus* extract (orally) at doses 250 and 750 mg/kg b.wt significantly reduced the myelosuppression and improved the WBC count, bone marrow cellularity as well as the number of maturing monocytes. Administration of *P.amarus* also increased the cellular glutathione (GSH) and glutathione-S-transferase (GST), thereby decreasing the effect of toxic metabolites of CTX on the cells. *P.amarus* administration was found to decrease the levels of phase I enzyme that metabolites CTX to its toxic side products. Administration of *P.amarus* did not reduce the tumor reducing activity of CTX. Infact, there was a synergistic action of CTX and *P.amarus* in reducing the solid tumors in mice. Results indicated that administration of *P.amarus* can significantly improve the therapeutic efficiency of CTX.

Key words: Chemo protection, cyclophosphamide, *P.amarus*, toxicity, antioxidant

INTRODUCTION

Cyclophosphamide (CTX) is a frequently using alkylating agent and is widely prescribing as anti-neoplastic drug for the treatment of breast cancer, lymphomas, childhood tumors, and many solid tumors (1). The most severe dose-limiting toxicity of CTX is a fulminant cardiac toxicity. Other toxic side effects of CTX are hematopoietic depression, hemorrhagic cystitis, gonadal dysfunction, alopecia, nausea and vomiting (2)

Minimizing the damage to normal tissues caused by chemotherapy has been instigated developments of methods to improve the therapeutic index (3). Several agents like WR-2721 (4), N-acetyl cysteine, mercaptoethane sulfonate (MESNA) (5) and mercaptoproponyl glycine have been tried as chemoprotecting agents but toxicity produced after repeated administration limited their clinical significance.

Recently there is an increase in interest in the search of potential drugs of plant origin that are capable of minimizing the toxicity induced by chemotherapy to normal cells with out compromising its anti-neoplastic activity. Traditional system of Indian medicine extensively uses the plant derived compounds and formulations to modulate the immune system of the host. These herbal formulations were found to be either less toxic or non-toxic. Extracts of garlic (6), *Viscum album* (7) and isolated compounds like curcumin (8) and multi herbal formulations like Septilin (9), Brahma Rasayana were found to have chemo protective activity in experimental animals as well as in patients receiving chemotherapy and radiation therapy (10,11).

Phyllanthus amarus Schum & Thonn, family-

Euphorbiaceae, is used in Ayurvedic system of medicine to combat many liver disorders (12) and is a well known anti-viral agent (13). *P.amarus* elicit a wide spectrum of pharmacological activities (14). It was found to be anti-oxidant (15), anti-diabetic (16), urolytic, antifungal, gastro protective (17), antitumor and suppress the expression of hepatitis B viral mRNA expression in cell lines. Simultaneous administration of *P.amarus* extract has been reported to inhibit hepatocellular carcinoma (HCC) induced by N-nitroso diethyl amine and increased the life span of animals harbouring HCC (18). It has also shown to inhibit carcinogenesis in animals induced by carcinogens such as 20-methyl chlorantherene, dimethyl benzanthracene and N-methyl N-nitrosoguanidine (19). The plant also possess anti mutagenic activity against a variety of known mutagens (20). We had also shown the effect of *P.amarus* as a radio protective agent in mice exposed to lethal dose of ^{3}-radiation (21). The present study was aimed to investigate the use of *P.amarus* as a chemo protective agent against cyclophosphamide and the effect of administration *P.amarus* on solid tumor bearing mice models treated with cyclophosphamide.

MATERIALS AND METHODS

Cyclophosphamide (Ledoxan®, Batch No.2155) was obtained from Dabur India Ltd, New Delhi, Nicotinamide adenine dinucleotide phosphate reduced (NADPH), GSH, 5-5' dithiobis (2-nitrobenzoic acid) (DTNB), and 1- chloro-2, 4-dinitrobenzene (CDNB) were obtained from Sisco Research Laboratories Pvt.Ltd, Mumbai, India. Bovine serum albumin (BSA) was obtained from E-Merck, Germany, Harris haematoxylin was purchased from Qualigens Chemicals, Mumbai. All other chemicals used in the present study were of analytical reagent grade.

Preparation of the plant extract

Aerial parts (stem and leaves) of authenticated *P.amarus* were collected from Thrissur district of Kerala State and were dried at 45^{0}C. A voucher specimen of the plant was identified and kept in the herbarium (voucher no. Eup-9) of Amala Ayurvedic Hospital and Research Centre. Dried parts of *P.amarus* were powdered and extracted (100g) twice with five times (500 ml) volume of 75% methanol by stirring overnight at the room temperature. The solution was then centrifuged at 2500 rpm to separate the supernatant and the supernatant was evaporated to dryness at 50^{0}C using a rotary evaporator under reduced pressure. The yield of the preparation was 7.9%. Portion of the extract was reconstituted in water before the experiment.

Animals

Inbred male BALB/C mice (6-8 weeks) weighing 25-30g were purchased from National Institute of Nutrition, Hyderabad. They were kept in well-ventilated cages under standard conditions temperature pressure and humidity. The animals were provided with normal mouse chow (Sai Durga Feeds, Banglore, India) and water *ad libitum*. All animal experiments conducted during the present study got prior permission and followed the guidelines of Institutional Animal Ethics Committee (IAEC).

Determination of the effect P.amarus on heamatological changes after CTX administration

24 mice were used for this experiment. Animals were randomly divided into four groups, six animals in each group. Group I was kept as normal animals. Group II-IV received CTX at a dose of 25mg /kg b.wt (i.p) every day for fourteen days. *P.amarus* treatment (750 and 250 mg/kg b.wt respectively, p.o)

was started for groups III and IV five days prior to CTX administration and continued for another 30 days. Body weights of all animals were recorded one day prior to CTX administration and every third day after CTX administration.

Blood was collected from the caudal vein into heparinised tubes one day prior to CTX administration and every third day thereafter and following parameters were checked. {a} Total W.B.C count (haemocytometer method) {b} Differential count and {c} Haemoglobin (cyanometh hemoglobin method).

Determination of the effect P.amarus on bone marrow cellularity and α-esterase activity after CTX administration

48 animals were randomly divided into four groups as given above having 12 animals in each group. Treatments for each group were same as described. On 3rd, 9th, 12th, and 30th day after CTX administration three animals from each group was sacrificed by cervical dislocation.

The bone marrow cells from both femurs were flushed into phosphate buffered saline containing 2% bovine calf serum. The number of bone marrow cells was determined using a haemocytometer and expressed as total live cells ($\times 10^6$)/femur. Bone marrow cells from the above preparation were immediately smeared on a clean slide and stained as per the method of Bancroft (22) to determine the presence α-esterase activity, which was expressed as number of positive cells/4000 cells.

Determination of the effect P.amarus on phase I and phase II enzyme levels after CTX administration

Livers from above animals were excised quickly and washed thoroughly with ice-cold saline (0.89%) and kept in –70°C till further analysis. On the day of analysis 10% homogenate of liver tissue was made in ice-cold Tris buffer (0.1 M, pH 7.4) and the homogenate was centrifuged at 4°C at 12000 rpm for 30mts. The supernatant was used in the analysis. Total protein content was estimated by the method of Lowry (23) using bovine serum albumin as the standard. The activity of phase I enzyme aniline hydroxylase activity was estimated by the modified method of Mazel (24). The activity of phase II enzyme GST activity was assayed by the method of Habig et al (25) and activity calculated using molar extinction co-efficient of the product, which was 9.6 $nM^{-1}cm^{-1}$. GSH levels were assayed by the method of Moron et al (26) using DTNB and intensity of yellow colour formed was measured at 412 nm using a spectrophotometer.

Effect of administration of P.amarus on solid tumor reduction in animals treated with CTX

Another set of 42 mice were randomly divided into six groups with seven animals in each group.

Group I	Control (untreated)
Group II	CTX alone
Group III	CTX + *P.amarus* 750 mg/kg b.wt p.o
Group IV	CTX + *P.amarus* 250 mg/kg b.wt p.o
Group V	*P.amarus* 750 mg/kg b.wt p.o
Group VI	*P.amarus* 250 mg/kg b.wt p.o

Dalton's lymphoma ascites (DLA) cells (1E 10^6 cells/ animal) were injected subcutaneously to the right hind limb of each animal. Treatment of *P.amarus* and CTX were started on the same day. CTX was administered at a dose of 10mg/kg b.wt (i.p) for fourteen days and *P.amarus* administration continued for another 30 days. (This lowered dose was used for the experiment to get only 50%reduction of tumor volume) The radii of developing tumor were measured using vernier calipers for 30 days and tumor volume was

calculated using the formula $V= 4/3\pi r_1^2 r_2$, were r_1 and r_2 denotes the radii of tumor at two different planes.

STATISTICAL ANALYSIS

Data was expressed as mean ± standard deviation (SD). Significance levels for comparison of differences were determined using Student's t-test and p value ≤0.05 was considered significant.

RESULTS

There was no significant change in the body weight of animals treated with CTX and CTX along with *P.amarus* treatment (data not shown)

Effect of P.amarus extract on heamatological parameters of CTX administered animals.

CTX administration significantly reduced the total WBC count in mice (Figure 1). Myelosuppression as seen from the WBC count was observed through out the period of CTX administration. But day 12 onwards *P.amarus* treated animals showed an increase in their WBC levels. On day 18 the CTX alone treated group showed a count of 3525 ± 294 while it was 5866 ± 265 in animals treated with CTX and *P.amarus* at a concentration of 750 mg/Kg b.wt indicating that the levels of W.B.C was significantly elevated after *P.amarus* treatment. Differential count and haemoglobin content did not show any significant variation (data not shown) in both treated and untreated animals.

Figure 2 shows the effect *P.amarus* administration on the bone marrow cellularity levels. Animals had a significantly lowered the bone marrow cellularity during CTX administration. But the continued administration of *P.amarus* increased the levels of bone marrow cellularity. On day 12 bone marrow cellularity was 6.63 ± 0.64 x10^6 cells for CTX alone treated animals where as animals treated with *P.amarus* (750 mg/kg b.wt) had an increased value of 11.16 ± 1.85 ($p \leq 0.005$) clearly indicating that bone marrow cell proliferation is significantly increased after *P.amarus* treatment.

The effect *P.amarus* treatment on the α-esterase activity of maturing monocytes is given in table 1. Levels of α-esterase positive cells were significantly decreased after CTX administration. *P.amarus* treatment elevated the number of α-esterase positive cells. On day 12 *P.amarus* treated group had 587.66 ± 18.61 ($p \leq 0.005$) positive cells/ 4000 cells while CTX alone administered group had only 205 ± 31.22 positive cells.

Effect of P.amarus extract on the levels of phase I and phase II enzymes in CTX treated animals

We have also studied the effect of *P.amarus* extract in phase I and phase II enzymes of animals treated with CTX. Phase I enzymes are involved in the activation of CTX where as phase II enzymes produces the detoxification of CTX derived metabolites. The effect of *P.amarus* administration on the levels of phase I enzyme aniline hydroxylase is shown in table 2.Administration of CTX elevated the levels of the enzyme significantly. *P.amarus* treatment lowered the levels of aniline hydroxylase as evident from lowered enzyme activity. On day 12 activity of *P.amarus* (750mg/kg.wt) treated group was 0.168 ± 0.016 ($p \leq 0.005$) while CTX alone treated group showed a value of 0.208 ± 0.001.
CTX administration was also found to decrease the levels of GSH (Table 3). Continuous administration of *P.amarus* elevated the levels of GSH in treated groups. On day 12 CTX alone treated animals had lowered GSH level of 6.69 ± 1.18 nmole/ml as compared to the GSH level of 14.40 ± 1.31 in *P.amarus* (750mg/kg b.wt) treated animals ($p \leq 0.005$).

The level of the phase II enzyme GST is shown in table 4. On day 12 animals administered with *P.amarus* showed a significant activity of 467.97 ±17.91 ($p \leq 0.005$) while CTX alone treated animals showed an activity of 298.60 ± 21.39 indicating that GST activity was elevated after *P.amarus* administration.

Effect of P.amarus on solid tumor reduction in animals treated with CTX

The effect of *P.*amarus on solid tumor reduction in animals treated with CTX is given in figure 3.The administered dose of CTX was found to be effective in controlling tumor growth. The administration of *P.amarus* did not have any inhibitory effect on CTX mediated tumor reduction. On the other hand *P.amarus* administration produced a synergistic effect on tumor growth inhibition. *P.amarus* administered at dose of 750mg/kg b.wt on CTX treated animals had lowest tumor volume among all the groups.

DISCUSSION

Alkylating agents were among the first compounds identified to be useful in cancer chemotherapy. All the alkylating agents have a common property of dissociating a positive charged, electrophilic alkyl group capable of attacking negatively charged electron rich, nucleophilic sites on most of the biological molecules. The chemotherapeutic usefulness derives from their ability to form a variety of DNA adducts that sufficiently alter DNA structure or function or both so as to have a cytotoxic effect. Many of them undergo a very complex activation process before it can generate reactive intermediates. Initial activation reaction of CTX carried out by microsomal oxidation system in liver produces 4-hydroxy CTX, a cytotoxic metabolite, which diffuses from hepatocytes into plasma and distributed throughout the body.4-hydroxy CTX is then further converted to some other cytotoxic metabolites and acrolein and phosphoramide mustard are among them (27,28). Phosphoramide mustard is known to cause myelosuppression. In the present study the myelosuppression caused by CTX is effectively prevented by *P.amarus*. The *P.amarus* treated animals also showed enhanced levels of bone marrow cellularity, which indicate that *P.amarus* stimulate the heamatopoetic system and this observation is further supported by the increased number of α-esterase positive cells, a marker of maturing monocytes.

Presently *P.amarus* was found to suppress the activity of phase I enzyme aniline hydroxylase. Phase I enzymes play a role in the activation of CTX. *P.amarus* was also found to significantly elevate the activity of phase II enzyme GST and levels of the one of the major cellular nonenzymatic antioxidant GSH, which involved in the detoxification of toxic electrophilic xenobiotics, hydrogen peroxide and free radicals. The metabolism of CTX in the body produces highly reactive electrophiles and the decreased value of GSH in CTX treated group is probably due to the electrophilic burden on the cells and also due to the formation of acrolein, which is known to deplete GSH content and DNA alkylation (29). Treatment with *P.amarus* reduces the electrophilic burden and thereby increases GSH levels. Elevated GSH levels in the cells further conjugate with the electrophiles and decreased their toxicity. The liver cytosolic detoxifying enzyme GST is also involved in the removal of toxic metabolites (30,31,32). GST level has been shown to increased by *P.amarus* treatment making cells more effective with respect to detoxification of toxic metabolites. *P.amarus* has been shown to be a good antioxidant *in vitro* as well as *in vivo* and a powerful scavenger

of oxygen radicals.

The solid tumor model clearly demonstrated that *P.amarus* does not interfere the anti tumor potential of CTX, on the other hand administration of *P.amarus* on CTX treated animals had a synergistic action on the inhibition of tumor growth. *P.amarus* contain several active ingredients. Presence of variety of tannins, several lignans like phyllanthin and hypophyllanthin, polyphenols, flavinoids such as quercetin, astragalin and some ellgitannins like catechin and epigallocatechin were isolated from *P.amarus* (33). Many plant phenolic compounds show excellent antioxidant activities. Although the exact mechanism of action *P.amarus* is not clear, the combined action of extract is manifested as a sum total of interactions between different ingredients. The degree of chemo protection will depend on the interactions of the ingredients singly or collectively with the cytotoxic agents.

In conclusion *P.amarus* exert its chemo protection by detoxification of CTX derived toxic metabolites, enhancing the recovery and repair process, accelerating the hematopoietic recovery and down regulating the activity of phase I enzyme and up regulating the activity of phase II enzymes and GSH. Moreover scavenging the free radicals or reactive metabolites by *P.amarus* with out affecting the antitumor efficacy of CTX would be beneficial to the host as well as will enhance the efficiency of the treatment. *P.amarus* has been found to inhibit the proliferation of cancer cells during the process of carcinogenesis. *P.amarus* extract was also found to produce significant protection against radiation. Hence *P.amarus* could act both as a chemo protector, tumour growth inhibiter and as an efficient radio protector. A detailed clinical study to fully exploit the potential of *P.amarus* in cancer is highly warranted.

Table 1: Effect of *P.amarus* treatment on the number of α - esterase positive cells of CTX administered animals

Group	3rd Day	9th day	12th day	30th day
Normal	941.00 ± 20.66	948.66 ± 24.78	916.66 ± 25.16	965.66 ± 29.14
CTX alone	258.33 ± 17.55	189.00 ± 11.53	205.00 ± 31.22	637.00 ± 33.51
CTX + *P.amarus* 750 mg/kg b.wt	483.00 ± 20.95*	527.66 ± 18.61*	593.33 ± 16.50*	878.66 ± 22.27*
CTX + *P.amarus* 250 mg/kg b.wt	410.66 ± 19.21*	451.66 ± 24.66*	469.33 ± 23.15*	843.33 ± 42.24*

* $p<0.005$ as compared with CTX alone group.
Values are expressed as positive cells/4000cells

Table 2: Effect of *P.amarus* treatment on the aniline hydroxylase levels of CTX administered animals

Group	3rd Day	9th day	12th day	30th day
Normal	0.220 ± 0.02	0.211 ± 0.01	0.216 ± 0.01	0.195 ± 0.02
CTX alone	0.266 ± 0.10	0.240 ± 0.02	0.208 ± 0.006	0.240 ± 0.10
CTX + *P.amarus* 750 mg/kg b.wt	0.188 ± 0.01*	0.218 ± 0.004*	0.168 ± 0.02*	0.195 ± 0.20
CTX + *P.amarus* 250 mg/kg b.wt	0.182 ± 0.01*	0.213 ± 0.01*	0.182 ± 0.02**	0.208 ± 0.003

* p≤0.005,** p≤0.01 as compared with CTX alone group.
Values are expressed as μmoles/min/mg protein

Table 3 : Effect of *P.amarus* treatment on the liver GSH levels of CTX administered animals

Group	3rd Day	9th day	12th day	30th day
Normal	9.23 ± 1.12	9.47 ± 0.57	11.56 ± 1.45	8.11 ± 0.96
CTX alone	6.21 ± 0.62	6.61 ± 1.57	6.69 ± 1.18	10.70 ± 1.51
CTX + *P.amarus* 750 mg/kg b.wt	14.91 ± 1.96*	18.86 ± 1.71*	14.40 ± 1.31*	12.02 ± 3.20
CTX + *P.amarus* 250 mg/kg b.wt	12.65 ± 1.32*	14.62 ± 1.52*	13.70 ± 1.12*	10.46 ± 1.29

* p≤0.005 as compared with CTX alone group
Values are expressed as nmol/mg protein

Table 4 : Effect of *P.amarus* treatment on the liver GST levels of CTX administered animals

Group	3rd Day	9th day	12th day	30th day
Normal	243.85 ± 18.21	212.43 ± 24.10	282.05 ± 27.89	249.23 ± 46.48
CTX alone	339.25 ± 15.34	324.49 ± 21.40	298.60 ± 21.39	309.95 ± 30.04
CTX + *P.amarus* 750 mg/kg b.wt	459.17 ± 37.95	543.53 ± 37.15*	467.97 ± 17.31*	289.46 ± 26.36
CTX + *P.amarus* 250 mg/kg b.wt	315.73 ± 40.52	419.78 ± 20.09	360.28 ± 20.57**	263.21 ± 6.25

*p≤0.005,** p≤0.01 as compared with CTX alone group

Values are expressed as nmoles of CDNB-GSH conjugate formed/min/mg protein

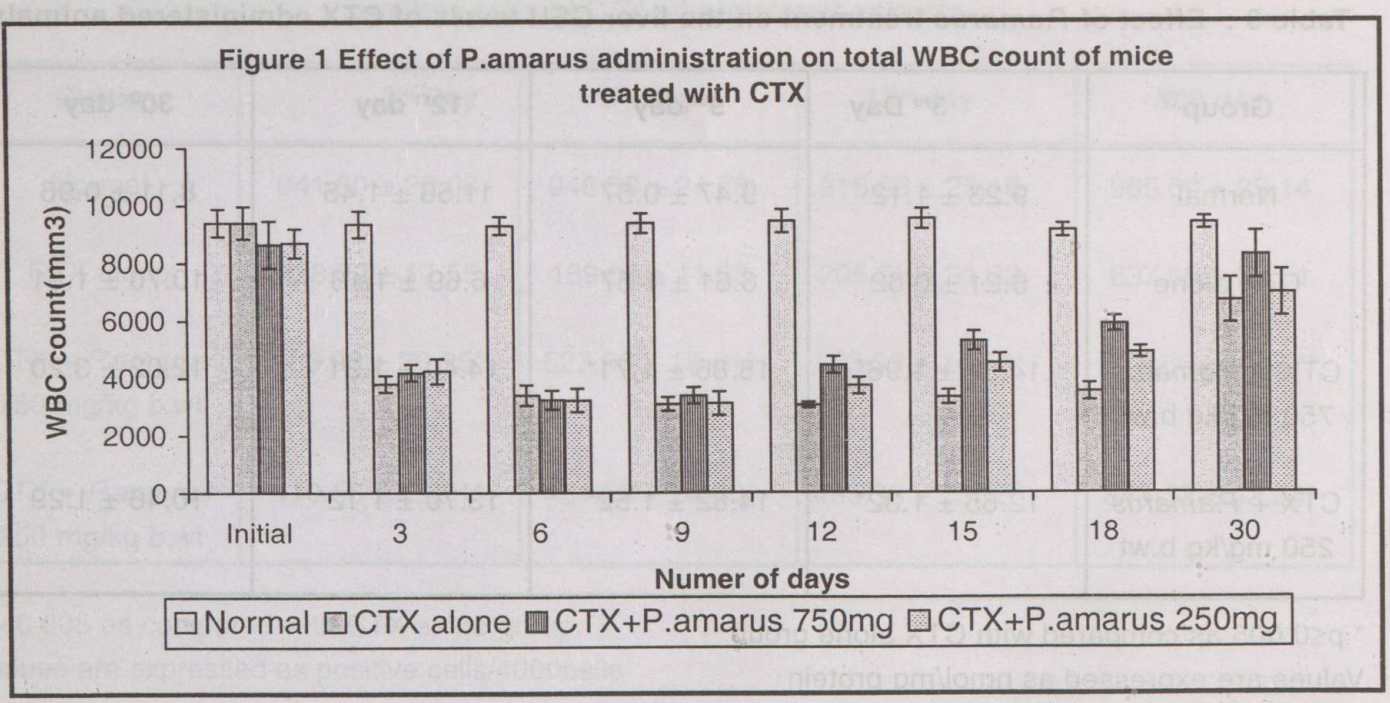


Figure 2 Effect of P.amarus administration on the bone marrow cellularity of CTX treated mice

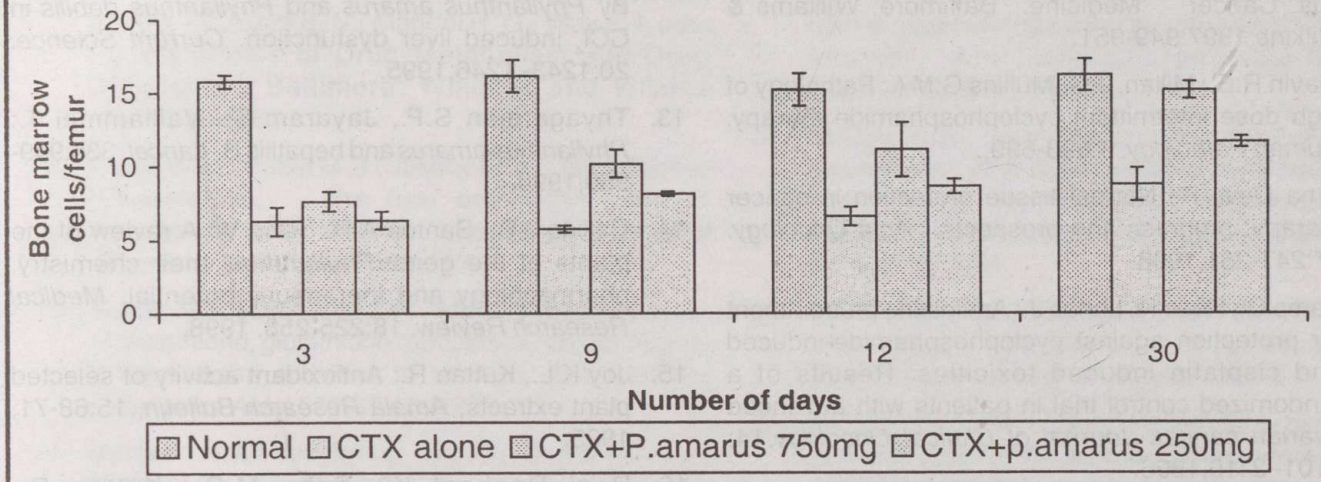
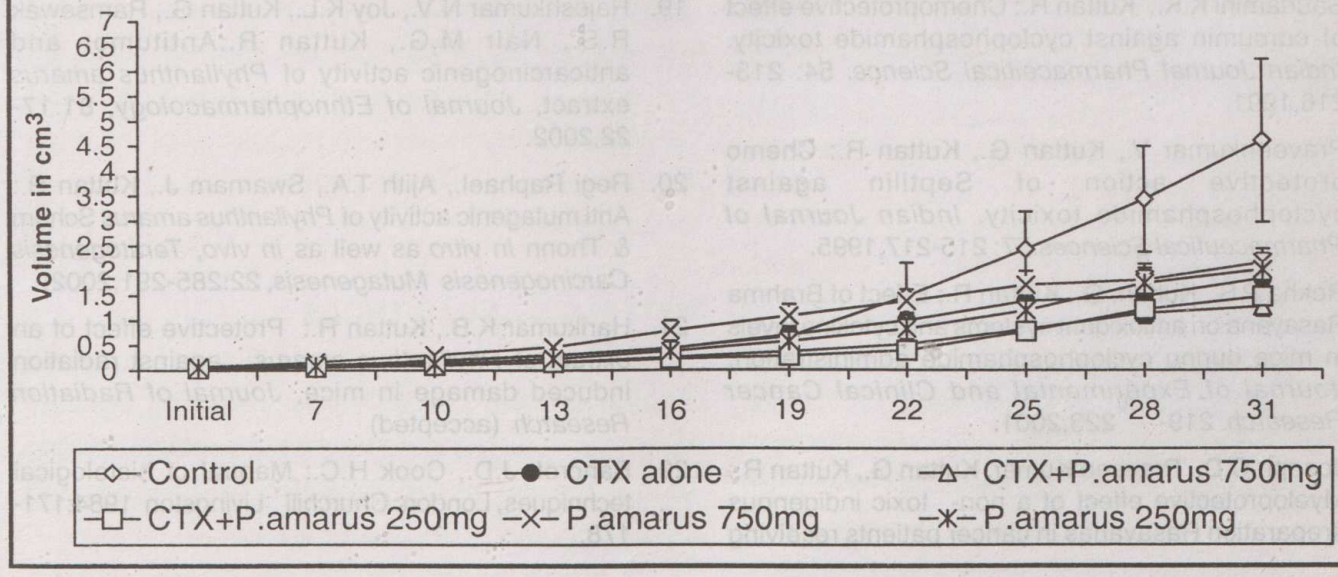


Figure 3 Effect of P.amarus administration on solid tumor treated with CTX in mice



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IMMUNOMODULATORY ACTIVITY AND TOXICITY STUDY OF NCV I AND AC II – DRUGS USEFUL AGAINST HUMAN IMMUNODEFICIENCY VIRUS

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ABSTRACT

The immunomodulatory activity of NCV I and AC II were studied in BALB/c mice. Administration of NCV I and AC II were found to increase the total white blood cells count in BALB/c mice. The maximum total WBC count in NCV I treated animals was 13875 ± 519 cells/mm^3 (percent increase 50%) and that for AC II was 13200 ± 829 cells/ mm^3 (percent increase 48%) on the 18th day. Administration of NCV I and AC II increased the bone marrow cellularity to 22.63 ± 0.513 x 10^6 cells/ femur and 21.2 ± 0.529 x 10^6 cells/femur as compared to normal which was 6909 ± 177. The number of α-esterase positive cells increased in NCV I and AC II treated groups from 720 ± 80 cells/ 4000 ells to 1299 ± 83 cells/4000 cells and 1297 ± 127 cels/4000 cells respectively. These results indicating its potentiating effect on the immune system.

INTRODUCTION

The human immunodeficiency virus kills the helper cells of the immune system. These cells, which are a part of the cell-mediated immune system, play a crucial role in cell-mediated immunity. This may cause immunosuppression in the body. As a result, the body offers a weak resistance to bacterial, viral and parasitical infections. There are several herbal preparations used in the indigenous system of medicines, which can enhance the body's immune status. NCV I and AC II were formulated at Amala Ayurvedic Research Centre which contain the plant materials with known immunostimulating activity such as *Tinospora cordifolia* (1) *Withania somnifera* (2) etc.

Using this preparation we have been treating HIV positive individuals (symptomatic and non-symptomatic patients) for the last 10 years. We have observed that in the case of symptomatic patients, administration of the drugs releaves the symptoms in these patients (3) and in the case of unsymptomatic patients the drugs delays the onset of symptoms. Drug did not have any effect in full blown AIDS patients. All these studies were done in patients and no attempt so far made to experimentally prove the efficacy of these drugs.

In the present study using animal models we have shown the activity of the drug in improving the immune status in the animals as seen from the haematology and bone marrow cellularity. We have done preliminary studies on the toxicity of the drug. We have also studied the toxicity of these drugs to these animals.

MATERIALS AND METHODS

BALB/c male mice (6-8 weeks), were purchased from Small Animal Breeding Station, Mannuthy. Animals were housed in ventilated cages and were given mouse chow (Sai Durga feeds and foods, Bangalore) and water ad libitum.

Harris haematoxylin was purchased from

Glaxo India Ltd., Bombay. Pararosaniline hydrochloride and α-nephthyl acetate were obtained from Loba Chennai, Mumbai. Alkaline phosphatase and Bilirubin kits were obtained from Span Diagnostics Ltd. (Surat) India. SGPT reagent kit was brought from CML Biotech (P) Ltd., Ernakulam, Kerala. Creatinine and Urea in serum was determined by commercially available kit from Biolab Diagnostics, Cochin.

All other chemicals used in these experiments were analytical regent grade.

All animal experiments were conducted according to the rules of Animal Ethics Commtitee, Govt.of India.

Preparation of extract

NCV I and AC II were purchased from Amala Ayurvedic Research Centre. 10 g of NCV I or AC II was stirred overnight in 500 ml distilled water. Filtrate was evaporated to dryness under vacuum at 50°C-55°C using a rotary evaporator under reduced pressure. The yield of the preparation of NCV I was 4.9 gm while that of AC II was 3.5 gm.

Determination of haematological parameters

BALB/C male mice (6-8 weeks) were used for the experiment. In each set of the experiment (NCV I and AC II) animals were divided into 3 groups (6 animals/group).

Group I	Normal
Group II	1g/kg b.wt.
Group III	250 mg/kg b.wt.

Group II and III were given 1g/kg b.wt. and 250 mg/kg b.wt. NCV I or ACII for 15 days orally. Blood was collected from the caudal vein into heparinised tubes one day prior to drug administration and continued every third day for 30 days. Following parameters were checked (a) body weight, (b) total WBC count (haemocytometer method) [4] (c) haemoglobin (cyanomethhemoglobin method) (d) differential count.

Determination of bone marrow cellularity

BALB/C mice (6-8 weeks old) were used for this experiment. For both the drugs NCVI and AC II, animals were divided into two groups (15 animals/group).

Group I	1 g/kg b.wt.
Group II	250 mg/kg b.wt.

Group I and II were given 1 g/kg b.wt. and 250 mg/kg b.wt. NCV I or AC II for 15 days orally. One day prior to drug administration and 8th, 16th, 24th and 30th day animals were sacrificed. Bone marrow was collected from both femurs into PBS containing 2% bovine calf serum and made into a single cell suspension. The number of cells were counted using a haemocytometer and expressed as total live cells ($x10^6$)/ femur [5,6]

From the above bone marrow preparation smears were made on clean glass slides and stained according to Bancroft's method [7] in order to determine α-esterase activity.

Effect of NCV I and AC II on liver function enzyme activity and renal function enzyme activity

One day prior to drug administration and 8th, 16th, 24th and 30th days, the blood was collected by heart puncture into vials and serum was separated and following parameters were checked in serum. Alkaline phosphatase, activity was checked by King and Armstrong's method [8,9]. SGPT activity in serum was done according to DNPH method [9].

Urea (Diacetyl monoxime method) and creatinine (Picrate Jaffe method) in serum were estimated using commercially available kit from Biolab Diagnostics, Cochin. Total Bilirubin was estimated by Malloy and Evelyn's method [10].

Thymus and spleen were excised from the animals, washed thoroughly in ice cold saline and weights were determined.

STATISTICAL ANALYSIS

Data was expressed as mean ± standard deviation (SD). Significance levels for comparison of differences were determined using student's 't' test.

RESULTS

Effect of NCV I and AC II on haematological parameters

Administration of NCV I and AC II increased total WBC count in normal BALB/C mice. Administration of these drugs showed a steady increase in the WBC count and when the drug is withdrawn there was a reduction in the WBC count. The maximum WBC count obtained in the NCV I treated animals was 13875 ± 829 on the 18th day (Figure 1). When compared to the normal animals haemoglobin content did not show a significant change in NCV I and AC II treated animals (Figure 2). Administration of both the drugs did not produce any change on lymphocyte-neutrophil ratio (data not shown). Body weight of animals did not show any significant change (Table 1).

Effect of NCV I and AC II on bone marrow cellularity and α-esterase activity

The effect of NCV I and AC II administration on the bone marrow cellularity and α-esterase positive cells is given in the Figure 3 and 4. Administration of NCV I and AC II increased bone marrow cellularity to 22.63 ± 0.513 x 10^6 cells/femur and 21.2 ± 0.529 x 10^6 cells/femur respectively while in normal group it was only 13.83 ± 0.832 x 10^6 cells/femur. The number of α-esterase positive cells is increased from 720 ± 80 to 1299 cells/4000 cells by NCV I and that of AC II was 1297 ± 127 cells/4000 cells.

Effect of NCV I and AC II on organ weight and liver function and renal function test.

Administration of both NCV I and AC II do not make any significant change in the weight of spleen and thymus.

There was no significant change in the activity of liver function enzymes such as ALP and SGPT and levels of total bilirubin indicating that these drugs did not have any influence in hepatofunction. Similarly change in the levels of urea and creatinine after drug administration was not significant indicating renal function are unchanged after drug administration.

DISCUSSION

Immunosuppression is the major set back in diseases like cancer, AIDS and in certain autoimmune diseases. So there is an increase in demand for compounds, which can stimulate the immune system. Synthetic immunomodulators have the side effects namely fever, neutropenia, anorexia, elevated transaminases and protein urea (11). Immunomodulators with minimal side effects upon continuous administration is of great use in clinical medicine. Use of plant products as immunomodulators is still in a developing stage.

Immunomodulators are agents that interefere with the actions of the immune system. They can be divided into three main groups;

immunosuppressive agents, immunostimulating agents and others immunomodulators. Haemopoietic growth factors, interleukin, interferons, tumour necrosis factors are immunomodulators. Immunomodulators have biphasic effect; some tend to stimulate immune system which are low, while some inhibit host defense parameters which are normal or already activated (12).

Haemopoietic growth factors were originally termed colony stimulating factors (CSFs) because they stimulate the formation of colonies of cells derived from individual bone marrow progenitors. They show a mutual connection, which is related to the hierarchy of haematopoiesis (13).

AIDS is the biggest challenge to medical sciences during this century. It has been shown that the HIV mainly affects CD4 lymphocytes. This produces immunodeficiency in the body. A similar condition like HIV/AIDS has been known in Ayurvedic system, which is known as Ojakshaya. Medicines for the Ojakshaya improve the metabolic process (agni). On the basis of the known literature we have formulated a preparation to counteract the Ojakshaya seen in HIV/AIDS (14).

The present study analysed the immunomodulatory effect of NCV I and AC II in BALB/C mice. The effect of NCV I and AC II on total WBC count indicates that they can stimulate the haematopoietic system. This observation is further supported by the increased levels of bone marrow cells and α-esterase positive cells. α-esterase positive cells is a marker of maturing monocytes.

Liver function enzyme activity and renal function test indicates that these NCV I and AC II were nontoxic drugs.

In conclusion both NCV I and AC II are nontoxic drugs with immunomodulatory activity. Further studies have to be conducted in order to find out the exact mechanism of action of these compounds in the stimulation of the immune system.

Table I : Effect of AC II and NCV I administration on Body Weight of treated mice

Group	Day 0	Day 30
Normal	26.8	31.65
AC II 1 g/kg b.wt.	27.3	29.2
NCV I 1 g/kg b.wt.	28.16	32.2

Table 2 : Effect of AC II and NCV I on Hepatic and renal function

Group	SALP		SGPT		Bilirubin		Urea		Creatinine	
	Day 0	Day 16	Day 0	Day 16	Day 0	Day 16	Day 0	Day 16	Day 0	Day 16
NCV I 1 g/kg b.wt.	15.04 ± 2.06	13.86 ± 1.69	60.93 ± 9.92	58.22 ± 4.41	0.792 ± 0.16	0.711 ± 0.137	39.96 ± 4.96	42.86 ± 7.24	0.653 ± 0.299	0.588 ± 0.392
AC II 1 g/kg b.wt.	14.14 ± 1.38	15.6± 0.518	54± 6.21	58.12 ± 8.28	0.517 ± 0.352	0.703 ± 0.189	37.53 ± 5.31	44.26 ± 3.06	0.588 ± 0.196	0.588 ± 0.196

Figure 1: Effect of AC II and NCV I administration 7on total WBC count of treated mice

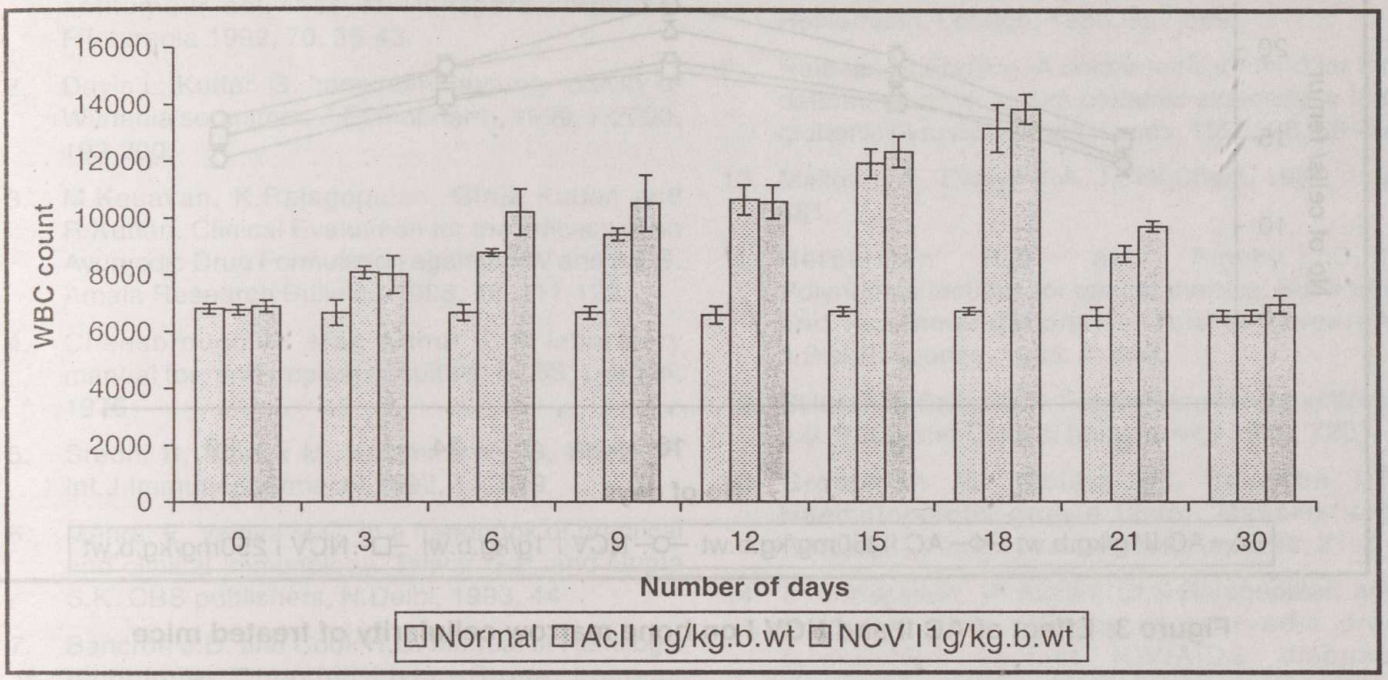
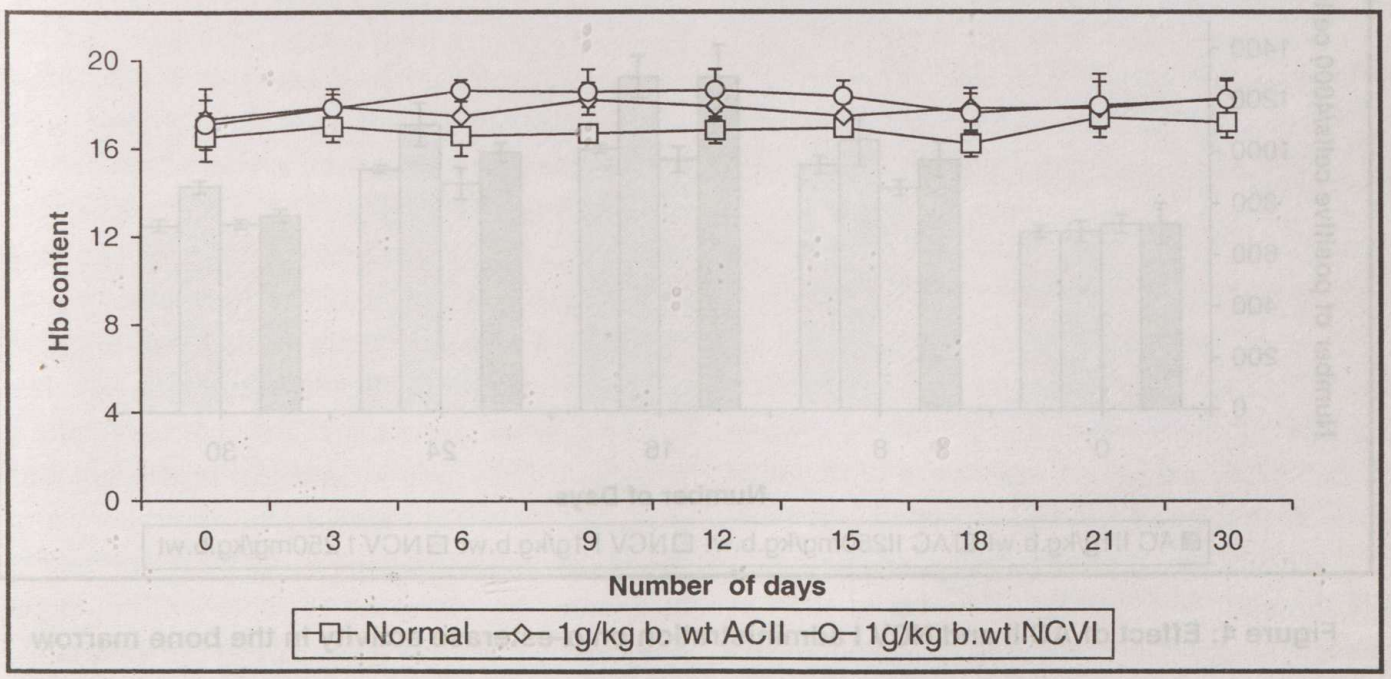


Figure 2: Effect of AC II and NCV I administration on Hb content in mice



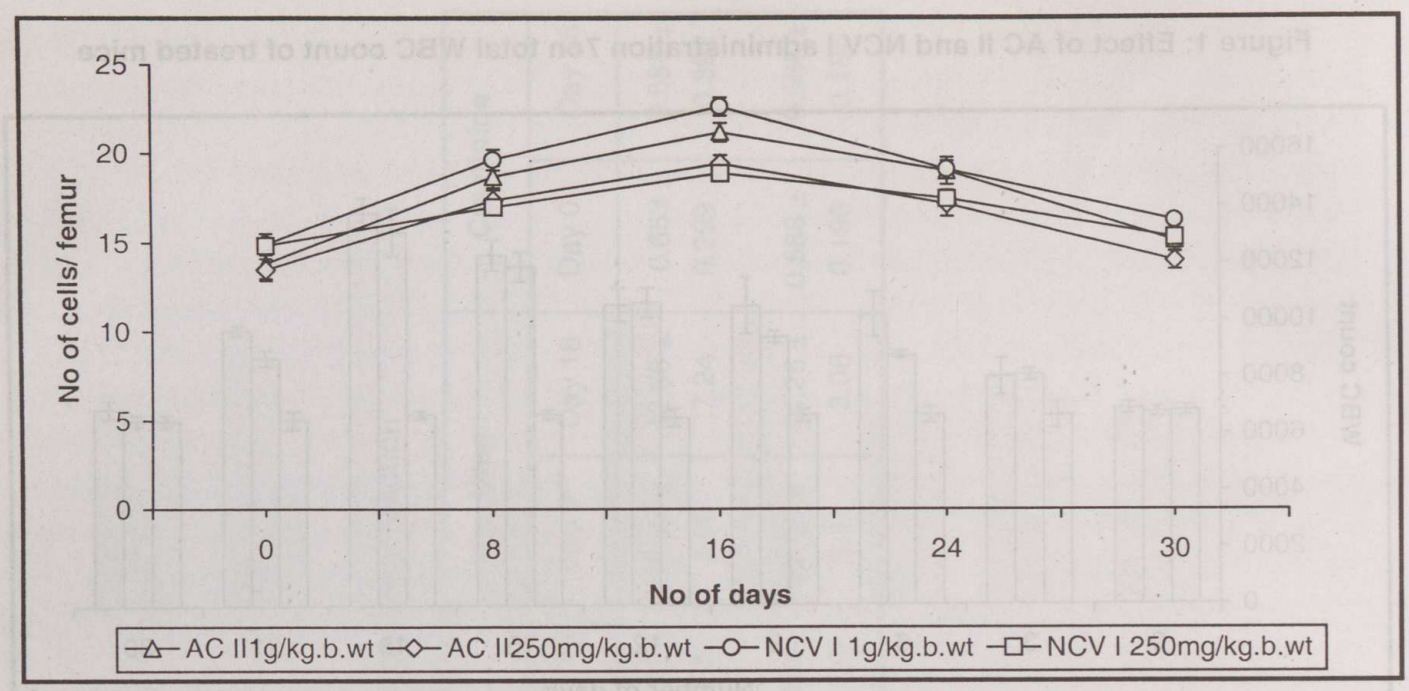


Figure 3: Effect of AC II and NCV I on bone marrow cellularity of treated mice

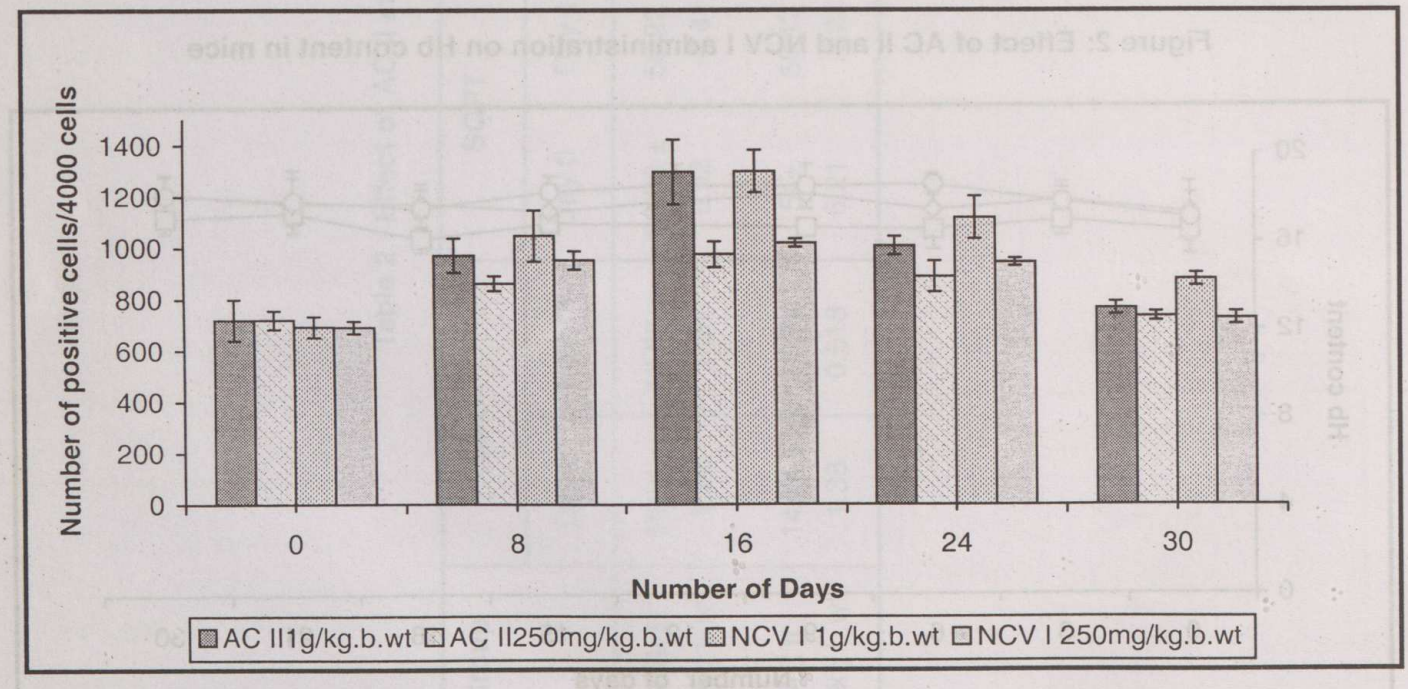


Figure 4: Effect of AC II and NCV I administration on α-esterase activity in the bone marrow

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ANTI-CLASTOGENIC ACTIVITY OF BETEL NUT EXTRACT AGAINST RADIATION INDUCED DAMAGE IN MICE

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ABSTRACT

Betel nut chewing has been reported to be one of the agents that is responsible for the induction of submucous fibrosis. Carcinogenic potential of tobacco is well known, role of betel nut in the induction of carcinogenicity is not documented beyond doubt. We have shown earlier that betel nut extract could reverse the mutagenicity induced in *Salmonella typhimurium*. In the present study, *in vivo* chromosomal aberrations and micronuclei tests were employed to assess the reversal of radiation-induced genotoxicity by betel nut extract. Administration of 0.5 g/kg b.wt. and 2.5 g/kg b.wt. of betel nut extract reduced the frequency of chromosomal aberrations ($p < 0.05$) and micronuclei formation ($p < 0.001$) induced in mouse bone marrow cells by radiation treatment. This result confirms the anticlastogenic activity of betel nut extract.

INTRODUCTION

Exposure to ionizing radiation may induce mutation, carcinogenesis, physiological disorders and lethality. Free radicals and reactive oxygen species produced by the interaction of radiation with water has been directly associated with these biological effects. Ionizing radiation either directly acts through the breakage of bonds of DNA or indirectly through ions and free radicals bringing about chromosomal changes (1). Micronuclei, an indicator of chromosomal damage is caused by exposure to genotoxic or carcinogenic agents predict the sensitivity to radiotherapy (2).

There have been contradictory reports on the carcinogenicity of betel nut. Extract as well as the isolated compound such as arecoline (3,4), arecaidine and their nitrosocompounds have been reported to produce DNA strand breaks in cultured cells (5). Betel nut chewing has been shown to produce submucous fibrosis leading to oral cancer. However epidemiological data on subjects using only betel nut(without tobacco) are scanty. On the otherhand, betel nut has active ingredients, which have been shown to prevent cancer. Catechin and epicatechin, which are known to be chemopreventive, are present at a very high concentration in betel nut extract (6,7). We have earlier reported that betel nut extract could reverse the mutagenicity induced to *Salmonella typhimurium* by direct acting mutagens as well as mutagens needing activation. It was also found to inhibit the mutagenicity induced by tobacco extract to the bacteria. Moreover extract was found to inhibit the urinary mutagenic induced by tobacco and benzo[*a*]pyrene in rats (8). In the present study, we show that chromosomal aberrations and micronuclei in mouse bone marrow cells, produced in animals by exposure to radiation, could be reduced by administration of betel nut extract.

MATERIALS AND METHODS

Tender betel nuts were collected from a private property. May-Grunwald and Giemsa were purchased from Hi-Media Laboratories, Bombay. Colchicin was obtained from Sisco Research Labroatories, Bombay. All other reagents used were of analytical grade.

Animals

Inbred strains of male Swiss albino mice (4-6 weeks old, weighing 18-20g) were purchased from Veterinary College, Mannuthy. They were housed in ventilated cages and fed with normal mouse chow (Sai Durga Foods and Feeds, India) and water *ad libitum.*

Radiation

Whole-body irradiation was given using Cobalt-60 teletherapy unit. Animals were kept in specially constructed restraining boxes with a capacity of holding seven mice and irradiated by Gamma rays.

Preparation of aqueous extract of tender betel nuts

Kernel of tender betel nuts was cut into small pieces. 100 g of betel nut kernels were boiled with 500 ml distilled water for 1 hr. Supernatant was evaporated to dryness under vaccum at 50°C and was diluted with water and used for the experiment.

Effect of betel nut extract on radiation induced micronuclei formation

In bred male Swiss albino mice (4-6 weeks, 18-20 g) were divided into 4 groups (4 mice/ group). The I group was kept as untreated normals. Group II was kept as irradiated control group. Group III and IV were fed with two different concentrations (0.5 g/kg b.wt. and 2.5 g/kg b.wt.) of betel nut extract for 5 consecutive days. All control and treated animals were received single exposure of whole body radiation (150 rads/ mouse) before the last dose of drug treatment. All animals were sacrificed 24 h after the radiation by cervical dislocation and both femurs were removed and bones were freed from muscles. The proximal ends of the femurs were carefully shortened with scissors until a small opening to the marrow canal became visible. Approximately 5 ml PBS and 100ul serum (FCS) were aspirated into a disposable syringe and the needle was inserted a few millimeters into the bone marrow canal. Bone marrow was flushed into a centrifuge tube containing FCS and mixed gently. Tubes were centrifuged at 1000 rpm for 10 min. The cell button was collected and smears were made. The air dried smears were stained using May-Grunwald Giemsa (undiluted) for 3min, followed by diluted May Grunwald in distilled water (1:1) for 2min and finally with diluted Giemsa (1:6 distilled water) for 10min. The slides were rinsed in distilled water, air dried and mounted in DPX. The slides were screened for 2000 polychromatic erythrocytes and corresponding normochromatic erythrocytes and also for the presence of micronuclei(9).

Effect of betel nut extract on radiation induced chromosomal aberrations

In bred male Swiss albino mice (4-6 weeks, 18-20g) were classified into 4 groups of 4 animals. Group I served as untreated normal. Group II was kept as irradiated control. Group III and IV were treated with 2 doses of betel nut extract (2.5 g/kg b.wt. and 0.5 g/kg b.wt.) orally. Before the last dose of drug administration all the control and treated groups received a single exposure of whole body radiation (300 rads/mouse). Colchicin (2 mg/kg b.wt., i.p) was injected 90 min. before the animals were sacrificed. 48 h after irradiation animals were killed by cervical dislocation and bone marrow were analysed for chromosome abnormalities (10,11). For this proximal ends of the femurs were

shortened with scissors to visualize the bone marrow canal. 0.5 ml of phosphate buffered saline (PBS) was aspirated into a disposable syringe and the needle was inserted into the bone marrow canal. The bone marrow was flushed out into a centrifuge tube containing PBS and mixed thoroughly.

Tubes were centrifuged at 1000 rpm for 10 min. The cell button was collected, mixed with a hyptonic solution of 0.075 M KCl prewarmed at 37°C and incubated for 20 min. at 37°C. The tubes were centrifuged and cell buttons were mixed with chilled methanol:acetic acid fixative (3:1). It was centrifuged and treatment with methanol was repeated thrice. The cell buttons were dropped into chilled slides held inclinaly such a way that the nuclei burst and the chromosomes are released. The slides are allowed to dry. Four slides were prepared from each animal and stained with Giemsa (2 ml Giemsa stock solution and 98 ml Sorenson's buffer). Slides were immersed in the stain for 5 min, washed with distilled water and air dried. Slides were observed under oil-immersion and screeed for metaphase spreads. A minimum of 100 metaphase spreads was scored for aberrations.

When breaks involved both the chromatids, it was termed 'chromosome type' aberration while chromatid type aberrations involved only one chromatid. If the deleted portion had no apparent relation to a specific chromosome, it was called fragment.

RESULTS

Effect of betel nut extract on radiation induced micronuclei formation

Radiation dose of 150rads was found to induce the formation of micronucleated polychromatic and normochromatic erythrocytes. The incidence of micronucleated (MnPCE) polychromatic erythrocytes was 2.9% ± 0.03 and that of micronucleated normochromatic erythrocytes(MnNCE)2.7%±0.22 and were high as compared to normals which were 0.16% and 0.18% respectively. In the group of animals treated with radiation along with betel nut extract (2.5 g/kg b.wt.), the incidence of MnPCE and MnNCE was 1.57% ± 0.2 ($p< 0.001$) and 1.6% ± 0.09 ($p< 0.001$) respectively, while for the 0.5 g/kg b.wt. treated group the MnPCE was 2.04% ± 0.3 ($p< 0.001$) and that of MnNCE was 2.02% ± 0.28 ($P < 0.001$) (Table 1). This data showed that micronucleated cells did not increase after treatment with betel nut extract but rather a significant reduction in the micronuclei formation of treated groups was noticed when compared to that of the irradiated control group.

Effect of betel nut extract on radiation induced chromosomal aberrations

Exposure of mice to a single dose of radiation (300 rads) produced higher incidence of chromosomal aberrations in Swiss albino mice as seen from the increased breaks and gaps as well as numerical aberrations and acentric fragments. The percentage of aberrant cells account to 38.25% ± 7.93 in case of the irradiated group as compared in normals which was 6.75% ± 3.8. Administration of betel nut extract, 2.5 g/kg b.wt. and 0.5 g/kg b.wt., reduced chromosomal aberrations to 27% ± 8.28 ($p< 0.05$) and 35.25% ± 4.5 respectively, indicating that pre-treatment of betel nut prior to irradiation produced decreased chromosomal aberrations confirming its anticlastogenic role (Table 2).

DISCUSSION

Epidemiological studies showed that cancer of the oral cavity and pharynx is related

with tobacco chewing while the role of betel nut in cancer causation is still doubtful. In fact, non-carcinogenic activity of betel nut was demonstrated by clinical studies. Khanolkar in 1944 reviewed distribution of oral cancer and chewing habits and concluded that betel leaf and betel nut had no etiological role in the development of mouth cancer but inclusion of tobacco made it carcinogenic (12). Ahluwalia and Duguid (13) reported that in Malaysia the incidence of oral cancer was rare in Malays who do not add tobacco in betel quid while it was higher in Indians who add tobacco.

DNA is considered as the most critical cellular target towards the lethal, carcinogenic and mutagenic effects of ionizing radiation (14). Ionizing radiation either break the DNA bands or produce reactive oxygen species. Reactions of oxygen radicals with DNA results in mutations, chromosomal aberrations and micronuclei formation. It has been reported that frequency of micronuclei and chromosomal aberrations increase with radiation dose through not linearly (15).

Shirname (16) showed that betel nut extract could produce mutagenicity to some strains of *S.typhimurium* but not to others. It was not mutagenic to Chinese hamster cell line and did not induce any micronuclei in bone marrow. There are reports on the mutagenicity and carcinogenicity of isolated alkaloids, arecoline and arecaidine from betel nuts. Arecoline was found to induce chromosomal aberrations and sister chromatid exchange in bone marrow of Swiss albino mice and induced micronuclei formation. Arecaidine was found to be mutagenic to *S.typhimurium* strains. Administration of arecoline and arecaidine to animal was reported to produce stomach tumours in animals (17). These studies indicate that betel nut extract as such is not carcinogenic but some of the isolated ingredients like alkaloids may be carcinogenic at high concentration.

Betel nut has significant quantity of catechin and epicatechin, which was shown to inhibit mutagenicity and carcinogenicity in several experimental systems. Catechin was also been shown to inhibit metastasis as it can reduce the action of metalloproteases needed for breaking of basement membrane. Catechins have potent antioxidant property that efficiently scavenges a variety of free radicals (18,19). Further studies of the biological effects of catechins in cell culture and *in vivo* indicates that this compound can inhibit lipid peroxidation (20,21). Catechins has also been known to have chemopreventive properties can inhibit carcinogenesis in the initiation, promotion and progression stages. Plant phenolics including catechins prevent tumour initiation because they reduce the levels of carcinogen-DNA adducts. Results shown in the paper also indicate that betel nut extract did not increase the genomic changes induced by radiation but rather could reduce chromosomal aberrations and micronucleated cells induced *in vivo* in mice indicating that it could also protect the normal cells from oxidative DNA damage and thus could prevent clastogenesis and carcinogenesis. These results are well in tune with our reported results indicating that betel nut extract could inhibit the mutagenicity in vitro in *Salmonella typhimurium* by different mutagens as well as in mice induced by tobacco and polyaromatic hydrocarbon.

Table 1 : Effect of betel nut extract on radiation induced micronuclei formation

	% MnPCE	% MnNCE	% MnPCE + % MnNCE
Normal	0.16 ± 0.025	0.18 ± 0.029	0.17 ± 0.015
Radiation	2.9 ± 0.03	2.7 ± 0.22	2.8 ± 0.14
Radiation + 2.5g/kg.b.wt.	1.57 ± 0.2***	1.6 ± 0.09***	1.6 ± 0.02***
Radiation + 0.5g/Kg.b.wt	2.04 ± 0.3***	2.02 ± 0.28***	2.03 ± 0.014***

$p < 0.001$***

Table 2 : Effect of betel nut extract on radiation induced chromosomal aberrations

	Chromatid		Chromosome			% of aberration
	Gap	Break	Gap	Break	Others	
Radiation	2.5	1.25	8.25	7.5	16.5	38.25 ± 7.93
Normal	0.75	0	3.5	2.5	0	6.75 ± 3.8
Radiation + 2.5 g/kg b.wt.	0.5	1.75	4.25	8.5	12	27± 8.28**
Radiation + 0.5 g/kg b.wt.	0	0.75	9.25	11	14.25	35.25 ± 4.5

$p < 0.05$**

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BRYOPHYTES OF KERALA: A POTENTIAL SOURCE FOR ANTI-CANCER AGENTS

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INTRODUCTION

Bryophytes are simple but a unique and fascinating group of non-flowering plants. Bryophytes come under two main groups called as mosses and liverworts (Hepaticae). They have amphibious adaptation to live both in water and on land; they are also found as epiphytic, epilithic and mountain plants. The life cycle of these groups of plants is characteristic with not so prominent sporophyte generation which is parasitic on the gametophyte. All mosses lack true roots, instead, they have root-like structures called rhizoids. Multidimensional aspects of bryophyte ecology have received attention all over the world in the last 50 years. However, studies in India in this field are meager (Manju and Madhusoodanan, 2002).

Bryophytes are intimately linked with civilization, culture, beliefs and ethics of humankind (Pant and Tiwari, 1990). Mosses have been used as stuffing, matting and bedding materials in Europe, Japan as well as in high-altitude Himalayan villages (Pant and Tiwari, 1990). Mosses are grown to adorn rocks, gardens etc. especially in Japan. Mosses are harvested and used in planters and wire-hanging baskets. Bryophytes are also in ethno-medical use from time immemorial in many parts of the world (Pant and Tiwari, 1990).

Today, bryologists and biochemists are actively engaged in the isolation of anti-tumour and anti-microbial compounds from bryophytes in Japan and certain other developed countries. In India, practically no published report is available regarding the anti-tumour property of any of the Indian bryophytes. In spite of the promising leads obtained in other countries and the rich bryophyte flora of India, no attempt has been made to look for anti-cancer agents among bryophytes. In this context, this mini-review is to highlight the potentiality of bryophytes in medicine with special reference to cancer, keeping in view with the urgent need to initiate studies in our country in this promising area.

BRYOPHYTES IN ETHNO-MEDICINE

Ancient Greek and Roman literature mentioned of medicinal properties to bryophytes. They are attributed the powers of healing wounds and 'cooling' the body. The decoction of a liverwort was believed to be effective in certain types of inflammation. When applied externally after pounding and mixing with honey, these herbs were believed to heal scabies, skin-flakes, burn and smallpox marks (Schusten, 1969). The power of curing liver diseases was also attributed to liverworts by certain ethnic groups (Pant and Tiwari, 1990). A paste is made with the circular patches of *Riccia* sp. and jaggery, and is applied on the skin for the treatment of ringworm infection

by the Himalayan highlanders (Pant and Tiwari, 1989). Several kinds of mosses including the species of *Philonotis, Bryum,* and *Mnium* as well as various matted hypnaceous forms of them were used to alleviate the pains resulting from burns by Gasaite Indians of Utah (Flowers,1959). The moss was crushed into a kind of paste and applied as a poultice. Natives of Alaska use moss by mixing it with grease to make a healing ointment that soothes (Miller and Miller, 1979). The northern Cheyenne Indians of Montana and inhabitants of Kumauni villages of Askot and Gargia (Pithoragarh district, North West Himalayas) use mosses for medicine. They mainly use this as an ointment for cuts and wounds (Pant and Tiwari, 1989).

Many bryophytes are used in Chinese medicine also (Ding, 1982). Some of these traditional Chinese bryophytes are also occurring in India. For example, *Phodobryum gigantium*, a moss used in Chinese medicine (Pant and Tiwari, 1990) is also occurring in Eravikulam National Park, Idukki district, Kerala (Manju and Madhusoodhanan, 2001). Genera like *Philonotis* and *Brym*, used in Chinese traditional medicine are also occurring in Kerala. Unfortunately, none of these bryophytes are screened for pharmacological activities.

BRYOPHYTE FLORA WITH SPECIAL REFERENCE TO KERALA

The geography and climate of Kerala are highly suitable for the growth and proliferation of mosses and liverworts. 56% of the total area of Kerala is occupied by the mountains of Western Ghats which are one among the 25 bio-diversity hotspots of the world. Even though the bryophytes form an important component in the forest ecosystem of Kerala, it was not subjected to thorough studies. A comprehensive study on the bryophytes of Kerala is lacking (Manju and Madhusoodhanan, 2002). However, we have important glimpses of bryophyte flora of Kerala. It is believed that approximately 500 species of bryophytes are likely to be occurring in Kerala (Manju and Madhusoodhanan, 2002). Srivastava and Sharma (2000) documented 29 species of liverworts and 73 species of mosses from Idukki and adjacent areas. Udar and Jain (1989) published 16 species of liverworts from various parts of Kerala. A recent exploration as a part of the detailed studies on the bryophyte flora of the state yielded about 250 species (Manju and Madhusoodhanan, 2000).

Kerala has 2 National parks and 12 Wildlife sanctuaries including a Tiger Reserve. These are bryophyte diversity-rich areas. Vora et al (1981) reported 83 species of mosses from the Silent Valley National Park, Palakkad district, Kerala. Among these, 43 are epiphytes, 37 grow on soil and rocks and 3 species are seen both as epiphytes and epilithics (Manju and Madhusoodhanan, 2002). Srivastava and Sharma (2000) documented 29 species of liverworts from Silent Valley National Park. A preliminary exploration for bryophytes in Eravikulam National Park, Idukki district, Kerala, revealed 16 genera with 19 species of liverworts and mosses. These included 6 new records for Kerala (Manju and Madhusoodhanan, 2001). Thus, Kerala has an attractive bryophyte flora which remains as virgin with regard to pharmacological studies.

Anti-Cancer activity in Bryophytes:

Anti-cancer and anti-microbial properties are two important pharmacological activities detected in bryophytes (Ando and Matsuo, 1989). Several workers in many developed countries,

especially in Japan, tested the extracts of many species of liverworts and mosses for their anti-cancer and cytotoxic properties (Dittmer, 1968; Ando and Matsuo, 1984). Extracts of several bryophytes are shown to have anti-tumourigenic activities. Some of the bryophytes shown to have anti-tumour / cancer activities and / or cytotoxicity to cancer / tumour cells are given in Table 1. The available studies suggest that bryophytes are a storehouse of anti-cancer agents with varying levels of activity. Further, isolated compounds were also tested for anti-cancer and cytotoxicity activities and important leads were obtained. Some of the compounds shown to have anti-cancer and / or cytotoxic activities to cancer cells are shown in table 2.

Sesquiterpinoids like costunolide and tulupinolide isolated from several liverworts (*Conocephalam supradecomplusam, C. conicum, Ferullania tumarisa, Marchantia polymorpha, Porcella japorica* and *Wiesnerella denudata*) effectively inhibited the growth of the human carcinoma of the nasopharynx in cell culture (Asakawa, 1981; Asakawa, 1982). Ent-kurane type diterpenoids isolated from *Jungermannia truncata* and *Jungermannia infusa* caused apoptosis in human leukemia cell lines and proteolysis of poly–(ADP ribose) polymerase, a hallmark of caspase activation. Caspase inhibitors abolished the appearance of DNA fragmentation and cytotoxicity, to a large extent, in the en-kurane treated cells (Nagashima et al 2002; Nagashima et al 2003). Thus, it appears that terpenoids from liverworts are promising agents for the induction of apoptotic cancer cell death.

Further, liverworts are known to contain chemicals, which inhibit 5-lipoxygenase and cycloxigenase (Asakawa, 1995). These compounds can influence cell proliferation, because some of the metabolites produced by the enzymes are known to stimulate cell proliferation (Subramoniam, 1997)

SECONDARY METABOLITES OF BRYOPHYTES:

Pharmacology and chemistry of bryophytes were almost not explored by Indian investigators. However, a perusal of world literature shows that bryophytes produce an array of secondary metabolites. Some of them are listed in Table 3. Bryophytes, liverworts in particular, are rich in terpenoids. Most of the liverworts possess characteristic cellular oil bodies, mainly composed of lipophilic terpenoids and aromatic compounds. The diversity of terpenoids in liverworts is amazing. For example, more than 17 diterpenoids have been isolated from the Japanese liverwort, *Jungermania truncata* (Nagashima et al, 2002). Similarly, more than 22 sesquiterpenoids have been isolated form the liverwort, *J. infusa* (Nagashima et al 2000). Some of the sesquiterpenoids reported are aromadendrane, acorane, cuparane, barbatane, costunolide, frullanolides, prelacinane, pinquisone and tulipinolide; diterpenoids are clerodane, halimane, labdane and ent-kurane, to name a few. It is of interest to note that anti-cancer activity has been reported in some of the di-and sesqui-terpenoids. These include costunolide, tulupinoside, ent-kurane (diterpenoid) and maytansinoids. These leads indicate that vast hope remains for the identification of invaluable chemotherapeutic agents from bryophytes.

Bryophytes are known to produce growth regulators and hormones having tremendous effect on the associated organisms in the bryophyte habitat. The influences of associated organisms such as algae and lichens on the

production of secondary metabolites by bryophytes are not studied. Production of secondary metabolites can possibly be dependent on or influenced by the availability of minerals and nutritional factors, seasonal variations, association of other organisms, physiological state, age and sex of plant, etc.

CONCLUSION

Bryophytes hold great promise for the future of anti-cancer drug development. It has been predicted that bryophytes could yield very important chemotherapeutic agents like Actinomycetes and *Pencillin* (Ding, 1982). The available world literature on bryophytes suggest that these simple organisms could produce very valuable anti-cancer agents. However, surprisingly, research in this line has not been initiated in our country. One of the reasons could be non-availability of trained bryophyte taxonomists for rapid identification of species. Multidisciplinary work involving bryologists, phyto-pharmocologists, oncologists, phytochesmists, etc. are required for screening and developing anti-cancer drugs from bryophytes. Search for cancer cell specific apoptosis inducing compounds among bryophytes, especially liverworts, is an interesting area to pursue.

Japanese chemists (taking the leading role) mostly followed a phytochemical approach in identifying the bio-activity of the isolates from the bryophytes. Activity guided isolation following phytotherapeutic approach could be more rewarding in the discovery of anti-cancer agents.

It is high time to initiate and establish pharmacological studies on bryophytes in India focusing development of standardized phytomedicine and / or isolated pure compounds as medicine. This should be one of the most priority areas in our country in the search for anti-cancer drugs from natural products.

Table 1 : Some of the bryophytes known to have anti-cancer activity and / or cytotoxicity to cancer cells

Sl. No.	Name of Bryophytes	Reference
1.	*Cladopodium crispifolium*	Spjut, 1986
2.	*Conocephalum conicum*	Asakawa, 1982Ohta et al, 1977Jalad et al, 1974
3.	*Conocephalum supradecomposam*	Asahawa, 1981; 1982
4.	*Diplophyllum* sp.	Asakawa et al, 1978
5.	*Frullania tamarisu*	Asakawa et al, 1982
6.	*Hyalocomium splendens*	Spjut, 1986
7.	*Jungermannia truncata*	Nagashima et al, 2002
8.	*Marchantia polymorpha*	Asakawa et al, 1982
9.	*Plagiomnium venustum*	Spjut, 1986
10.	*Polytrichum* sp.	Spjut, 1986
11.	*Porella japonica*	Asakawa et al, 1982
12.	*Wiesnerella denudata*	Asakawa et al, 1994

Table 2 : Compounds from bryophytes known to have anti-cancer / cytotoxic properties

Sl. No.	Compounds	Reference
1.	Benzonapthoxanthenones (*Conocephalum conicum*)	Zheng et al, 1994
2.	Costunolide (*C. supradecomposam*)	Asakawa, 1982
3.	Cinnamoylbibenzyls	Zheng et al 1994
4.	Diplophyllin (*Diplophyllum* sp.)	Sakai et al, 1988; Asakawa et al, 1978
5.	Diterpenoids (*Jungermannia truncata*)	Nagashima et al, 2002; Nagashima et al, 2003
6.	Maytansinoids	Sakai et al, 1978
7.	Tulupinoside (*C. conicum*)	Asakawa, 1982
8.	Zaluzanin (*C. conicum*)	Asakawa, 1982

Table 3: Secondary metabolites identified in Bryophytes (Pant and Tiwari, 1990; Huneck et al 1983; Markham and Porter, 1978; Nagashima et al, 2003)

1	Monoterpenoids
2	Diterpenoids
3	Sesquiterpenoids
4	Glycosides
5	Chalcone
6	Flavone glycosides
7	Macrocyclic flavonoids
8	Aromatic and Phenolic compounds
9	Benzonapthoxanthenones
10	Bis-bibenzyls
11	Sugar alcohols
12	Prenylquinones
13	Aminoacid derivatives
14	Fatty acid derivatives
15	5-Tocopherol

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HUMAN EPIDERMAL RECEPTOR : A PROMISING MARKER IN CANCER THERAPY.

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Molecular markers in clinical oncology can be divided into diagnostic markers, which distinguish one disease from another, prognostic markers, which are associated with the clinical behaviour of a tumor, and predictive markers which are used to predict outcome of therapy and to aid in the selection of optimal treatment. Diagnostic and prognostic markers, though important in clinical management, are deterministic in nature, in that the natural course of a cancer is not likely to be changed because of knowledge of that marker status. However more excitement within the last decade has been centered on predictive markers, many of which are also the targets for specific therapeutics. The importance ofcourse is that the ascertainment of these predictive markers may guide treatment selection that can change the course of a disease.

The varying efficacy and toxicity of traditional cancer therapies has driven the development of novel target based agents. Members of the HER (Human Epidermal Receptor) family, in particular epidermal growth factor receptor (HERI|EGFR) are attractive therapeutic targets because they are overexpressed and |or dysregulated in many solid tumors. Activation of HERI | EGFR mediated through ligand binding triggers a net work of signaling processes that promote tumor cell proliferation, migration, adhesion and angiogenesis and decrease apoptosis. Therefore, inhibiting HERI | EGFR activity could effectively block downstream signaling events and consequently, tumorigenesis. Various approaches are being investigated to target members of the HER family, particularly HERI | EGFR and HER 2.

The HER family consists of four closely related transmembrane receptors, epidermal growth factor receptor (HERI | EGFR, HER 2, HER 3 and HER 4. These receptors are structuraly similar, although they have distinct characteristics that dictate their signaling specificity. Each receptor has an extracellular ligand binding domain, a transmembrane region and an intracellular cytoplasmic domain (Fig 1). The amino terminal extracellular domain of HER|EGFR has two cysteine rich regions that form the ligand binding domain. The transmembrane region is a single alphahelix that anchors the receptor to the cell.

The cytoplasmic domain contains a TK region and carboxy terminal tail that contains atleast five tyrosine autophosphorylation sites. Importantly, the TK domains of HER 2 and HER 4 show approximately 80 % homology to that of HERI | EGFR whereas HER 3 lacks intrinsic TK activity. In addition to these receptors a mutant receptor, EGFR III is commonly detected on many types of human tumors. EGFR VIII lacks residues 6-276 in the extracellular ligand binding domain.

This alteration results in ligand independent constructive activation of the mutant receptor protein.

Various ligands can bind to HERI | EGFR, HER 3 & HER 4. These ligands have different specificities for each receptor, resulting in different cellular effects. Ligand binding induces HER 1 | EGFR homodimerisation as well as heterodimerisation with other HER receptors. Importantly, HER 2 does not bind to any known ligand, but is the preferred heterodimerisation partner for other HER 2, HER 3 homodimers are TK deficient and cannot initiate signal transduction. Epidermal growth factor receptor dimerisation induces TK catalytic activity, which leads to autophosphorylation in several tyrosines within the receptors carboxyl terminal tail. The resulting phosphotyrosines (Y992, Y 1068, Y 1086, Y 1448 and Y 1173) act as docking sites for a number of signal transducing enzymes and adaptor proteins . Two major pathways are involved in HER signalling, the ras-raf—mitogen activated protein kinase (MAPK) and the phosphatidyl inositol 3 kinase (P 13 K) Akt pathways. The various post receptor signaling cascades activated through ligand binding and dimer formation result in different cellular effects. For instance, the MAPK pathway is important mainly for cell proliferation and the PI 3 K has a prominent role in cell survival. The mutant variant, EGFR VIII, also activates downstream signaling, but does nost require ligand binding. It is clear that signaling by HER | EGFR in tumor cells is highly modulated by the level of coexpressed HER receptors, as well as local receptor ligands.

HER I |EGFR is perhaps the most widely studied member of the HER family, and extensive research provides compelling evidence for using HER 1 | EGFR as a target for anticancer therapy. Abnormal receptor activation or dysregulation of the HER 1 | EGFR signal transduction pathway can result from a number of different mechanisms that are potentially relevant to the growth and | or development of human carcinomas.

Several studies suggest that the level of HERI | EGFR expression correlates with poor disease prognosis and reduced survival. However, there is no consensus on the correlation between expression and prognosis for most tumors. This may be explained by the difficulties in quantifying HERI | EGFR expression and the inherent heterogeneity of human tumors.

Another important mechanism of abnormal signaling by HERI | EGFR in tumor cells is the overproduction of HERI | EGFR ligands, such as transforming growth factor 2 (TGF - 2). Overproduction of ligands can increase receptor activation in an autocrine manner , leading to enhanced transformation. The establishment of an autocrine ligand receptor system causes independent growth of tumor cells and is believed to be a vital step in the development of hormone resistant tumors particularly of the breast and prostate.

EGFR VIII is the most common HERI | EGFR mutant. EGFR VIII has a truncated extracellular domain, is constitutively activated and is resistant to downregulation by endocytosis. EGFR VIII is overexpressed in some solid tumors, particularly in high grade gliomas mainly as the result of gene amplification. The presence of EGFR VIII is believed to confer a more malignant tumor phenotype. Importantly, preclinical studies show that EGFR VIII can enhance tumor invasion in the absence of HERI | EGFR ligands (28,29) Immunohistochemical studies show that EGFR VIII ,is present in other epithelial tumors such as breast, prostate and non-small cell lung cancer(26)

however, its clinical significance in these tumor types remain unproven.

HERI | EGFR acts as a point of integration for signals arising from G protein coupled receptors and cytokine receptors. Consequently HERI | EGFR can cross talk with various heterologous receptors activated by neurotransmitters lymphokines and stress inducers (30).

Members of the HER family are established therapeutic targets for the development of novel anticancer agents. In view of this, several approaches are being used to block these receptors. The mechanism of action in Figure 2 and table 1 summarises the compounds currently in clinical development. Preclinical and early clinical data from trials with erlotinib and other agents show that these inhibitors are well tolerated and could benefit patients with a variety of cancers. Further understanding of the HER family signaling pathways and their interactions with other networks within tumor cells are necessary to optimize the clinical development of these targeted agents. Finally a predictive marker that will help select patients for treatment with

HERI|EGFR inhibitors is sorely needed to maximize the information derived from ongoing clinical studies.

Table 1 : Incidence of HERI|EGFR and HER2 Overexpression and\or Dysregulation in Selected Human Tumors

Tumor Type	HERI|EGFR Expression (%)	HERI|EGFR Mutation (%)	TGF-a Expression (%)	HER 2 Expression (%)
Breast	14-91	78	40-70	15-30
Colorectal	25-77	NA	50-90	11-20
Esophageal	35-88	NA	46-88	NA
Glioblastoma	40-60	57	NA	NA
HNSCC	95	NA	88	NA
NSCLC	40-80	16	85-100	0-35
Ovarian	35-70	73	55-100	0-32
Pancreatic	30-50	NA	95	19-45
Prostate	41-100	NA	NA	14-86

Abbreviations: HERI|EGFR, epidermal growth factor receptor, TGF, transforming growth factor, HNSCC, head and neck squamous cell cancer, NSCLC, non-small cell lung cancer, NA, data not available.

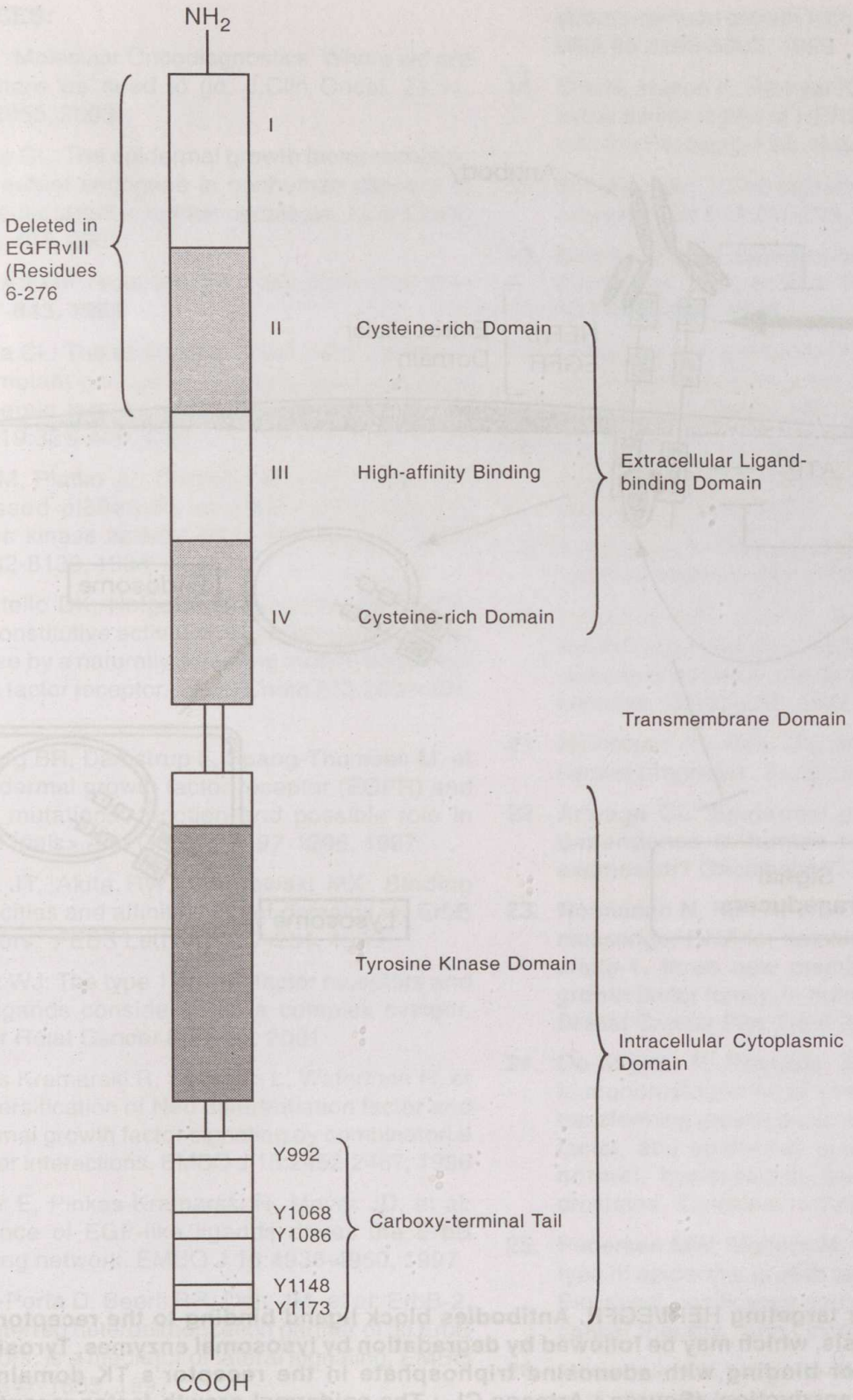

Fig I. Structure of HERI/EGFR. (reprinted from *Int J Biochem Cell Biol*, vol 31, A. Wells, EGF receptor, pp 637-643, 1999, with permission from Elsevier.[4])

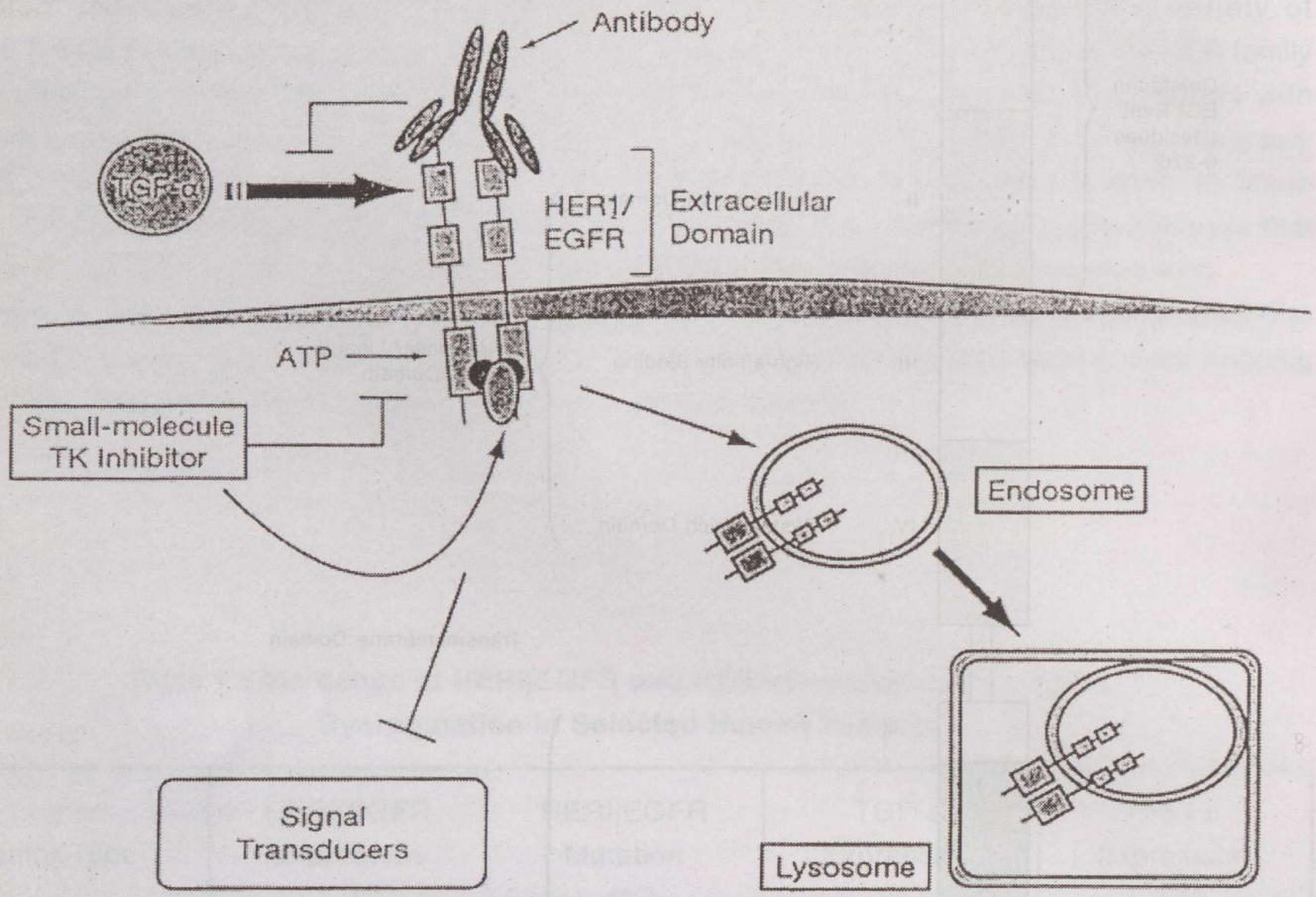

Fig 2. Approaches for targeting HERI/EGFR. Antibodies block ligand binding to the receptor inducing HERI/EGFR endocytosis, which may be followed by degradation by lysosomal enzymes. Tyrosine kinase inhibitors compete for binding with adenosine triphosphate in the receptor's TK domain, blocing downstream signal transduction. (Source : Arteaga CL : The epidermal growth factor receptor : From mutant oncogene in nonhuman cancers to therapeutic target in human neoplasia. *J Clin Oncol* 19:32s-40s, 2001. Reprinted with permission of the American Society of Clinical Oncology.[7])

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ALTERED EXPRESSION OF MUC1 MUCIN AND E-CADHERIN IN BENIGN AND NEOPLASTIC UTERINE CERVIX CARCINOMA.

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ABSTRACT

Neoplastic transformation of epithelial cells is commonly associated with altered synthesis of transmembrane glycoproteins. Although the two glycoproteins Muc1 and E-cad is associated most consistently with epithelial tissues, no detailed study concerning their interactions and the relationship with invasive and metastatic potential have been investigated. Therefore, in the present study, immunohistochemistry was performed using monoclonal antibodies against MUC1 and E-cad in biopsy specimens of cervical carcinoma and this was compared to the expression profiles observed in benign cases. In the neoplastic cells from squamous cell carcinoma, high levels of cytoplasmic Muc1 expression and low levels of E-cad expression was noticed .In addition, the present study also indicates a correlation between the expression of the two proteins and some clinicopathologic features of aggressiveness of cervix carcinoma. These data suggest that Muc1 and E-cad have an important role in differentiation, invasion and metastasis of cervical neoplasia and hence the two proteins can be used as biomarkers for predicting the outcome of the disease.

Key words: Muc1, E-cad, Uterine cervix carcinoma, Immunohistochemistry

INTRODUCTION

Mucins are high molecular weight glycoproteins that contain oligosaccharides and are the major components of the viscous mucous gel covering the surface of epithelial tissue. In general, mucins have the unique function of protecting and lubricating epithelial surfaces but in recent years they have also been implicated in additional diverse roles, such as growth, fetal development, epithelial renewal and differentiation, epithelial carcinogenesis and metastasis (1).Among the fourteen mucin genes identified, the human DF3/MUC1 is one of the best known transmembrane glycoprotein with a large extra cellular domain and a cytoplasmic domain. While the exact function of MUC1 mucin in the normal cell is still a matter of debate, they have had considerable impact as markers in many human carcinomas (2). In recent years, monoclonal antibodies (MAbs) against MUC1 mucin have been generated that recognize epitopes on the polypeptide or differences in the degree of glycosylation. Aberrant expression of the Muc 1 mucin has been seen in breast carcinomas and other neoplasms, such as colon and pancreatic cancers (3, 4) and has been related to invasiveness and poor prognosis (5, 6, and 7). E-Cadherin protein is reported to act as a master molecule in maintaining the architecture and in differentiation of normal epithelium (8).Although MUC1 and E-cad expression is associated most

consistently with epithelial tissues, no detailed study concerning the consequences of their interactions on cell signalling pathways, and the relationship with invasive and metastatic potential of these proteins in benign and neoplastic uterine cervical carcinoma tissues have been reported to date. Hence the purpose of the present study is to find out the relationship between the expression patterns of MUC1 and E-cad proteins during the progression from early to advanced carcinomas and their relationship with the clinicopathological factor.

MATERIALS AND METHODS

STUDY POPULATION

Patient Selection:

The patient population for this study consisted of 47 individuals presenting with cervical carcinoma at the Institute of Obstetrics and Gynaecology, Madras Medical College, Egmore, Chennai, India. In addition to this ten benign cervical tissues were obtained from patients undergoing hysterectomy for benign diseases of the corpus uteri with normal cervical cytology. Informed consent regarding the use of tissue specimens for research purpose was obtained from all patients. The clinical details of the patients were collected from the medical records maintained in the hospital and also by personal interview.

Sample Collection:

Biopsy specimens were obtained prior to the initiation of any treatment. All tissue samples were fixed in 10% neutral buffered formalin and were embedded in paraffin for routine histologic procedures.

The patients with cervical carcinoma were clinically staged according to the criteria of the International Federation of Gynaecology and Obstetrics (FIGO). The morphology of each biopsy was carefully evaluated on routine processed Haematoxylin-Eosin stained sections. Histologic examination of biopsies was assigned according to the WHO classification. The benign biopsies revealed Erosion Cervix, Basal Cell Hyperplasia, Chronic Cervicitis and the carcinoma biopsies were classified as Well Differentiated Squamous Cell Carcinoma (WDSCC) and Moderately Differentiated Squamous Cell Carcinoma (MDSCC) tumors. No lesion of the poorly differentiated type was available in the sample studied. Patients displaying positive and negative lymph node metastasis were also compared in this study. Accordingly, the samples included Erosion Cervix-3, Basal Cell Hyperplasia-4, Chronic Cervicitis-3, WDSCC-18, and MDSCC-29.

Study Design:

Antibodies used:

Mouse monoclonal antibody DF3, DAKO, Denmark and Goat polyclonal antibody CDH1, Santa Cruz, California was used at 1/50 and 1/30 dilution respectively. All the dilutions were done in 1X (PBS) phosphate buffered saline buffer (pH=7).

Immunohistochemical study:

The avidin-biotin peroxidase complex (ABC) method was used for immunostaining. 4μ sections were cut from paraffin blocks, collected on silanized glass slides and immunostained for MUC1 Mucin and E-Cadherin. To block endogenous peroxidase activity, the specimens were treated with 0.3% hydrogen peroxide in pure methanol for 30 minutes. The sections were incubated in 3% Bovine Serum Albumin (BSA) for 30 minutes to block non-specific binding. Thence

the sections were incubated overnight with primary antibody at 4°C, washed and sequential incubations were carried out with a biotinylated secondary antibody (30 minutes), washed and incubated again with avidin-HRP conjugated tertiary antibody (30 minutes). The peroxidase reaction was visualised by incubating with AEC for 10 minutes in the dark and counterstained with haematoxylin. All washings were done in 1X PBS buffer. To ensure antibody specificity, control sections were incubated with PBS instead of the primary antibody.

Evaluation of immunostaining:

Sections were examined under the light microscope. The staining pattern was assessed without the prior knowledge of histopathological diagnosis. The staining was categorized as follows: negative - 1; mild positive - 2; moderate positive - 3; and intense positive - 4 respectively. The staining pattern was evaluated by an independent investigator and confirmed in consultation with the pathologist. The staining score was expressed as mean value ± SE.

Statistical analysis:

For histological type comparisons of the staining intensities of the two respective antibodies, the mean staining score was calculated using Student's'– test. Expression of the various proteins was analysed between the different histological groups using non-parametric Mann-Whitney U Test and P value < 0.05 was considered significant. Spearman Bivariate Correlation Analysis was performed for analysing the correlation between the various parameters studied using histological types and metastases. Odds ratio and the corresponding 95% confidence intervals were calculated for risk analysis.

RESULTS

MUC1 / Mucin Expression:

The labeling of MUC1/DF3 antibody in hyperplastic epithelium exhibited moderate membranous staining of MUC1 in the basal, parabasal and the superficial cells (Fig. 1A). In chronic cervicitis, mild cytoplasmic expression was observed in the epithelial compartments. In well differentiated cases the differentiated cells exhibited moderate to intense staining whereas in moderately differentiated cases the protein expression has been found to be very intense and cytoplasmic throughout the tissue section. MUC1 staining was intense in almost all the invasive cases (Fig.1C, E &Table 3).

Relationship between the expression of MUC1 mucin and several clinicopathologic features:

MUC1 protein showed a consistent up-regulation in carcinoma cases and the expression rate differed significantly when compared to benign cases especially in the WDSCC (p 0.013) and MDSCC (p 0.037) types. Aggressive lesions were characterized by dominant cytoplasmic staining when compared to membranous staining in benign cases (Table 3). Staining of higher intensity was observed in higher stages of the disease and in node positive cases (Table 1). A reverse trend was noticed in the staining pattern of Muc1 and E-cad with majority of Muc1 positive lesions being negative or mildly positive for E-cad.

E-cadherin Expression:

In hyperplastic epithelium, the specific E-cad membranous distribution was localized mostly in the superficial layer with occasionally tissues showing positivity in the basal cells. A striking alteration of the immunohistochemical pattern of E-cad distribution pattern was apparent in chronic

cervicitis lesions where they showed intense cytoplasmic expression. A heterogeneous distribution of staining was noticed in the epithelial lesions. The immunostaining appeared to be less intense whenever cell-cell contact appeared looser. Interestingly, the invasive carcinoma showed a consistent down regulation of E-cad expression. In the E-cad positive cancer cases, very mild cytoplasmic immunostaining for E-cad was distributed throughout the carcinoma cell nests.

Relationship between the expression of E-cadherin protein and several clinicopathologic features:

In this study E-cad protein generally showed a significant down regulation in carcinoma cases when compared to all stages (P value 0.0001) .The highly differentiated cases showed high cytoplasmic immunoreactivity when compared with E-cad membranous staining in benign cases (Fig. 1B,D,F & Table 3). Although not significant, the expression rate of E-cad in patients with positive lymph node metastasis was found to be low when compared to negative lymph node metastasis (Table 2).

DISCUSSION

Treatment of metastatic tumors is one of the most puzzling problem facing clinicians .The majority of patients (80%) present an advanced disease at the time of diagnosis, because of the characteristic rapid progression of the tumors and late detection ability. At present, Hysterectomy with limited lymph node dissection is generally chosen even for early cervical carcinoma. A biological marker that estimates the malignant potential at an early stage would help to determine whether minimally invasive surgery is indicated for this malignancy. Several investigators have identified bio-markers for predicting malignant potential in cervical cancer. However, only a few can be used to aid this decision. Hence this study is focused on two metastatic marker proteins, associated with invasion and metastasis, secreted by the tumor cell.

In this regard, Muc1 and E-cad are found to be important tumor marker proteins involved in the complex stages of tumor invasion and metastasis.The results of this study suggest that abnormal expression of Muc-1 and E-cad occur in cancer which is reported to be due to altered glycosylation (8, 9). Further, an inverse relation in pattern of expression has been noticed in the case of Muc1 and E-cad. This supports the reports of anti-adhesive property for Muc1 (10, 11) wherein mucin is reported to accelerate tumor invasion via impairment of the cell-cell adhesion molecule, E-cadherin. Hence, the findings of the current study suggest that Muc1 mucin and E-cad expression may be useful markers of malignant potential.

The changes in cellular localization observed in this study seem to be important as they provide information on the role of these glycoproteins in cellular malignant transformation. This phenomenon has also been noticed by others (12) who suggested the localization of proteins to correlate with tumor progression, invasion and metastasis. Heterogeneity has been described both in the proportion of tumor cells expressing Muc1 and E-cad and in its cellular localisation. These two cell surface glycoproteins contain transmembrane domains and are synthesized as membrane proteins (13, 14). As in the case of all epithelial cells, the normal squamous cells of uterine cervix exhibit Muc1 in the entire cell membrane, whereas in glandular epithelial cells Muc1 mucins are expressed only on the apical side

of cells. In carcinoma cells we observed that this polarisation is lost and Muc1 mucins are translocated to the cytoplasm. This clearly implies that aberrant expression of Muc1 destabilizes cell-cell and cell-extracellular matrix interactions and therefore may be an important co-factor in the transition from pre invasive to invasive lesion. In addition to this, various studies have pointed out that the cytoplasmic accumulation of E-cadherin may reflect abnormal transport to the cell surface or abnormal re-uptake of the molecule from the cell surface back into the cytoplasm (15, 16, and 17). The presence of a cytoplasmic and therefore non-functional E-cadherin molecule is associated with loss of differentiation in squamous cell carcinoma (18).

In addition, the present study also brings out a correlation between the expression of the two proteins and some clinicopathologic features of aggressiveness of cervix carcinoma. The frequency of Muc1 up-regulation and E-cad down regulation in tumors showed strong correlation with high pathologic grade (MDSCCNKLC), advanced stage (111B-FIGO) and with positive lymph -node metastasis. Present observations also show an up regulation of Muc1 with concurrent downregulation of E-cad expression and a translocation of these proteins from membrane to cytoplasm with lesser differentiation / keratinisation. Therefore the findings of the current study suggest that Muc1 and E-cad have important roles in differentiation, invasion and metastasis of cervical neoplasia. As a result, the examination of these bio-markers might thus be useful for studies aimed at preventing metastasis and assessment of pre-operative cervical biopsy specimens of cervical neoplasia and are expected to provide useful information for predicting the outcome of the disease at an early stage.

Table 1 : Relationship of MUCIN-1 expression with clinicopathological characteristics

Variables	Muc1 positive (%)	Muc1 negative (%)	Expression Mean value	P value
Benign	6 (60)	4 (40)	2.10 ± 0.35	0.004*
Cancer	41 (87.2)	6 (12.8)	3.45 ± 0.14	
Histology				
WDSCCKLC	15 (83.3)	3 (16.7)	3.28 ± 0.27	0.013*
MDSCCNKLC	26 (89.7)	3 (100)	3.63 ± 0.17	
Stage				
IA&IB	10 (83.4)	2 (16.7)	3.33 ± 0.35	0.019*
IIA&IIB	9 (81.8)	2 (18.2)	3.36 ± 0.36	0.022*
IIIA&IIIB	28 (87.5)	4 (12.5)	3.53 ± 1.02	0.0001*
Nodal status				
Negative	31 (73.6)	11 (26.2)	2.98 ± 0.20	0.011**
Positive	22 (95.7)	1 (4.3)	3.74 ± 0.14	

* Comparison with benign lesion
** Comparison with positive and negative groups with reference to Muc-1 expression

TABLE 2 : Relationship of E-cadherin with clinicopathological characteristics

Variables	E-cad positive (%)	E-cad negative (%)	Expression Mean value	P value
Benign	10 (100)	0 (0)	3.00 ± 0.15	0.0001*
Cancer	39 (83)	8 (17)	1.95 ± 0.07	
Histology				
WDSCCKLC	16 (88.9)	2 (11.11)	2.00 ± 0.11	0.0001*
MDSCCNKLC	23 (79.3)	6 (20.7)	1.93 ± 0.11	0.0001*
Stage				
IA&IB	10 (83.4)	2 (16.7)	1.92 ± 0.15	0.0001*
IIA&IIB	8 (72.7)	3 (27.3)	1.82 ± 0.18	0.0001*
IIIA&IIIB	27 (84.4)	5 (15.6)	1.40 ± 0.57	0.0001*
Nodal status				
Negative	34 (81)	8 (19)	2.14 ± 0.12	0.568**
Positive	21 (91.3)	2 (8.7)	2.04 ± 0.09	

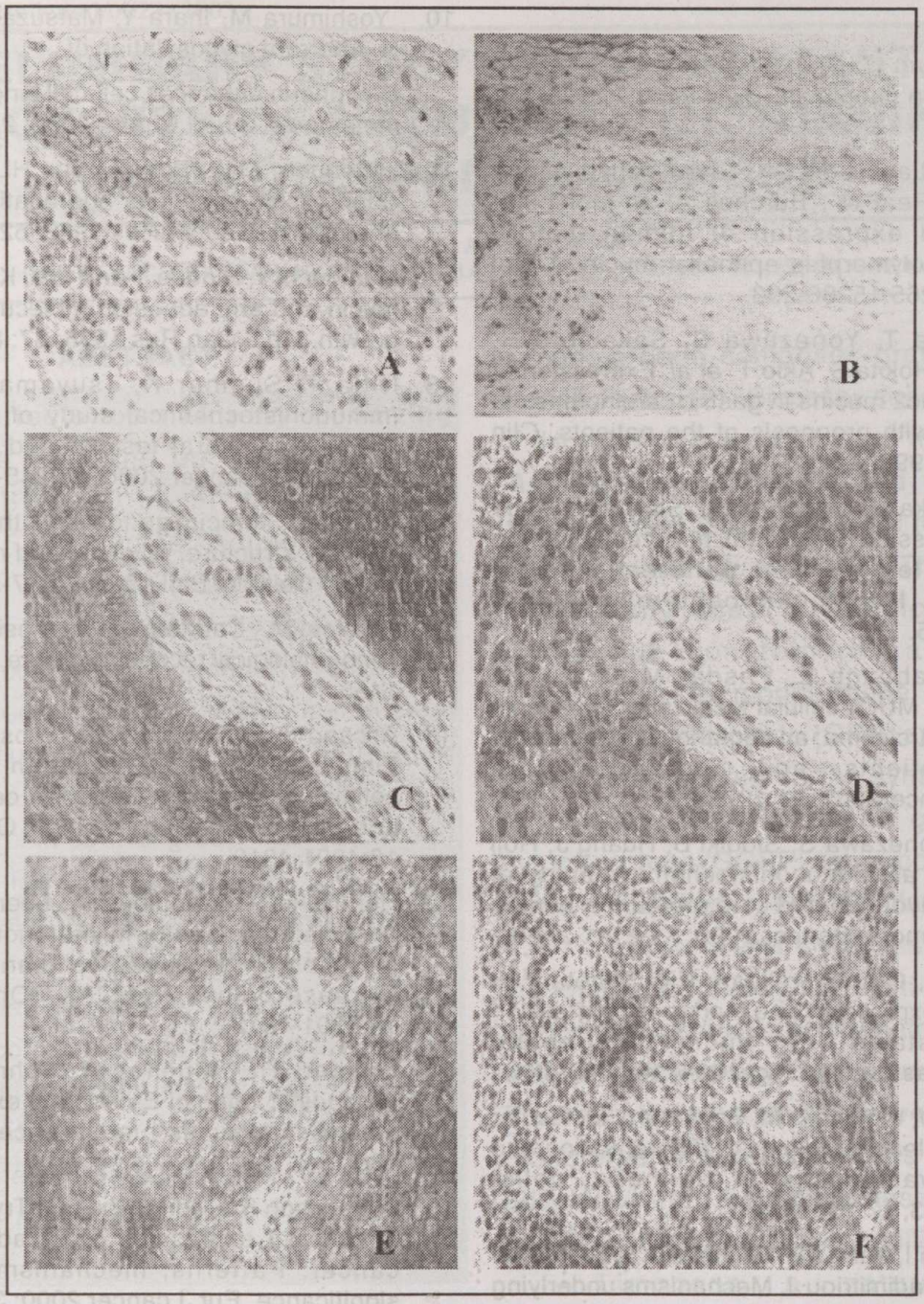
* Comparison with benign lesion

** Comparison with positive and negative groups with reference to E-cad expression

TABLE 3 : Expression of MUC1 and E-cad in relation to their cellular localization

(Number of cases and percentage)

MUC1	Negative	Apical	Memb.	Cyto + Memb	Cyto	E-CAD	Negative	Memb	Cyto
Benign (10)	4 (40%)	3 (30%)	3 (30%)	0 (0%)	0 (0%)	Benign (10)	0 (0%)	6 (60%)	4 (40%)
SCC (47)	6 (12.8%)	0 (0%)	0 (0%)	20 (42.5%)	21 (44.7%)	SCC (47)	8 (17%)	0 (0%)	39 (83%)



1A : Hyperplastic lesion showing membraneous staining for Muc1 in the basal and parabasal cells (X 40)

1B : Hyperplastic lesion showing membraneous staining for E-cadherin in the basal and parabasal cells (X 40)

1C : Well differentiated invasive lesion showing intense positivity for Muc1 antigen (X 40)

1D : Well differentiated invasive lesion showing mild positivity for E-cadherin (X 40)

1E : Moderately differentiated invasive lesion showing intense positivity for Muc1 (X 40)

1F : Moderately differentiated invasive lesion showing mild occasionally positive cells with anti-E-cadherin antibody (X 40)

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PRELIMINARY STUDY OF ANTIMETASTATIC ACTIVITY OF NATURALLY OCCURRING MONOTERPENE LIMONENE

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ABSTRACT

The effects of naturally occurring monoterpene limonene on lung metastasis induced by B16F-10 melanoma cells were studied in C57BL/6 mice. Administration of monoterpene limonene (100 μmoles/kg body wt. 10 doses i.p.) remarkably reduced the metastatic tumour nodule formation by 65%. The result correlated with the biochemical parameters such as serum sialic acid, lung collagen hydroxyproline and uronic acid contents. Serum sialic acid level in control group was 126.8 μg / ml serum which was significantly lowered in limonene treated (49.3 μg/ml serum) animals. Uronic acid level was also inhibited to 56% in limonene treated animals. Histopathological studies also correlated with these above results. These results indicate that naturally occurring limonene could inhibit the metastatic progression of B16F-10 melanoma cells in mice.

Key Words: Metastasis; Limonene, B16F-10 melanoma

INTRODUCTION

Monoterpenes are compounds found in the essential oils extracted from many plants including fruits, vegetable, spices, and herbs. The compounds contribute the flavor and aroma of plants from which they are extracted (1). Monoterpenes are non-nutritive dietary components widely consumed by man. Limonene, a monoterpene comprises grater than 90% of orange peel oil (2). Bioassay directed fractionation of dill seed oil and caraway oil from the plants of '*Anethum graveolens L*' and *'Carum carvi L'* (*Umbelliferae*) respectively led to the isolation of limonene and carvone (3). These plants are traditionally used for medicinal purposes. Monocyclic monoterpenes are synthesized metabolically by monoterpene synthases using geranyl pyrophosphate (GPP) as substrate. GPP is also the precursor in the synthesis of farnesyl pyrophosphate (FPP) and geranyl geranyl pyrophosphate (GGPP) two important compounds in cell metabolism of animals plants and yeast (1).

Monoterpene cyclases produce cyclic monoterpenes through a multi-step mechanism involving a universal intermediate, a terpinyl cation that can be transformed to several compounds (1). Pharmacokeinetic studies have indicated that a monoterpene perril alcohol is subject to rapid adsorption from the gastrointestinal tract followed by efficient metabolism to perillic acid (4,5).

Limonene a naturally occurring monoterpene has chemopreventive and chemotherapeutic activity against many rodent solid tumour types (6,7). The chemopreventive activity of limonene during initiation can be attributed to the induction of phase 1 and phase II enzymes with resulting carcinogen detoxification (8). The chemopreventive activity of limonene during promotion/ progression may be due in part

to inhibition of the post-transitional isoprenylation of growth controlling small G proteins (9). These are several evidences for induction of apoptosis by perillic acid and the experiments, confirmed that perillic acid create a block on the cell cycle progression G (0)/G (1) phase (10). Both limonene and perillic alcohol (reduced form of perillic acid) are being evaluated in phase I clinical trials in advanced cancer patients (2).

Metastasis is the dissemination of tumour cells from primary tumour site to distant site. The process of metastasis is a cascade of linking sequential steps involving multiple host tumour interactions. This complex process requires the cells to enter circulation, arrest at the distant vascular bed and proliferate as secondary colony. There are several evidences exists for each of these sequential steps of metastasis (11,12,13) and these sequential steps offers insight into therapeutic and prevention targets.

In the present study we are reporting the antimetastatic activity of naturally occurring monoterpene limonene by inhibiting the lung metastasis induced by B16F10 melanoma cells in C57BL/6 mice.

MATERIALS AND METHODS

Animals:- C57BL/6 mice (20-25g body wt, 6-8 weeks old males) were purchased from National Institute of Nutrition, Hydrabad, India. The animals were fed with mouse chow (Sai Feeds India) and water *ad libitum.*

B16F-10 melanoma a highly metastatic cell line was obtained from National Centre for Cell Sciences (NCCS), Pune, India. The cells were maintained in DMEM, supplemented with 10% FCS and antibiotics.

Monoterpene Limonene was purchased from Sigma Chemicals, St.Louis, MO, USA. Hydroxyproline was purchased from Sigma Chemicals, St.Louis, MO, USA. and N-acetyl neuraminic acid was purchased from Sisco Research laboratory Bombay. All other reagents were of analytical reagent quality.

Drug, dose and route of administration:- Monoterpene Limonene was suspended in light paraffin oil and administered at a concentration of 100 μmoles /kg body wt. Monoterpene Limonene was intrapertonially administered from the same day of tumour inoculation and were continued for 10 consecutive days.

All the animal experiments were performed according to the rules of Animal Ethical committee, Govt. of India.

Effect of naturally occurring monoterpene limonene on metastatic lung tumour nodule formation.

C57BL/6 mice (20-25 g) were grouped into three (8 nos/group). All the animals were injected with B16F10 melanoma cells (10^6 cells/animal) through lateral tail vein. Group I, was treated with 10 doses of limonene. Group II treated with 10 doses of paraffin oil and group III kept as untreated. The animals were sacrificed on 21st day lung were excised and serum was collected. The lungs were used for the morphological examinations of metastatic tumour nodule appearing on the surface and for the estimation of collagen hydroxyproline content (14) hexosamine content (15) and uronic acid content (16). Serum was used for determining the sialic acid levels (17) and gammaglutamyl transpeptidase (GGT) levels(18) .

Effect of naturally occurring monoterpene limonene on the survival rate of metastatic tumour bearing animals.

A similar set of experiment was conducted and the death of animals due to tumour burden

was recorded. The increase in life span compared to the control animals was calculated using the formula T-C/Cx 100 where T is the life span of treated and C is life span of tumour alone animals.

*Histopathological analysis.*Lung tissues were fixed in 10% formalin, dehydrated and embedded in paraffin wax. Section (4 μm) were stained with eosin and hematoxylin.

Statistical analysis. Experiments were performed twice and the results were expressed as mean ± standard deviation .Statistical evaluation was done by student's t-test.

RESULTS

Effect of monoterpene limonene on the metastatic lung tumour nodule formation. The effect of monoterpenes on the inhibition of pulmonary tumour nodule is shown in table I. Untreated control animals developed massive number of tumour nodules. Administration of monoterpene limonene could remarkably inhibit the tumour nodule formation (65%).

Effect of monoterpene limonene on the survival rate of metastatic tumour bearing animals.

The effect of monoterpene on the survival rate of tumour bearing mice is shown in table I. There was an increase in the life span of animals treated with monoterpene limonene. The increase in life span for treated animals (50.7%).

Effect of monoterpene limonene on lung collagen hydroxyproline hexosamine and uronic acid content.

The effect of monoterpene limonene on lung collagen hydroxyproline hexosamine and uronic acid content as shown in table II. Control animals showed an increased level of lung collagen hydroxyproline (20.9 μg/mg protein) which was significantly reduced in animals treated with limonene (7.5 μg/mg protein).

The uronic acid levels in the lungs was significantly lowered in limonene (164 μg/100 mg tissue) treated animals compared to the control animals (373 μg/100 mg tissue). The effect of monoterpenes limonene on hexosamine levels in lungs is given in table II. The hexosamine content was lowered in limonene (0.88 μg/100 mg tissue) treated animals compared to the control (2.08 μg/ 100 mg tissue).

Effect of naturally occurring monoterpene limonene on serum sialic acid levels.

The effect of serum sialic acid levels of metastatic tumour bearing animals is given in table III. The serum sialic acid levels were high in the case of control animals (126.8 μg/ml serum) which was significantly lowered to 49.3 μg/ml serum by the administration of limonene.

Effect of naturally occurring monoterpene limonene on serum GGT levels.

The effects of limonene on serum GGT levels are shown in table 3. GGT levels were higher in control animals (115.8 nmol p-nitroaniline/ml serum) which was significantly reduced to 58.04 nmol p-nitroaniline/ml serum by limonene administration.

Effect of naturally occuring monoterpene limonene on the lung architecture.

Lungs in the control animals (Fig.1a) showed infiltration of the neoplastic cells around the main bronchioles extended to the pleura. Metastatic tumour bearing animals treated with terpenoid compound limonene showed a significant reduction in tumour mass. Aveoli and pleura were remarkably tumour cell free in the case of limonene (Fig.1c) treated groups.

DISCUSSION

In the present study we analysed the effect of monoterpene limonene on the inhibition of lung metastasis induced by B16F-10 melanoma cells. Invasion and metastais are the most insidious and life threatening aspect of cancer. Metastasis of cancer cells is a threat to the available cancer therapy and is a major reason for the treatment failure. Medicines available presently are cytoreductive drugs which are non-specific.

Earlier we showed that metastasis could be inhibited by phenolic compounds such as catechin and curcumin (19) which were found to produce artificial cross links with collagen (20). Several other naturally occurring compounds such as viscum album (21) diallyl sulphide, diallyl disulphide and allyl methyl suphide (22) have shown to possess antimetastatic activity. Monoterpenes are one of the most promising group of compounds of plant origin and they have shown to possess anticarcinogenic activity (23). Most of them used as chemopreventive agents (24).

Introducing highly metastatic B16F-10 melanoma cells into C57BL/6 mice through the tail vein can produce metastatic tumour colonies on lungs. The intraperitoneal administration of monoterpene limonene to metastatic tumour bearing animals produced 65% reduction in the pulmonary lung tumour nodule formation in limonene treated animals. During lung fibrosis collagen is deposited in the lungs. The index of lung fibrosis was evaluated by estimating the lung collagen hydroxyproline content. The characteristic of this defect is an increased accumulation of extracellular matrix proteins in the aveolar walls specifically collagen resulting in a reduction in pulmonary function. The treatment of limonene was more effective in reducing lung fibrosis, which was confirmed by the lowered levels of lung collagen hydroxyproline content.

Sialic acid, a family of acetylated derivative of neuraminic acid occurs as terminal component of carbohydrate chains of glycolipids and glycoproteins. Increased level of ^{2}1-6 branched N linked oligosaccharides have been observed in many metastatic tumour cells (25) and progression from a tumorigenic to metastatic phenotype in both rodent and human cancers has been associated with corresponding up regulation of sialic acid content (26).Uronic acid and hexosamines are the basis structures of sialic acids (27). The index of tumour cell proliferation was also evaluated by estimating hexosamine and uronic acid contents. The lungs of the metastatic tumour bearing animals showed higher levels of uronic acid and hexosamine. Administration of limonene significantly reduced the elevated contents of uronic acid and hexosamine. Total sialic acid levels in serum have been recognized as a valuable non specific monitor of tumour burden in melanomas.(28). The increased sialic acid level s in the control metastatic tumour bearing animals was significantly reduced in the animals treated with monoterpene limonene.

Highly elevated levels of GGT are observed in patients with either primary or secondary neoplasms. GGT, a marker of cellular proliferation was increased in the serum of tumour bearing animals compared to normal animals. Here we considered GGT as a metastatic tumor marker. The increased serum GGT levels were reduced by treatment with limonene. All of these results correlated with the histopathological analysis and increase in life span of treated animals. The above experimental evidences show

that the monoterpene limonene could inhibit the metastatic growth of B16F-10 melanoma cells in mice model.

The exact mechanism of action of the monoterpenes limonene in the inhibition of metastasis is not clearly known at present. These are several reports on the inhibition of farnelysation of ras oncoprotenes by certain monoterpenes (29). Further detailed investigations are needed to determine the exact mechanism of the antimetastatic activity of the monoterpenes limonene.

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Table I :Effect of Monoterpene limonene on lung tumour colony formation and survival rate

Treatment	% inhibition on lung tumour formation	% of increase in life span (T-C/C x 100)
Tumor alone	0%	0%
Vehicle	0%	0%
Limonene	65%	50.7%

B16F-10 melanoma cells ($1x10^6$) were injected in to each animal via lateral tail vein. Monoterpene limonene was administered intraperitoneally for 10 days. Control animals were treated with vehicle paraffin oil. Animals were sacrificed on 21st day and lung tumour nodules counted. For survival study death due to tumour burden was recorded, and the life span was calculated.

Table 2 : Effect of monoterpene limonene on lung collagen hydroxyproline, hexosamine and uronic acid levels of metastatic tumor bearing animal

Treatment	Lung collagen hydroxy-proline(μg/mg protein)	Hexosamine (mg/100 mg tissue)	Uronic acid (μg/100 mg tissue)
Normal	1.9 ± 0.1	0.4 ± 0.01	25.4 ± 2.6
Tumor alone	20.9 ± 1.8	2.2 ± 0.25	373 ± 52.4
Vehicle	21.58 ± 4.7	2.2 ± 0.26	352 ± 50.8
Limonene	7.5 ± 1.1*	0.88 ± 0.17*	164.5 ± 13.1*

B16F-10 melanoma cells ($1x10^6$) were injected into each animal via lateral tail vein. Limonene was administered intraperitoneally for 10 days. Control animals were treated with vehicle paraffin oil. Animals were sacrificed on 21st day and lung excised.

±SD(Standard Deviation)
* $P < 0.001$

Table 3 : Effect of monoterpene limonene on serum sialic acid and serum GGT levels of metastatic tumour bearing animal

Treatment	Serum sialic acid (ug/ml serum)	GGT (10^{-9} mol p-nitraniline/ ml serum)
Normal	21.3 ± 0.5	24 ± 0.17
Tumor alone	126.8 ± 10.3	115.8 ± 7.16
Vehicle	109 ± 4.1	131.66 ± 20
Limonene	49.3 ± 4.7*	58.04 ± 23*

Experimental design is as given in table I. Animals were sacrificed on 21st day blood was collected by heart puncture and serum separated.
±SD(Standard Deviation)
* $P < 0.001$

1(a) Control 1(b) Normal 1(c) Limonene treated

Fig.1 Histopathological analysis

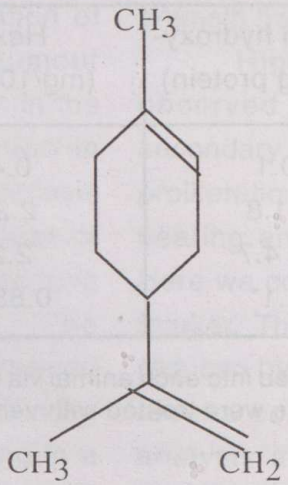

Fig.2 Structural formula of limonene

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ROLE OF EXTRA CELLULAR MATRIX IN ANGIOGENESIS

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The mammalian organs are divided into a series of tissue compartments separated by the extra cellular matrix (ECM) unit consisting of the basement membrane (BM) and its underlying interstitial stroma. The stroma is present in spaces between epithelial, endothelial, and smooth muscle cells and BMs are located closely associated with the cell's surface. Cells grow, move, differentiate and function in intimate contact with ECM. The ECM is secreted locally and assembled into a network in the space surrounding the cells. Several types of macromolecules are associated with ECM that includes fibrous proteins like collagen and elastin, adhesive glycoproteins such as fibronectin and laminin, proteoglycans and hyaluronans. In 1938, Clark et al were among the first to suggest a possible role for ECM in angiogenesis. In recent years the role of ECM in angiogenesis and the enzyme system responsible for its continuous remodeling have received considerable attention (1).

Early studies of the ECM investing new capillaries revealed that angiogenesis was dependent on the synthesis of key ECM molecules and precise degradation of specific components in the BM (2-4). *In vitro* studies of the organization of new capillaries derived from primary endothelial cell (EC) cultures or endothelial cell lines has revealed the importance of ECM in the morphology, proliferation, cytoskeletal organization and shape of EC. (5,6,7) The ECM transmits a number of important morphogenic signals to EC and growing capillaries. Alterations in the secretion of collagen or the deposition and assembly of other ECM proteins have been shown to promote the regression of growing capillaries in several model systems (5-12). The ECM has been shown to induce changes in gene expression and the secretary phenotype of EC during morphogenesis. These include, among other changes alteration in fibronectin, laminin and collagen during tubular reorganization, initiation of the expression of Type I collagen, and the up regulation of a secreted protein acid rich in cysteine (SPARC). (8,13,14). It has been shown that in cultures of EC that spontaneously give rise to capillary-like structure; type I collagen is a necessary substrate for the attachment and spreading of endothelial cell during the formation of tubes. SPARC is thought to play an important role in inducing changes in the cytoskeleton that promote cell rounding and migration and initiate reorganization of endothelial cell.

Angiogenesis is a tremendously complex and intricate process and is essential in normal physiology and progression of certain pathology like cancer. It is a multi step process and involves complex interactions among endothelial cells (EC), immune/inflammatory cells and ECM. The process begins with local degradation of basement membrane and invasion of EC into the perivascular space and adjoining matrix to form a

'perivascular fibrin matrix' (capillary sprout). The provisional fibrin matrix probably provides a more fluid matrix that is supportive of the angiogenesis process (15). These sprouts elongate by further EC migration at the tip and proliferation at the base. Subsequently remodeling occurs, BM is laid out, pericytes smooth muscle cells and other vessel supporting cells are recruited and a patent vessel is formed. Ultimately the tip of these tubes connects to create loops that are capable of conducting blood flow.

Endothelial cells are normally quiescent while they are bound to capillary BM, which is composed of type IV collagen, laminin, heparan-sulphate proteoglycans (HSPG), perlecans, nidogen/entactin, SPARC/BM-40/osteopontin, type XV collagen, type XVIII collagen and other molecules. This indicates that the primary signals originating from capillary BM inhibit proliferation and promote an environment that facilitates appropriate cell-cell adhesion (16). It is important to remember that the structure of the vascular BM is quite complex at this stage. It is highly cross-linked, and only certain domains of various constituents are exposed to (and can interact with) endothelial cells in this assembled form. By contrast, when the BM constituents are being assembled or disassembled, endothelial cells interact with the different domains of these molecules. This is an important concept, because it means that the same set of proteins, in different structural configurations, can impart distinct functional influences on the vascular endothelial cells at different stages of the angiogenic process. The angiogenic response is induced by growth factors such as vascular endothelial growth factor (VEGF), basic Fibroblast Growth Factor (bFGF), platelet-derived growth factor (PDGF), chemokines and others. First, the capillary BM is degraded by several matrix-degrading enzymes (17-19), such as MMPs. During tumour angiogenesis, the immune cells that accumulate around the neoplastic cells even before angiogenesis is initiated can also produce these factors. This vascular BM degradation serves multiple purposes, which include the liberation of endothelial cells to migrate and proliferate from their cell-surface anchors (integrins), the liberation of sequestered growth factors (VEGF and bFGF) and the detachment of the pericytes that surround and support the blood vessels.

INTEGRINS

Integrins have been shown to function *in vivo* in vasculogenesis and angiogenesis. Injection of a neutralizing antibody against the β_1 subunit blocked formation of an aortic lumen in quail embryos (20). Brooks et al. (21) have provided evidence that $\alpha_v\beta_3$ is required for blood vessel growth. An antibody (LM609) against the $\alpha_v\beta_3$ integrin complex inhibited normal vessel growth, and also FGF-2-stimulated or tumor-induced angiogenesis in the CAM assay, but did not disrupt pre-existing vessels. The mechanism by which anti $\alpha_v\beta_3$-mAb disrupts angiogenesis appears to involve apoptosis. A single intravascular injection of a cyclic RGD peptide antagonist of $\alpha_v\beta_3$ integrin, or of the LM609 monoclonal antibody, led to the rapid regression of human tumors transplanted into the CAM (22). Although antibody against $\alpha_v\beta_3$ inhibited angiogenesis induced by FGF-2, anti-$\alpha_v\beta_3$ antibodies inhibited angiogenesis induced by VEGF, suggesting that integrins used to produce new blood vessels can differ, depending on the angiogenic stimuli (23).

MATRIXMETALLOPROTEINASES (MMPS)

Matrix metalloproteinases are a family of

zinc-dependent endopeptidases. MMPs are classified into subfamilies based on their substrate preferences. Those include gelatinases, collagenases, stromelysins, elastases, MT-MMPs (membrane type-MMPs) and a group of unnamed members, in which gelatinases (MMP 2 and MMP 9) are important in angiogenesis aspect. The members and substrates of MMPs are shown in Table 1.

The MMPs are synthesized as inactive Zymogens (Pro-MMPs). They are kept inactive by an interaction between a cysteine-sulphydryl group in the propeptide domain and the Zinc ion bound to the catalytic domain: activation requires proteolytic removal of the propeptide domain. Most of the MMPs are activated outside the cell by other activated MMPs or serine proteinases. (24).

MMPs could regulate angiogenesis. Both endogenous and synthetic MMP inhibitors reduce tumour angiogenesis in animal experiments (25,26) indicating that MMPs are important positive regulators. MMPs might simply act by degrading the ECM, which would allow endothelial cells to invade the tumour stroma. Indeed, cleavage of collagen type-I is required for endothelial-cell invasion of the- ECM and for vessel formation (27). MMP-2, -9 and -14 directly regulate angiogenesis, and MMP-19 might also be important as it is expressed in blood vessels (28). Down regulation of MMP-2 expression in cancer cells decreases angiogenesis in a chicken chorioallantoic membrane model (29). Furthermore, tumour angiogenesis and growth is reduced in MMP-2 deficient mice compared with wild type mice (30). Cleavage of collagen type IV by MMP-2 exposes a cryptic, $\alpha_v\beta_3$ integrin binding site within the collagen. Blockage of this new site with an antibody decreases migration of endothelial cells and in vitro angiogenesis (31). MMP -9 has been shown to be important for angiogenesis in transgenic model of tumour progression — the K14-HPV 16 Skin Cancer model (32). MMP-9 acts by increasing the bioavailability of the proangiogenic factor VEGF (33), although it is not known exactly how. MMP-14 is also thought to promote tumour angiogenesis: antibodies directed against the catalytic domain of MMP-14 block endothelial-cell migration, invasion and capillary-tube formation *in vitro*. MMP-14 can degrade the fibrin matrix that surrounds newly formed vessels (34), thereby potentially allowing the endothelial cells to invade further into the tumour tissue, and MMP -14 and MMP -9 null mice have impaired angiogenesis during development (35,36), supporting a role for these MMPs in angiogenesis.

MMPs also produce fragments that are angiogenesis inhibitors. Cleavage of Plasminogen by MMP -2, -3, -7, -9, -12 generates angiostatin (37-39), and MMP-3, -9, -12, -13 and -20 might be involved in the generation of endostatin, a C-terminal fragment of the basement membrane collagen type XVIII (40). Both angiostatin and endostatin reduce endothelial –cell proliferation and, in addition, endostatin might inhibit endothelial –cell invasion by acting as an inhibitor of MMP-14 and MMP- 2 (41). MMP-12 might also inhibit tumour angiogenesis by cleavage and shedding of cell surface bound urokinase type plasminogen activator reseptor, which is required for endothelial cell invasion into fibrin (42).

The detached endothelial cells are now in direct contact with interstitial provisional matrix components, such as vitronectin, fibronectin, type I collagen and thrombin. Pro-angiogenic growth factors induce endothelial cells to produce many of these matrix molecules (43). So, 'provisional' matrix provides proliferative cues to the endothelial cells, whereas the 'assembled' BM matrix provides

growth-arresting cues. Additional structural studies are required to evaluate the molecular identity of such cues, but the induction phase and resolution phase of angiogenesis are controlled by the temporally ordered structure of the matrix and its compositional changes.

SUMMARY

Vascular BM and stroma components are required for the initiation and resolution of angiogenesis. The precisely controlled interactions during angiogenesis involve ECM proteolysis and these proteolytic activities also activate and/or release important angiogenic cytokines such as VEGF and bFGF. Although most of these components sustain the growth, survival and health of vascular endothelium, 'cryptic domains' (these domains are hidden within a folded or assembled protein structure) within these large proteins also possess antiangiogenic activities. In addition biologically active degradation products of ECM such as angiostatin from plasminogen, endostatin from collagen can regulate angiogenesis. A balance of proteases and antiproteases, in a highly regulated spaciotemporal pattern is required for proper neovessel formation and remodeling. In this regard, proteases, matrixmetalloproteinases (MMPs) and vascular integrins have encouraged as key mediators of angiogenic and angiostatic actions mediated by ECM. Macrophages and mast cells also involve during induction and propagation of angiogenic cascade and mediate this effects through secretion of cytokines and growth factors, release of proteases and activation of fibroblasts. Fibroblasts are chiefly responsible for production of ECM and release of MMPs for selective degradation and organization of ECM.

Table 1. The matrix metalloproteinase family.

Subfamily	Name	MMPs	Main substrates
Interstitial Collagenases	Fibroblast Collagenase	MMP-1	Fibrillar collagen
	Neutrophil Collagenase	MMP-8	Fibrillar collagen
	Collagenase-3	MMP-13	Fibrillar collagen
	Collagenase-4	MMP-18	Fibrillar collagen
Gelatinases	Gelatinase A	MMP-2	Gelatin, type IV collagen, fibronectin, elastin, laminin
	GelatinaseB	MMP-9	Gelatin, elastin, fibronectin, vitronectin
Stromelysins	Stromelysin-1	MMP-3	Gelatin, fibronectin, casein, laminin, elastin, MMP-2/ TIMP-2
	Stromelysin-2	MMP-10	same as above
	Stromelysin-3	MMP-11	Fibronectin, laminin, gelatin, aggrecan
	Matrilysin	MMP-7	Fibronectin, vitronectin, laminin, gelatin, aggrecan
Elastases	Metalloelastase	MMP-12	Elastin, gelatin, collagen IV, fibronectin, laminin, vitronectin, proteoglycan
Membrane-Type MMPs	MT1-MMP	MMP-14	proMMP-2, procollagenase 3
	MT2-MMP	MMP-15	pro-MMP-2
	MT3-MMP	MMP-16	proMMP-2
	MT4-MMP	MMP-17	Unknown
Other MMPs		MMP-19	
	Enamelysin	MMP-20	Amelogenin

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IMMUNOMODULATORY ACTIVITY OF ALLYL ISOTHIOCYANATE

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ABSTRACT

Administration of Allyl isothiocyanate (AITC) was found to stimulate the immunological response in Balb/c mice. Treatment with five doses of AITC (25μg/dose/animal,i.p.) was found to enhance the total WBC count13,100cells/mm^3 on 9th day. Bone marrow cellularity (22.36 x 10^6 cells/femur) as well as α-esterase positive cell number also increased after treatment with AITC(1397cells/ 4000cells). Treatment with AITC along with the antigen (SRBC) produced an enhancement in the circulating antibody titre and the number of plaque forming cells (PFC) in the spleen. Maximum number of PFC (1329PFC/10^6 spleen cells) was obtained on the 5th day. These results indicate the immunomodulatory activities of the naturally occurring isothiocyanate, Allyl isothiocyanate (AITC)

Key Words:
Allyl isothiocyanate, Immunomodulation, immunosupression

INTRODUCTION

The ability of tumour cells to escape from the immune surveillance mechanism of the body is one of the major reasons for the rapid progression of human cancers . Cancer cells may secrete immunosuppressive factors to modify the host immune responses. These factors can suppress immune responses, thereby impairing the inflammatory responses, chemotaxis of phagocytes and the complementary cascade.

Most of the cancer chemotherapeutic drugs have side effects. Immunosuppression is one of the major side effects of chemotherapy as well as radiation therapy (1). Side effects produced by both of these conventional therapies of cancer are nausea, vomiting, mucosal ulceration, etc. Drug that could alleviate these side effects will be highly useful in cancer treatment. Use of plant products as immunomodulators getting more and more importance in the field of cancer research. Some of the plants with known immunomodulatory activities are *Viscum album* (2), *Panax ginseng* (3), *Tinospora cordifolia* (4) etc. Components such as polysaccharides, lectins, (5), proteins and peptides (6) present in plants have been shown to stimulate the immune system. Administration of an extract from the powdered root of the plant *Withania somnifera* was found to stimulate immunological activity in Balb/c mice (7).

Isothiocyanates, which occur in cruciferous vegetables as their glucosinolate conjugates are considered to be broad spectrum inhibitors of metabolic activation of several nitrosamines including the tobacco-specific nitrosamines (NNK) and N'nitrosornicotine. Isothiocyanates have been shown to be chemopreventive agents for carcinogenesis. Phenyl and benzyl isothiocyanates inhibited carcinogenesis induced by carcinogens such as diethyl nitrosamine (DEN),

dimethyl benzo(a)anthracene(DMBA) or benzo(a)pyrene(BP)(8).

Green and yellow vegetables including cabbage, Brussels sprouts and other cruciferous vegetables contain several organosulphur compounds including isothiocyanates and dithiolethiones (9). It has been shown that the organosulphur compounds inhibit chemically induced tumorigenesis in fore stomach (10), lung (11), oesophagus (12), liver and colon (13).

So far no systematic studies have been reported to prove scientifically the immunomodulatory effects of Allyl isothiocyanates (AITC) Therefore, in the present study, we aimed to analyze the immunomodulatory activities of this naturally occurring isothiocyanates in normal Balb/c mice.

MATERIALS AND METHODS

Animals:

Balb/c mice (20-25g) were purchased from National institute of Nutrition, Hyderabad, India. Animals were kept in well ventilated cages and fed with normal mouse chow (Sai, India) and water *ad libitum*.

Sheep red blood cells (SRBC) was collected in Alsever's solution from local slaughter house. Pararosaniline hydrochloride and α-naphthyl acetate were purchased from Glaxo India Ltd. Bombay. Allyl isothiocyanate (AITC) was purchased from Merck- Schuchardt, Munchen, Germany.

Drug administration

Allyl isothiocyanate was suspended in light liquid paraffin and administered intraperitoneally at a dosage of 25μg/dose/animal in all experiments.

Determination of the effect of isothiocyanates on the haematological parameters

Two groups (6Nos./group) of Balb/c mice were used for the study of which one group was treated with Allyl isothiocyanate (AITC) consecutively for five days. The second group was kept as untreated control. Blood was collected from caudal vein and parameters such as total WBC count (haemocytometer), differential count (Leishman's stain) and haemoglobin level (cyanomethemoglobin) were recorded prior to drug administration and continued every third day for 30 days.

Determination of the effect of allyl isothiocyanate on the organ weights

Two groups of Balb/c mice (6Nos/group) were used in this study. One group was kept as untreated control. The other group was treated with AITC for five consecutive days. Body weights of the animals were recorded before sacrifice. Animals were sacrificed 24h after the last dose of drug and weight of the vital organs such as liver, spleen, thymus, lungs and kidneys were recorded and expressed as relative organ weights.

Determination of the effect of allyl isothiocyanate on the circulating antibody titre

Balb/c were divided into two groups (6 Nos./group). Group I was treated with five consecutive does of allyl isothiocyanate. Along with the 5th dose, all animals were immunized with SRBC (0.1ml, 20%). Group II animals were treated with SRBC only and kept as control. Blood was collected from the caudal vein every third day after drug administration and continued for a period of 30 days. Serum was separated, heat inactivated at 56^{0}C for 30 minutes and used for the estimation

of antibody titre by the method of Singh et al (3) using SRBC as antigen.

Determination of the effect of allyl isothiocyanate on the antibody producing cells

Balb/c mice were divided in to 2 groups (6 nos./group) and all the animals were immunized with SRBC (2.5x 10^8 cells). Group I animals were kept as control with out any drug treatment. Group II animals were treated with 5 consecutive doses of AITC(25μg/dose/animal) and SRBC was administered along with the 5th dose of the drugs. The animals were sacrificed on different days starting from the third day after immunization up to the 9th day, spleen was processed to single cell suspension and the number of plaque forming cells was determined by the Jerne's plaque assay (14).

Determination of the effect of allyl isothiocyanate on bone marrow cellularity and α-esterase activity:

Balb/c mice were divided into 2 groups (3 Nos/group). One group was kept as control with out any drug treatment and the other group was treated with five consecutive doses of Allyl isothiocyanates (25μg/dose/animal). Animals were sacrificed 24h after the drug treatment and the bone marrow cells were collected from the femur into the medium containing 2% FCS (foetal calf serum). The bone marrow cell number was determined using a haemocytometre and expressed as total live cells /femur. Bone marrow cells from the above preparation was smeared on clean glass slides and stained with Harris haematoxylin to determine the non-specific α-esterase activity according to the method of Bancroft and Cook (15).

STATISTICAL ANALYSIS

All experiments were performed twice and the results were expressed as mean ± standard deviation. Significance was calculated by the student's 't' test.

RESULTS

Effect of allyl isothiocyanate on the haematological parameters

Administration of AITC increased the total WBC count in normal Balb/c mice (Figure-I). The maximum WBC count obtained in the AITC treated animals was 13,100 cells/mm^3 on 9th day. Haemoglobin content was also increased in AITC treated group from 17.10g/dl to 20.6g/dl on the 12th day. However, administration of AITC produced no effect on lymphocyte-neutrophil ratio. (Data not shown).

Effect of allyl isothiocyanate on the organ weight

Effect of AITC on the organ weight is given in Table I. There was increase in the weight of thymus in AITC treated animals(0.17±0.01g/100g body wt.) compared to normal (0.08±.04g/100g body wt.). The size and weight of spleen was also enhanced significantly by the administration of AITC (0.37±0.03g/100 body wt.) compared to normal (0.33g/100g body wt.). The weight of kidney is slightly increased by the treatment of AITC (1.22±0.22g/100g body weight) compared to normal (1.15±0.06g/100g body weight). There was no significant change in the weight of other vital organs such as liver, and lung.

Effect of allyl isothiocyanate on the circulating antibody titre

The effect of AITC on the circulating

antibody titre is shown in Figure II. The maximum titre value of 1024 and 512 was observed on 12th day in AITC treated animals. The control animals showed the maximum antibody titre value of only 128 on the 15th day.

Effect of allyl isothiocyanate on the antibody producing cells

Administration of AITC was found to significantly enhance the number of antibody producing cells in spleen (Figure-III). The maximum number of antibody producing cells in AITC treated group (1329 cells/10^6 spleen cells) was observed on the 5th day while control animals had a maximum of 268 plaque forming cells/10^6 spleen cells.

Effect of allyl isothiocyanate on the bone marrow cellularity and α-esterase positive cells

The effect of AITC administration on the bone marrow cellularity and ±-esterase positive cells is given in Table-II. Administration of AITC increased the bone marrow cellularity to 22.36x 10^6 cells/femur, while in normal group it was only 18.6x10^6 cells/ femur. The number of α-esterase positive cells was also found to be increased in AITC (1397/4000cells) treated animals compared to controls (911 cells/4000cells).

DISCUSSION

Immunosupression is a major drawback in conventional therapies of cancer such as radiation and chemotherapy (1). Both of these methods have side effects such as nausea, vomitting, alopecia, etc. Drugs that do not have these side effects will be highly useful in cancer therapy. Use of plant products, as immunomodulators is still an interesting area in the research field. Some plant products such as *Viscum album* extract (6) and the powdered extract of the plant *Withania somnifera* (7) are highly suggestive remedies in immunosuppressive conditions as proved in our laboratory. Some herbal preparations, such as Rasayanas, which are used in the indigenous system of medicine, also have been proved to possess immunomodulatory activities (16).

Here, in the present study, immunomodulatory activities of a naturally occurring isothiocyanatehave been explored. The effects of thiscompound on total WBC count and bone marrow cells significantly indicating that this compound could stimulate the blood cell forming machinery of the body. Stem cell differentiation is also stimulated because there is an increased presence of α-esterase positive bone marrow cells in AITC treated group. AITC could stimulate the humoral immune response, as there is a significant enhancement in the circulating antibody titre and plaque forming cells. The increased titre remained several days indicating that there is a sustained immunological activity even after the treatment. Treatment with AITC could also stimulate the production of immune cells, as there is a marked enhancement in the weight of spleen and thymus. The above results indicate that this naturally occurring isothiocyanate stimulate the stem cell proliferation and its differentiation. These might play an important role as immunostimulant and could be used as adjuvants during cancer therapy.

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Table 1 : Effect of AITC and PITC on relative organ weights[a]

Treatment	Relative organ weights (g/100g body. wt.)				
	Spleen	Thymus	Liver	kidney	Lungs
AITC	0.37±0.03	0.17±0.01*	4.85±0.17	1.22±0.02	0.57±0.04
Normal	0.33±0.02	0.08±0.04	4.47±0.96	1.15±0.06	0.60±0.19

[a]Animals were treated with five doses of AITC (25mg/dose/animal)
*P<0.001

Table 2 : Effect of AITC on bone marrow cellularity and α-esterase activity[a]

Treatment	Bone marrow Cellularity (cells/femur)	α-esterase activity (No.of α-esterase positive cells/4000cells)
Normal	18.16 x10^6 ±0.24	911± 39.4
AITC	22.36 x 10^6±0.83	1397.3± 61.9*

Treated animals received five doses of AITC (25µg/dose/animal)
*P<0.001

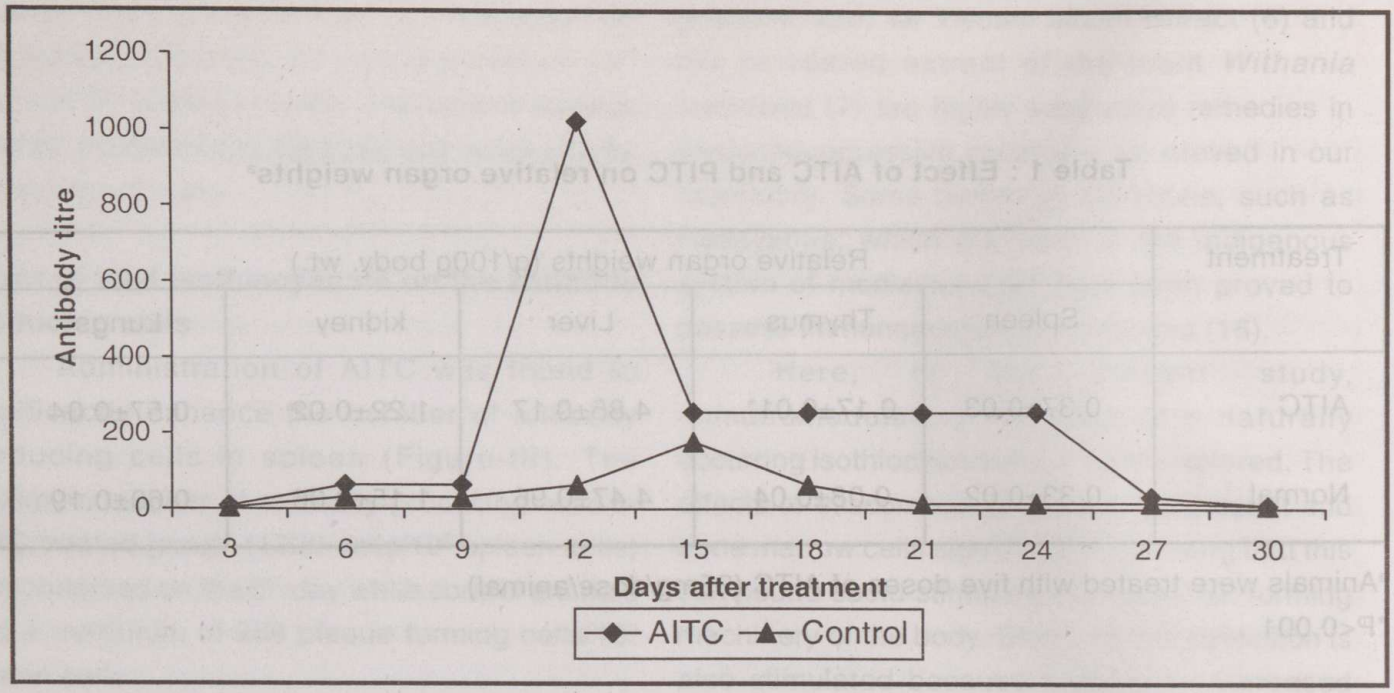

Fig. 2 : Effect of AITC on antibody titre

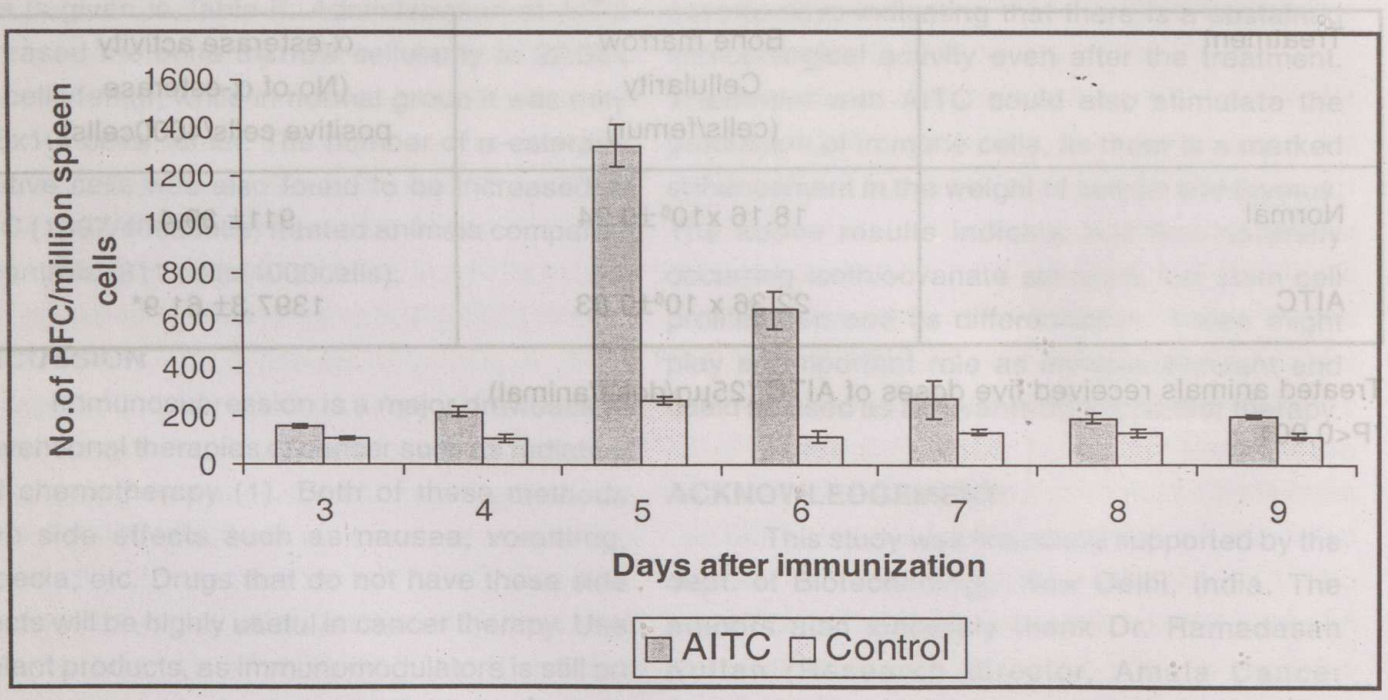

Fig. 3 Effect of AITC on plaque forming cells in spleen

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EFFECT OF PIPERINE ON THE INHIBITION OF NITRIC OXIDE (NO) AND TNF-α PRODUCTION.

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ABSTRACT

Effect of piperine which is an alkaloid present in plants such as *Piper nigrum and Piper longum* on the production of NO and TNF- ± level was analyzed using *in vitro* as well as *in vivo* systems. The level of nitrite in the LPS stimulated Balb/C mice (95.3 μM) was reduced in the piperine treated animals (25 μM) significantly. Nitrite level in the Con-A treated control animals (83.1 μM) was also significantly reduced to 18.5 μM in the piperine treated mice. The drastically elevated levels of TNF-α in the LPS stimulated animals (625.8 ρg/ ml) was lowered in the piperine treated animals (105.8 ρg/ ml). Piperine also inhibited the Concanavalin-A induced TNF-α production. Piperine could inhibit the nitrite production by in vitro activated macrophages (116.25 μM) to the normal level (15.67 μM) at concentration of 5 μg/ ml.

Key words: Piperine, TNF-α, LPS, Nitric oxide, Concanavalin A.

INTRODUCTION

Nitric oxide (NO) is a highly reactive molecule because it is considered to be an important defense mechanism in the nonspecific immune system of the body (1). Although NO acts as an important mediator of tissue damage in inflammatory diseases, elevated NO levels are thought to play a central role in tissue damage observed during septic shock (2,3).Inducible nitric oxide synthase (iNOS) is the enzyme responsible for the enhanced nitric oxide production during inflammation. NO has the potential to interact with oxygen, metals and other free radicals (4) which also can form peroxynitrite ($ONOO^-$) and dinitrogen trioxide (N_2O_3), following an interaction with the haeme proteins represents the activation of soluble guanyl cyclase and cyclooxygenase (COX). This interaction is important in the regulation of a proinflammatory process (5,6,7). High concentrations of NO have deleterious effects, so it is necessary that the production of NO is tightly regulated (8).

Tumor necrosis factor- α (TNF- α) is produced by activated macrophages and monocytes. TNF- α can induce various immune responses to cells as the result of the release of soluble mediators such as PGE_2, leukotrienes, platelet-activating factor, NO, reactive oxygen intermediates, induction of gene expression and cytotoxicity. Depending on the cell type, one or more of these effects can be observed and these responses may be interrelated (9).

Lipopolysaccharide (LPS) is a major component present in the cell wall of gram negative bacteria. LPS is presumably the most efficient and certainly the most studied inducer of TNF- α (10). Concanavalin-A (Con-A) is a lectin isolated from *Canavalia ensiformis*(Jack bean). Con A-have a three dimensional structure, which

has been helpful in interpreting experiments on lymphocyte mitogenesis. (11,12). So it may activate T-lymphocytes and induces the release of IFN-^{3} and other cytokine production (13).

Black pepper (*Piper nigrum*) is being used as spice from ancient time throughout the world. Black pepper and long pepper (*Piper longum*) also have been used as an important component of indigenous system of Indian medicines for the treatment of bronchitis , gastro-intestinal diseases, rheumatism , neuralgic pain and asthma (14,15). One of the major component of pepper is an alkaloid piperine (1-piperoyl piperidine) which has been reported to enhance the bioavailablity of drugs by inhibition of glucuronidation in the liver (16) and small intestine (17). Piperine is also a potent inhibitor of mixed function oxygenase system also (18). The present study was designed to investigate the effect of piperine on the inhibition of NO and TNF- α production in both *in vitro* as well as *in vivo* systems.

MATERIALS AND METHODS

Animals

Four to six week old Balb/c mice were purchased from National Institute of Nutrition, Hyderabad. The animals were fed with normal mouse chow (Sai feeds, India) and water *ad libitum*. MEM and RPMI-1640 were purchased from the Hi-media Lab, Mumbai. Con A was purchased sigma chemicals. TNF-α elisa kit was purchased from Assay designs Inc, USA.

Dug Usage

Piperine was a gift from Dr. Majeed. M, Sami chemicals, Banglore. The drug was suspended in 0.1% gum acacia for *in vivo* studies. For *in vitro* studies the drug was suspended in DMSO(stock preparation) and further diluted with the medium.

Effect of piperine on the production of NO and TNF- α by macrophages (*in vitro*)

Macrophages were elicited by injecting 5% sodium caseinate intraperitoneally in Balb/c mice. Peritoneal macrophages were isolated by lavage from peritoneum with 5 ml of sterile saline. Macrophages were washed with PBS and resuspended in RPMI-1640 with 10% FCS. The cells were plated in 96 well culture plate and incubated for 2 hours at 37^{0} C, in a 5% CO_2. After incubation non adherent cells were removed and the adherent macrophages were incubated (2 X 10^5 cells/ well) in complete culture medium (RPMI-1640, 10% FCS, 100 μg/ml streptomycin and penicillin, 2 mM glutamine). Macrophages were cultured with or with out LPS (5 μg/ml) in the presence and absence of piperine (2.5 μg/ml, 5 μg/ml) for 24 hours at 37^{0}C with 5% CO_2 . After 24 hours the plates were centrifuged and the supernatant was used for the estimation of NO and TNF- α.

Estimation of nitrite (In vitro)

Nitrite in the culture supernatant was determined by the Griess reaction (20). Briefly 100 μ sample was incubated with equal amount of Griess reagent (1 part of 0.1% of N (1-naphthyl)-diamine dihydrochloride in distilled water and 1 part 1% sulfanilamide in 5% concentrated H_3PO_4) and incubated for 10 min at room temperature. Absorbance were estimated at 540 nm. The amount of nitrite was calculated from the $NaNO_2$ standard curve.

Determination of the effect of piperine on the production of NO and TNF-α by the stimulated macrophages (*In vivo*)

Balb/c mice weighing 20-25g were separated into 5 groups (5 nos/group). Group-I animals were

treated with LPS (250 μg in 0.5 ml PBS) alone, group-II animals were treated with LPS (250 μg in 0.5 ml PBS) and piperine (200 μ moles/ Kg body weight/ animal/ dose) intraperitoneally. Group-III treated with Con-A (100 μg in 0.5 ml PBS) alone and group-IV treated with Con-A (100 μg in 0.5 ml PBS) and piperine (200 μ moles/ Kg body weight/ animal/ dose) intraperitoneally. Group-V was kept as untreated normal animals. Piperine treatment was started four days prior to the LPS and Con-A treatment. Six hours after the LPS and Con-A treatment blood was collected from the tail vein and serum was separated.

Effect of piperine on serum nitrite levels of *In vivo* stimulated animals

Nitrite level which is an indicator of NO synthesis was measured in serum after reducing nitrate to nitrite using bacterial nitrate reductase by microplate assay method based on Griess reaction (19). To the serum, equal amount of Griess reagent (1 part of 0.1% of N (1-naphthyl)-diamine dihydrochloride in distilled water and 1 part 1% sulfanilamide in 5% H_3PO_4) was added and incubated for 10 min at room temperature. The absorbance was determined at 540 nm(20). Amount of nitrite was calculated from the $NaNO_2$ standard curve.

Estimation of serum TNF-α by ELISA

Serum TNF-± was estimated using the ELISA kit according to the manufacture's procedure (21).

STATISTICAL ANALYSIS

Results are expressed as Mean±SD. The student's t- test was used to make a statistical comparison between the groups. Results with P< 0.001 were considered as statistically significant.

RESULT

Effect of piperine on the release of nitrite by activated macrophages (*In vitro*)

Effect of piperine on the release of nitrite by activated macrophages is shown in Table-3. Macrophage cultures stimulated with LPS (5 μg/ ml) for 24 hours produced elevated levels of nitrite (116.25 μM). Normal unstimulated macrophages produced low amount of nitrite (24.66 μM). Piperine at a concentrations of 2.5 μg/ml, 5 μg/ ml, reduced the nitrite production to 46.67 μM and 15.67 μM respectively in the LPS stimulated macrophages. This significant reduction indicates the dose dependent activity of piperine.

Effect of piperine on serum nitrite levels of LPS stimulated animals

Effect of piperine on the serum nitrite is shown in Table-1. Drastically elevated concentrations of serum nitrite after the LPS stimulation (95.3 μM) was significantly reduced to 25 μM in the piperine treated animals, which is similar to that of the normal animals (23 μM).

Effect of piperine on serum nitrite levels of Con-A stimulated animals

Effect of piperine on the serum nitrite levels of Con-A stimulated animals also shown in Table-1. Highly increased levels in the Con-A stimulated animals (83.1 μM) was significantly reduced in the piperine treated animals (18.5 μM).

Effect of piperine on the serum TNF-α level of LPS stimulated animals

Effect of piperine on the serum TNF-α level is shown in the Table-1. Elevated levels of serum TNF-α in the LPS stimulated animals (625.8 μg/ml) was significantly reduced in the piperine treated animals (105.8 μg/ml).

Effect of piperine on the serum TNF-α level of Con-A stimulated animals

Effect of piperine on the serum TNF-α is shown in Table-2. Elevated levels of serum TNF-α in the Con-A stimulated animals (585.3 ρg/ml) was significantly reduced in the piperine treated animals (85.8 ρg/ml).

DISCUSSION

The present study demonstrated the effect of piperine on the inhibition of nitrite and TNF-α both *in vitro* as well as *in vivo*. Earlier studies have shown that LPS can induce the production of TNF-α in macrophages (10). Con-A is a mitogenic lectin which can stimulate the production of IFN-γ by the T-lymphocytes. TNF-α might be directly involved in inducible nitric oxide synthase activation in macrophages and acting as an additional signal for synergistic induction of NO formation. There are some reports demonstrating the ability of endotoxin to stimulate the T-cells and induce the secretion of IFN-γ may also stimulate the cells and induce the production of NO and TNF-α (24).

Inducible nitric oxide synthase mediate NO production, which have critical role in carcinogenesis. Excessive amount of NO has been implicated in the pathogenesis of many disease processes like septic shock, cardiomyopathy, chronic degenerative disease and rheumatoid arthritis(6,27). Tumor associated monocytes and macrophages are release cytokines like TNF-α, which enhance the oxidative metabolism of human mononuclear phagocytes (28). This results in the release of reactive oxygen intermediates including superoxide and hydrogen peroxide, that may cause cytostasis or cytotoxicity (29).

It is reported that piperine inhibits the cytochrome P-450 activity and aryl hydrocarbon hydroxylase (AHH) activity (16). Cytochrome P-450 mediates the heterocyclic amine (HCA) formation. These heterocyclic amines are hepatocarcinogens and it also induce the colon, mammary gland tumors in animals (30). N-hexane derivatives of piperine have 5-lipoxygenase and cyclooxygenase –1 (COX-1) inhibitory potential. Constituents of pepper have inhibitory activity on prostaglandin and leukotriene biosynthesis *in vitro* (31). We don't know the exact mechanism of action of piperine on the inhibition of NO and TNF-α. Further studies have to be conducted to elucidate this activity.

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Table-1 : Effect of piperine on serum nitrite and TNF-α levels of LPS & Con-A stimulated animals

Treatment	Concentration of nitrite (μM)	Concentration of TNF-α(ρg/ml)
Normal	23.0 ± 0.18	34.0 ± 15.0
LPS (Control)	95.3 ± 5.8	625.8 ± 29.2
LPS+ Piperine	25.0 ± 4.9*	105.8 ± 11.3*
Con-A	83.1 ± 9.7	585.3 ± 23.02
Con-A+Piperine	18.5 ± 0.18*	85.8 ± 12.8*

Animals were treated with piperine, 4 days prior to the LPS or Con-A stimulation.
After 6 hours the blood was collected serum used for nitrite and TNF-α estimation estimation.
Values are expressed as Mean ± SD. *P<0.001 statistically significant from the control.

Table 2 : Effect of piperine on nitrite produced by LPS stimulated macrophages

Treatment	Concentration of nitrite ((μM)
Macrophage alone	24.66 ± 0.48
Macrophage + LPS	116.25 ±11.38
Macrophage +LPS+ Piperine (2.5μg/ml)	46.67 ± 5.25*
Macrophage +LPS+ Piperine (5 μg/ml)	15.67 ± 1.69*

Peritoneal macrophages were harvested and 2 X 10^5 cells were plated in 96 well culture plate. The cells were cultured in the presence and absence of LPS as well as piperine.
Values are expressed as Mean±SD. *p<0.001 statistically significant from the control.

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EFFECT OF *PIPER LONGUM* LINN. ON B-16 F10 MELANOMA CELLS INDUCED METASTASIS

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ABSTRACT

The effect of *Piper longum* extract on the inhibition of lung metastasis induced by B16F-10 melanoma cells was studied in C57BL/6 mice. The extract was administered by two different modalities. A reduction in tumor nodule formation was shown by simultaneous (47%) and prophylactic (41%) mode of administration. Increased lung collagen hydroxyproline (21.13 μg/mg protein) in the metastasised lungs of control animals compared to normal animals (0.98 μg/mg protein) was significantly reduced in treated animals. The elevated level of uronic acid (349.5 μg/100 mg tissue) and hexosamine contents (4.03mg/100mg lung lyophilised tissue) in metastatic control animals were significantly reduced in the animals treated with alcoholic extract of *Piper longum*. Similarly the elevated levels of serum sialic acid and serum gamma glutamyl transpeptidase activity in the untreated control animals was significantly reduced in the animals treated with the extract. The lifespan of the *Piper longum* treated animals were also significantly increased.

Key words: *Piper longum*; B16F-10 melanoma; metastasis; hydroxyproline;hexosamine.

INTRODUCTION

The process of cancer metastasis is multifaceted and complex since it is dependent upon the interaction of invasive tumor cells and their products (1,2). Therapeutic treatment modalities of chemotherapy, radiotherapy and surgery, while effective for some patients with solid malignancies, cannot be successfully employed for others with progressive out growth of metastasis (1). Cisplatin and other drugs used in conventional cancer chemotherapy are limited by their serious side effects of nephrotoxicity, acute cochleor ototoxicity and peripheral neuropathy. The key factor for toxicities is immune suppression. Due to these acute toxicities it has become necessary to develop second-generation drug, which are equally effective but less toxic (3).

In our laboratory, interesting studies have been carried out using Iscador, which is an extract of the plant *Viscum album* and we have reported its antimetastatic activity (4). We have also reported the antimetastatic activity of Curcumin, which is present in the plant *Curcuma longa* (5).

Piper longum Linn. an important medicinal plant is used in traditional medicine by many people in Asia and Pacific islands especially in Indian medicine (6). *Piper longum* is a component of medicines reported as good remedy for treating gonorrhea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infections, chronic gut related pain and arthritic conditions (7). Other reported beneficial effects of *Piper longum* include analgesic and diuretic effects, relaxation of muscle tension and alleviation of anxiety (8). Constituents of piper species have inhibitory activity on prostaglandin and leukotriene biosynthesis *in vitro* (9).However, no study on the antimetastatic

activity of *Piper longum* has been reported. Inorder to verify the anecdotal claims that *Piper longum* has numerous phytochemical benefits, we have investigated the antimetastatic activities of the plant.

MATERIALS AND METHODS

Animals

C57BL/6 mice were purchased from National Institute of Nutrition (Hyderabad, India). The animals were maintained in ventilated cages, fed with mouse chow (Sai feeds, Bombay, India) and water *ad libitum*.

All the animal experiments were performed according to the rules and regulations of the Animal Ethical committee, Govt. of India.

Cells

B16F10 melanoma cells, a highly metastatic cell line was obtained from National Centre for Cell Science, Pune, India. The cells were maintained in DMEM supplemented with 10% FCS and antibiotics. The cells were also grown as subcutaneously transplantable solid tumor in C57BL/6 mice.

Hydroxyproline and glucoronic acid lactone were purchased from Sigma Chemicals, St.Louis, USA. N-acetyl neuraminic acid and papain were obtained from SISCO Research Laboratory, Bombay, India. DMEM was purchased from HiMedia Laboratory, Bombay, India. All other chemicals used were analytical reagent grade.

DRUG PREPARATION

Source: Authenticated *Piper longum* was obtained from Amala Ayurvedic Centre.

Alcoholic extract :100 g of dried fruit powder was stirred overnight in 70% methanol (1L), centrifuged at 10,000 rpm (7,275g) for 10 minutes at 4°C and supernatant was collected. Methanol was removed in vacuum and the yield obtained was 26%. The extract was resuspended in PBS (pH 7.2). Phytochemical analysis of the extract showed the presence of alkaloid.

DRUG ADMINISTRATION

The extract was administered (10mg/dose/animal) in two different modalities (intraperitoneally) as follows.

a) Simultaneous administration: - The extract was given to the animals simultaneously with metastatic tumor cells and continued for 10 consecutive days.

b) Prophylactic administration: - Animals were treated with 10 consecutive doses of the extract prior to tumor induction.

Determination of the effect of *Piper longum* extract on the inhibition of tumor cell growth.

B16F-10 melanoma cells (5000 cells/well) were plated in a 96 well flat bottom titre plate and incubated at 37°C in 5% CO_2 atmosphere. After 24 hours different concentrations (10-100 µg/ml) of the extract was added and the incubation was continued for 48 h under the same conditions. Cell viability was determined by the MTT assay (10).

Determination of the effect of *Piper longum* on the lung tumor nodule formation

C57BL/6 mice weighing 20-25g were separated into 3 groups (14 mice/group). The animals were induced metastasis by injecting B16F-10 melanoma cells (10^6 cells/animal) via the lateral tail vein (11). Group I and group II animals were treated with extract by two different modalities and the third group was kept as untreated control. After 21 days of tumor inoculation, eight animals from each group were sacrificed, lungs were excised and the lung tumor

nodules were counted. The lungs were used to estimate the levels of lung collagen hydroxyproline, lung uronic acid, and lung hexosamine (14-16) and were also used for histopathological analysis. Blood was collected by heart puncture, serum separated and used to estimate serum sialic acid (17) and gamma-glutamyl transpeptidase (GGT) level (18).

Determination of the effect of *Piper longum* on the survival rate of metastatic tumor bearing animals

Six animals from each group of the previous experiment were observed for their survival rate and the percentage of increase in life span (ILS) was calculated using the formula % ILS = T-C/C x 100 where 'T' and 'C' represent the number of days treated and control animals survived respectively.

Determination of the effect of *Piper longum* on the inhibition of hydroxyproline, lung uronic acid content and lung hexosamine content

Lung collagen hydroxyproline content was determined by the method of Bergman and Loxley (12). Lungs were homogenised and hydrolysed using 6N HCl. The hydrolysate was evaporated to dryness, residue dissolved in water, neutralised and used for the assay by chloramine-T method. A standard graph was plotted using hydroxyproline standard.

The uronic acid present in the lungs was estimated by the method of Bitter and Mair (13). The lungs were digested with crude papain and hydrolysed (14). The hydroylysate was treated with sulphuric acid. Uronic acid level was estimated in the presence of carbazole reagent at 530 nm. A standard graph was plotted using glucoronic acid lactone.

The hexosamine content in the lung tissue was estimated by the method of Elson and Morgan (15). Lyophilized tissue samples were hydrolysed with 2N HCl and the hydrolysate was evaporated to dryness. The residue was dissolved in water and treated with 2% acetyl acetone. Hexosamine was estimated in the presence of Ehrlich's reagent (16). A standard graph was plotted using glucosamine standard.

Determination of the effect of *Piper longum* on the inhibition of serum sialic acid and serum gamma glutamyl transpeptidase (GGT) level

Protein-bound serum sialic acid levels were estimated by thiobarbituric acid assay (17). The serum was hydrolysed using 0.2N sulphuric acid. The hydrolysate was oxidised with per-iodic acid and incubated at 37°C for 30 minutes. After terminating oxidation using sodium arsenate, 6% thiobarbituric acid was added. Sialic acid was estimated at 549 nm with reference to 632 nm after adding DMSO. Sialic acid content was determined from standard graph plotted using n-acetyl neuraminic acid.

Serum gamma glutamyl transpeptidase (GGT) levels were estimated, as it is an abundant membrane bound enzyme. The enzymatic assay was performed by measuring the release of P-nitroaniline from gamma glutamyl p-nitroanilide in the presence of an acceptor (glycyl-glycine) (18). The GGT content was determined from the graph plotted using p-nitro aniline standard.

Histopathological studies

Lung tissues were fixed in 10% formaldehyde, dehydrated and embedded in paraffin wax. Sections (4 μM) were stained with eosin and haematoxylin.

STATISTICAL ANALYSIS

Results were expressed as the mean ± standard deviation. Statistical evaluation was done by student's t-test.

RESULTS

A) Effect of Piper longum on the Inhibition of tumour cell growth

Alcoholic extract of *Piper longum* was found to be 100% cytotoxic to B16F-10 cells at a concentration of 250 µg/ml and it was non-toxic at a concentration of 50µg/ml (Table 1).

B) Effect of Piper longum on lung tumor nodule formation

There was a reduction in lung tumor nodule formation when the animals were treated with alcoholic extract of *Piper longum* in different modalities (Table II).

The untreated control animals developed a massive number of tumor nodules on their lungs and were assigned an arbitrary number of 250 (10). Among the different modalities of drug administration, a moderate inhibition of tumor nodule formation was observed when the extract was administered simultaneously (47%) and prophylactically (41%).

C) Effect of Piper longum on the survival rate of tumor bearing animals

The effect of alcoholic extract of *Piper longum* on the survival rate of metastatic tumor bearing mice is shown in Table II. Control metastatic tumor bearing mice survived only up to 32 days. When the extract was administered simultaneously and prophylactically the lifespan of the animals was increased to 61.34%, and 34.20% respectively.

D) Effect of *Piper longum* on the lung collagen hydroxyproline content, lung uronic acid and the lung hexosamine content

The effect of alcoholic extract of *Piper longum* on the lung collagen hydroxyproline content is shown in Table III. Tumor bearing animals showed a drastically increased level of lung collagen hydroxyproline (21.13 µg/mg protein) compared to normal animals (0.98 µg/mg protein). This elevated level was reduced when the animals were treated with the extract of *Piper longum*. The maximum inhibition was observed when the extract was administered simultaneously (12.35 µg/mg protein). Prophylactic administration of the extract also showed an inhibition in the level of lung collagen hydroxyproline content (14.2 µg/mg protein).

The effect of *Piper longum* extract on lung uronic acid content is shown in Table III. Uronic acid content of normal animals is 35 µg/100 mg tissue. The tumor bearing control animals showed elevated levels of uronic acid in their lung tissue (349.5 µg/100 mg tissue), which was reduced, in the treated animals. Treatment of the extract simultaneously with the tumor cells reduced the uronic acid level to 155.6 µg/100 mg tissue. Prophylactic treatment of *Piper longum* extract also showed reduction in uronic acid level to 157.5 µg/100 mg tissues. These reduced levels of uronic acid indicate the decreased lung fibrosis in drug-treated animals.

The effect of alcoholic extract of *Piper longum* on the lung hexosamine content is shown in Table III. The hexosamine content of the normal animals is 0.53 mg/100 mg lyophilised tissue. There was an increased level of lung hexosamine content in the control tumor bearing animals (4.03mg/100 mg lyophilised tissue) and a reduced

level in the treated animals. Simultaneous administration of *Piper longum* extract showed inhibition of lung hexosamine content (2.05 mg/ 100 mg lyophilised tissue) followed by prophylactic mode of drug administration (2.2 mg/100 mg lyophilised tissue). The reduced level of lung hexosamine also indicates a decreased tumor burden.

E) Effect of *Piper longum* on the serum sialic acid and GGT levels

The effect of alcoholic extract of *Piper longum* on the serum sialic acid level of metastatic tumor bearing animals is shown in Table IV. The serum sialic acid level was drastically elevated in the control group (102.9 µg/ml serum) compared to normal animals (23.2 µg/ml). Simultaneous as well as prophylactic treatment of *Piper longum* extract showed a reduction in the sialic acid levels (54.6 µg/ml and 58.4 µg/ml serum respectively).

The effect of *Piper longum* extract on serum GGT levels is shown in Table I. The higher content of GGT in the control animals (105.9 nmole p-nitroaniline/ml serum) was significantly reduced by the simultaneous and prophylactic treatment of *Piper longum* extract to 51.3-nmole p-nitroaniline/ml and 57.4 nmole/p-nitroaniline/ml respectively.

F) Effect of *Piper longum* on lung architecture.

Histopathological analysis of lung tissue showed infiltration of neoplastic cells around main bronchiols and along the pleura in the control tumor bearing animals (Fig 1). The tumor nodules were composed of polygonal tumor cells with a prominent nucleus. Intracellular melanin deposition and a clear area of necrosis were also present. Lung tissue of *Piper longum* treated animals showed a reduction in tumor mass and there were reduced number of tumor cells in alveoli and pleura.

DISCUSSION

The main objective of this study was to focus on the antimetastatic activity of alcoholic extract of *Piper longum*. The deadly aspect of metastatic tumor progression has been the observation that highly malignant tumor cell subpopulations are often resistant to therapy with chemotherapeutic, cytoreductive anticancer drugs (19). The use of natural products against metastasis is less toxic and more effective. There are several plant products studied in our laboratory, which have the efficacy to inhibit the lung metastasis induced with B16F-10 melanoma cells, which also possess the immunostimulatory effect. Some of them are Brahma Rasayana (20), *Tinospora cordifolia* (21), genestein(22) etc.

The alcoholic extract of *Piper longum* has been found to have an inhibition of tumor nodule formation. Administration of the extract simultaneously as well as prophylatically with the tumor cells were found to inhibit the metastatic spread of B16F-10 melanoma cells in the mice. Maximum inhibition of metastasis was observed when it was administered simultaneously followed by prophylactic administration compared to the untreated control. Tumor nodules are the metastatic colonies of B16F10 melanoma cells and it promotes the lung fibrosis and collagen deposition in the lung. This inhibition of tumor nodules correlated with an increase in the lifespan of the metastatic tumor bearing animals. During lung fibrosis collagen is deposited massively in the alveolus of lungs. A part of collagen is hydroxyproline (23), which results the reduction in pulmonary function. In the extract treated group

there was a reduction in the lung collagen hydroxyproline content than the control tumor bearing animals. In the tumor cells, oxidation of the primary alcohol group of aldoses of sugar derivatives occurs, yielding uronic acid. This uronic acid leads to the formation of glucuronic acid lactone. In the presence of glucuronic acid lactone, propyl hydroxylase enzyme converts the prohydroxyproline to hydroxyproline. Hexosamine is a significant compound in lung tumor cells. It plays an important role in the synthesis of N-acetyl neurominic acid (sialic acid), which is a component of glycolipids present on the surface of tumor cells (23). Metastatic property of tumor cells highly upregulated with the elevated levels of sialic acid content (24) Gamma glutamyl transpeptidase (GGT), a marker of cellular proliferation, was increased in the serum compared with its level in normal animals. All these results may explain the increase in life span of metastatic tumor bearing animals treated with the alcoholic extract of *Piper longum*. Histopathological analysis also shows a difference in the lung architecture. Further studies have to be conducted in order to elucidate the actual mechanism of action of *Piper longum* for these activities.

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Table 1 : Cytotoxicity of Piper longum on B16F-10 cells in culture

Concentration in µg/ml	% of cytotoxicity
250	100.0
200	77.5
100	68.6
50	53.9

B16F-10 melanoma cells 5000 cells/well were plated in a 96 well flat bottom titre plate with different concentrations of drugs and cell viability was determined by MTT assay.

Table 2 : Effect of *Piper longum* on lung tumour nodule formation and survival rate of metastatic tumor bearing animals

Treatment	No.of nodules per lung	% inhibition of tumor nodule	% of increase in life span (% ILS)
Control	250	-	-
Piper longum			
a) Simultaneous	131 ± 23.4*	47.6*	61.34*
b) Prophylactic	147 ± 25.7*	41.2*	34.2*

B16F-10 melanoma cells (10^6) were injected via the lateral tail vein. Alcoholic extract of *Piper longum* (10mg/dose/animal) was administered intraperitoneally for 10 consecutive days in different modalities. Controls were treated with vehicle gum acacia. Values are the mean ± SD. Statistically significant from untreated controls.
*P < 0.005

Table 3 : Effect of Piper longum on the lung collagen hydroxyproline, lung uronic acid, lung hexosamine levels of tumor bearing animals.

Treatment	Lung collagen Hydroxyproline (µg/mg protein)	Lung uronic acid (µg/100 mg tissue)	Lung hexosamine (mg/100 mg lyophylised tissue)
Normal	0.98 ± 0.07	35 ± 1.9	53 ± 0.03
Control	21.13 ± 2.03	349.5 ± 29.7	4.03 ± 0.41
Piper longum			
a) Simultaneous	12.35 ± 1.6*	155.6 ± 10.1*	2.05 ± 0.30*
b) Prophylactic	14.20 ± 1.5*	157.5 ± 16.4*	2.20 ± 0.26*

Experimental design is given in Table-II. Animals were sacrificed on the 21st day and their lungs were excised. Values are the mean ± SD. Statistically significant from untreated control.
*P < 0.001

Table 4 : Effect of *Piper longum* on the serum sialic acid and serum GGT level of metastatic tumor bearing animals

Treatment	Serum sialic acid (µg/ml serum)	Serum GGT (nmol p-nitroaniline/ml serum)
Normal	23.2 ± 1.3	25.7 ± 2.1
Control	102.9 ± 16.3	105.9 ± 12.3
Piper longum		
a) Simultaneous	54.6 ± 6.5*	51.3 ± 4.5*
b) Prophylactic	58.4 ± 4.5*	57.4 ± 5.2*

Experimental design is given in Table-II. The animals were sacrificed on the 21st day, blood collected by heart puncture, and serum separated. Values are the mean ± SD. Statistically significant from untreated control.
*P < 0.001

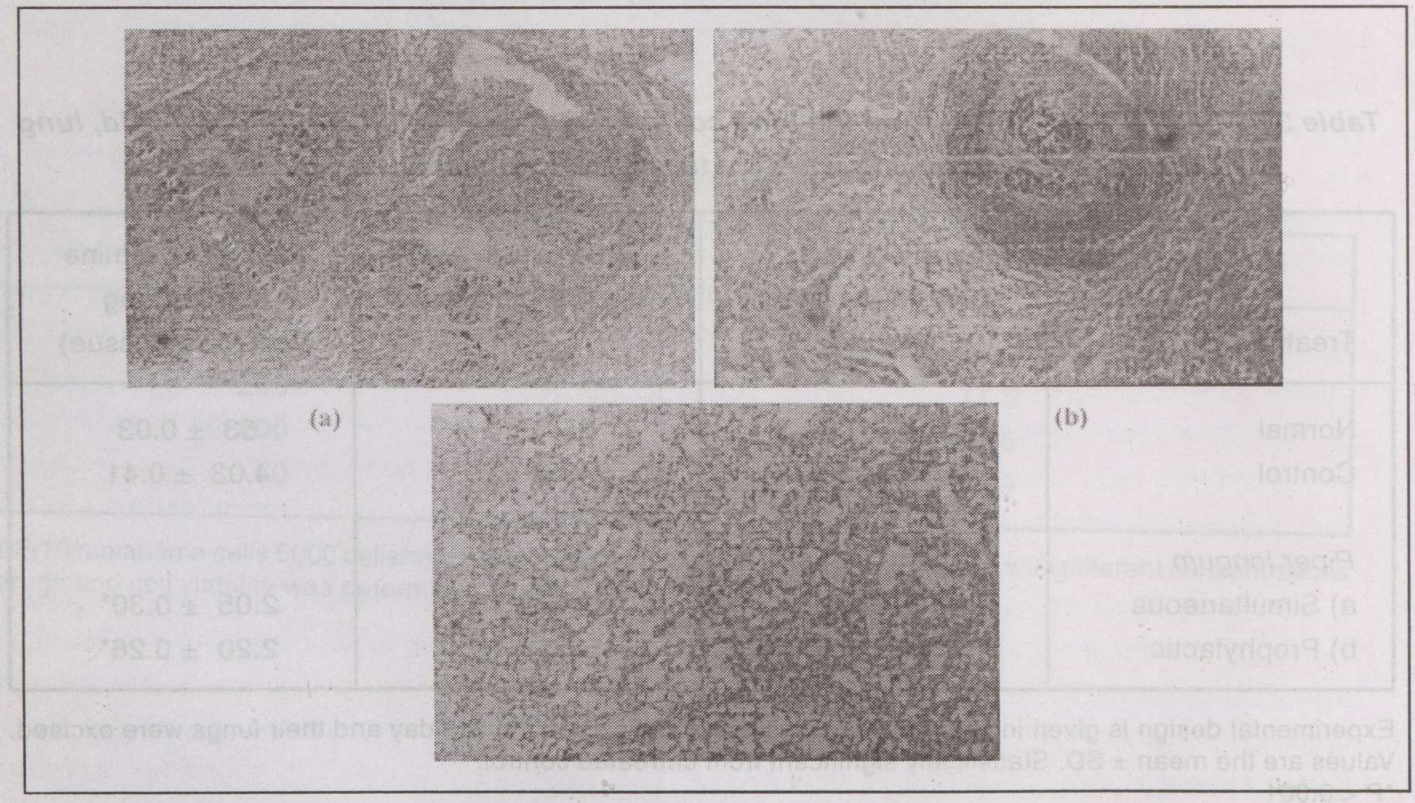

Fig.1. Histopathological analysis of lungs from metastatic tumor bearing mice.
a) Control (b) Normal (c) Treated with simultaneous administration of alcoholic extract of *Piper longum*.

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ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF *PUNICA GRANATUM*, LINN

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ABSTRACT

The methanolic extract of *P.granatum* fruit rind possess significant in vitro antioxidant and anti-inflammatory activities. The concentration needed for 50% inhibition of superoxide radical production, hydroxyl radical generation, nitric oxide radical formation and lipid peroxidation were183.3, 94, 53, and 88.54 μg/ml respectively. The anti-inflammatory activity of the extract was evaluated in Carrageenan, BSA (Bovine Serum Albumin) and Dextran induced acute and formalin induced chronic inflammatory models in Balb/c mice. The extract showed significant anti-inflammatory activity in both the models comparable to control group.

Key Words: *P.granatum* Antioxidant, Anti-inflammatory.

INTRODUCTION

Inflammation is the immediate defensive mechanism or reaction to an injury, which may be caused by infection, chemical or physical agents (Janis Kuby, 1997). It involves pain, heat, redness and swelling and loss of function of affected part. In some cases the inflammation may lead to the development of chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel diseases, psoriasis etc. Reactive Oxygen Species (ROS) and free radicals are thought to act directly as cellular messengers and elicit an inflammatory response. ROS and free radicals also activate a series of enzyme system including protein kinases, protein phosphatases, transcription factors and heat shock proteins and increase the extend of inflammation. Supplementation of non-toxic antioxidants may have a protective role in these conditions.

From ancient times medicinal plants have been proved to be powerful therapeutic agents for the treatment of cancer, ulcer, inflammation etc. Approximately 60% of the world's population almost relies entirely on plants for medication (Fransworth, 1994) and several medicinal plants have been proved to be useful in the treatment of these diseases. Although some medicinal plants have been proved to be useful as therapeutic agents, several other plants are still awaiting discovery.

P.granatum (Punicaceae) L., commonly called as pomegranate is a small tree used medicinally in Europe, Mauritania, Indo China, West Indies, Guiana, Brazil, and La Reunion & South Africa. This plant is used in folklore medicine for the treatment of various diseases such as hepatic damage, snake bite, ulcer, arthritis etc (Kirthikar, 2000). This plant also shows a potent in vivo antioxidant and gastro protective activities against Aspirin and Ethanol induced gastric ulceration models (Ajaikumar et al, 2003).

The present study was aimed at evaluating the in vitro antioxidant and antiinflammatory activities of *P. granatum* fruit rind.

MATERIALS AND METHODS

Plant material and drug preparation

P.granatum fruits were purchased locally from Thrissur. The air dried, powdered material was extracted with 70% methanol by stirring at room temperature for 24 hours. The extract was filtered, concentrated and evaporated to dryness. The dried extract suspended in distilled water and used for further studies.

Animals

The in vivo anti-inflammatory effect of fruit rind of *P.granatum* was assessed using Balb/c mice (22-26 g) were supplied by the Small animal breeding station of Kerala Agriculture University, Mannuthy, India. The animals were grouped in ventilated cages and maintained at 22-28^{0}C, 60-70% relative humidity, 12h light and dark cycle. The animals were fed with standard mouse chow (Lipton, India) and water *ad libitum.*

In vitro antioxidant activities

Superoxide radical scavenging activity

The effect on the superoxide radical production was checked using the nitroblue tetrazolium (NBT) reduction method of Mc Cord and Fridovic,1969 . The reaction mixture contained: EDTA (6 mM; with 3mg NaCN), riboflavin (2 mM), NBT (50 mM), plant extract (from 1 to 100 μg/ml) and phosphate buffer (67mM, pH 7.8) in a final volume of 3 ml. The tubes were uniformly illuminated with an incandescent lamp for 15 min, and the optical density was measured at 530 nm before and after illumination.

Hydroxyl radical scavenging activity.

Hydroxyl radical scavenging was measured by studying the competition between deoxyribose and the test compounds for hydroxyl radicals generated from the Fe3+ / ascorbate / EDTA / H_2O_2 system. The reaction mixture contained: deoxyribose (2.8 mM), $FeCl_3$ (0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), ascorbate (0.1 mM), KH_2PO_4-KOH buffer (20 mM, pH7.4), and the extract (from 1 to 100 μg/ml) in a final volume of 1 ml. After incubation for 1 h at 37° C, the deoxyribose degradation was measured as TBARS by the method of (Ohkawa et al, 1979).

Nitric oxide radical inhibition activity.

Nitric oxide, generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which were measured by Griess reaction (Green et al,1982; Marcocci et al, 1994). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and the compound (from 1 to 100 μg/ml) was incubated at 25° C for 150 min. After incubation, 0.5 ml of the reaction mixture was removed and 0.5 ml of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylethylene diamine dihydrochloride) was added. The absorbance of the chromophore formed was reviewuated at 546 nm.

Inhibition of lipid peroxide formation (Induction by Fe2+/ ascorbate system).

The reaction mixture containing rat liver homogenate (0.1 ml, 25% w/v) in Tris-HCl (30 mM), Ferrous ammonium sulphate (0.16 mM) and ascorbic acid (0.06 mM) and different concentrations of the compound (from 1 -100 μg/ml) in a final volume of 0.5 ml was incubated for 1 h at 37^{0}C (15) and the resulting thiobarbituric acid reacting substance (TBARS) was measured by the

method of Ohkawa (Ohkawa et al, 1979). A 0.4-ml aliquot of the reaction mixture was treated with sodium dodecyl sulphate (SDS) (0.2 ml, 8.1%), thiobarbituric acid (1.5 ml, 0.8 %), and acetic acid (1.5 ml, 20%, pH 3.5), made to a total volume of 4 ml by adding distilled water, and kept in a water bath at 95° C for 1 h. After cooling, distilled water (1 ml) and 5 ml of n-BuOH/pyridine 15:1 (v/v) were added. After shaking and centrifugation, the organic layer was separated and the absorbance measured at 532 nm.

In vivo Anti-inflammatory activities

Acute and chronic anti inflammatory activity were evaluated. The former was done by the method of carrageenan, BSA and Dextran induced paw oedema in mice and later by formalin induced oedema in mice hind paw.

Carrageenan induced paw oedema in mice :

Animals were divided in to three groups of five animals in each group. In all groups acute inflammation was induced by subplantar injection of 0.02 ml of freshly prepared 15% suspension of carrageenan in normal saline in right hind paw of mice . One group was kept as the control the second group received 250 mg /Kg and the third group 500 mg /Kg of methanol extract orally 1 hr prior to the subplantar injection of carrageenan .The paw thickness was measured using vernier calliper at 0 and three hors after carrageenan injection

Dextran induced paw oedema in mice :

Animals were divided in to three groups of five animals in each group. In all groups acute inflammation was induced by subplantar injection of 0.02 ml of freshly prepared 1% suspension of dextran in normal saline in right hind paw of mice . One group was kept as the control the second group received 250 mg /Kg and the third group 500 mg /Kg of methanol extract orally 1 hr prior to the subplantar injection of dextran.The paw thickness was measured using vernier calliper at 0 and three hours after dextran injection

BSA induced paw oedema in mice ;

Animals were divided in to three groups of five animals in each group. In all groups acute inflammation was induced by subplantar injection of 0.02 ml of freshly prepared 1% suspension of BSA in normal saline in right hind paw of mice . One group was kept as the control the second group received 250 mg /Kg and the third group 500 mg / Kg of methanol extract orally 1 hr prior to the subplantar injection of BSA. The paw thickness was measured using vernier calliper at 0 and three hours after BSA injection

Formalin induced oedema in mice hind paw

Animals were divided into three groups of five animals in each group. In all groups ; chronic inflammation was induced by subplantar injection of 0.02 ml of 2% formalin in the right hind paw of mice . One group was kept as control while the second group received 250 mg/Kg and the third group 500 mg/Kg of methanol extract orally one hour prior to formalin injection and the administration of the extract was continued for 6 consecutive days. Degree of inflammation was measured using vernier calliper before and 6 days after formalin challenge.

STATISTICAL ANALYSIS:

The data were statistically analyzed using Student's t test and P values less than 0.05 were

considered significant. All data were represented as mean ± SD.

RESULTS

Invitro antioxidant activities

Inhibition of superoxide radical production

Extract of *P .granatum* fruit rind were found to scavenging the superoxides generated by riboflavin photo reduction method. The concentration of the extract needed for 50% inhibition of superoxide radicals was found to be 61.1μg/ml (Table I). the concentration of known antioxidants such as curcumin needed for the same effect was 6.5 μg/ml. this indicates the antioxidant activity of *P.granatum* fruit rind extract is quite potent.

Inhibition of hydroxyl radicals

Degradation of deoxyribose by hydroxyl radicals generated by Fe3+ / ascorbate / EDTA / H_2O_2 system was found that a concentration of 94 μg /ml of the extract is needed for 50% inhibition whereas concentration of curcumin needed for the same effect was 2.8 μg /ml (Table I).

Inhibition of Nitric oxide radicals

Nitric oxide radicals generated from sodium nitroprusside at physiological pH was found to be inhibited by the methanolic extract of *P.granatum* fruit rind. The concentration of the extract needed for 50% was found to be 53μg/ml (Table I).

Inhibition of Lipid peroxidation

The addition of methanolic extract of *P.granatum* fruit rind was found to inhibit lipid peroxides generated by the induction of Fe2+/ ascorbate and Fe3+/ADP/ascorbate in rat liver homogenates. The concentration of the extract needed for 50% inhibition was 88.54μg/ml and that of curcumin was 6.4μg/ml (Table I).

Anti-inflammatory activities

The result of carrageenan, dextran, BSA and formalin induced mice oedema which indicate the anti-inflammatory activity of alcoholic extract of *P.granatum* fruit rind and are presented in the Table II . It was found that the extract significantly inhibited the oedema formation in dose related manner. At the dose 500 mg/Kg body weight shows inhibitory effect (70.7, 39.2, 79.7 and 87.8% for carrageenan, dextran, BSA and formalin induced models respectively) on the oedema formation.

DISCUSSION

The present study reports for the first time the antioxidant and anti-inflammatory activity of *P.granatum* methanolic extract. The present study shows that the extract could inhibit carrageenan, dextran, BSA and formalin induced acute and chronic inflammation in a dose dependent manner.

The administration of the extract showed a significant decrease in carrageenan, BSA and formalin induced inflammation models whereas the extract showed little effect in dextran induced paw oedema.

Free radicals have been demonstrated to be involved in the triggering of several diseases such as atherosclerosis, cancer, ulcer, inflammatory diseases etc (Halliwell and Gutteridge). Superoxide radical regulates metabolites capable of signaling and communicating important information to the cellular genetic machinery. Over production of Superoxide radical takes place in various chronic inflammatory cases, induced by drug, toxin, stress,

tissue injury and heavy exercise. Hydroxyl radicals and nitric oxide radicals are also involved in inflammatory processes. Oxygen Derived Free Radicals may be released extracellularly from leukocytes after exposure to chemotactic agents, immune complexes or a phagocytic challenge. Extracellular release of low levels of these potent mediators can increase the expression of chemokines (eg: IL-8), cytokines and endothelial leukocyte adhesion molecules, amplifying the cascade that elicits the inflammatory response (Remick and Villarete, 1996).

Inflammation mainly caused by the generation of free radicals hence, the administration of antioxidants may have a protective role in these conditions. Most of the anti-inflammatory drugs act as antioxidants and scavenge free radicals generated during inflammatory processes. It is also reported that the administration of Superoxide dismutase (SOD) or other scavengers of free radicals has been observed to decrease inflammation in some animal models (Baret et al, 1984). The present study revealed that methanolic extract of *P. granatum* fruit rind shows potent activity in scavenging Superoxide, hydroxyl and nitric oxide radicals in vitro. We have also reported that the administration of *P. granatum* methanolic extract increases the SOD, catalase and GSH levels in aspirin and ethanol induced ulcer (Ajaikumar et al, 2003). These are potent scavengers of free radicals.

P.granatum is reported to contain alkaloids such as pelletriene, pseudopellitriene and the preliminary phytochemical screening of the plant showed the presence of flavonoids and terpenes. These compounds have several biological properties including protective effects through several mechanisms such as antioxidant effects. Hence, in the present study it is proved that the anti-inflammatory activity of *P. granatum* fruit rind may be due to its potent antioxidant properties.

CONCLUSION

In conclusion the findings of our study provide some scientific basis for the traditional use of *P. granatum* fruit rind for managing inflammatory pains. However, further experimentation is necessary to isolate the active principle and to understand the correct mechanism of action of the compound.

Table I

Test material	IC_{50} value in μg/ml			
	Superoxide	Hydroxyl	Nitric oxide	Lipid peroxidation
P.granatum pericarpextract	61.1±2.67	94.1±3.23	53.0±5.01	88.54±7.52
Curcumin	6.5±0.88	2.8±0.47	ND	ND

ND - Not Determined

Values are ± SD of triplicates

Table 1 : Effect of methanolic extract of *P.granatum* pericarp on anti-inflammatory activities

Groups	Treatment (mg/Kg)	Initial paw thickness (cm)	Paw thickness after 3 hr (cm)	Increase in paw thickness (cm)	Inhibition (%)
Carrageenan model					
Control	Vehicle	0.194±0.008	0.360±0.023	0.164±0.010	-
P.granatum	250	0.196±0.011	0.278±0.013	0.082±0.008	50
P.granatum	500	0.196±0.008	0.244±0.011	0.048±0.013	70.7
Dextran model					
Control	Vehicle	0.188±0.009	0.306±0.014	0.112±0.008	-
P.granatum	250	0.178±0.021	0.268±0.008	0.090±0.007	19.6
P.granatum	500	0.144±0.005	0.212±0.088	0.068±0.006	39.2
BSA model					
Control	Vehicle	0.186±0.011	0.334±0.018	0.148±0.015	-
P.granatum	250	0.158±0.013	0.254±0.011	0.096±0.009	35.1
P.granatum	500	0.168±0.020	0.198±0.013	0.030±0.008	79.7
Formalin model*					
Control	Vehicle	0.194±0.088	0.334±0.023	0.140±0.045	-
P.granatum	250	0.196±0.011	0.301±0.014	0.104±0.014	27.7
P.granatum	500	0.196±0.008	0.213±0.006	0.017±0.008	87.8

* Paw thickness was measured after 6days
** P values <0.05 compared to control

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INTENSITY MODULATED RADIOTHERAPY - CANCER CARE FOR THE NEWER GENERATION

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Intensity modulated Radiotherapy (IMRT)is a state -of- the- art cancer treatment method that delivers high dose of radiation directly to cancer cells in a very targeted way, much more precisely than is possible with conventional radiotherapy .IMRT can deliver high radiation dose directly to cancer cells while sparing more of the surrounding healthy tissue.

IMRT uses computer generated images to plan and then deliver tightly focused radiation beams to cancerous tumors than is possible with conventional radiotherapy. With this capability clinicians can deliver a precise radiation beam (dose) that conforms as closely as possible to the shape of the tumor.

IMRT can be used to treat tumors that might have been considered untreatable in the past due to close proximity of the vital organs and structures. Treating such tumors requires tremendous accuracy, for example in the case of head and neck tumors IMRT allows radiation to be delivered in away that minimize exposure to the spinal cord, optic nerve, salivary glands and other important structures. In the case of prostate cancer in the exposure to the bladder or rectum can be minimized. Usually IMRT is being used to treat tumors in the brain, breast, head and neck, liver, lung ,nasopharynx, pancreas, prostate, uterus etc.

A powerful computer program optimizes a treatment plan based on a physicians dose instructions and information about tumor size, shape and location in the body. A medical linear accelerator, equipped with a special device called Multi leaf collimator that shapes the radiation beam, delivers the radiation in accordance with the treatment plan. The equipment can be rotated around the patient to send the radiation beams from the most favorable angles for giving the tumors a high dose while preventing important healthy tissues.

RECOMMENDATIONS FOR IMRT

- It provides a precise and effective way of delivering radiation therapy
- High dose of radiation can be delivered directly to tumors and cancer cells, while surrounding organs and tissues are protected.
- Lower doses to healthy normal tissues may mean fewer complications or side effects .
- Physicians can treat cancers that were previously untreatable with radiation therapy.
- With IMRT radiation oncologists are no longer confused to use a single, large, uniform beam of radiation that may cause treatment side effects by treating more of the normal tissues near the tumor.
- IMRT can be a non-invasive alternative to surgery in some cases.
- With early detection, IMRT can eradicate tumors before the cancer spreads.
- IMRT can be done on an out patient basis.
- IMRT tangles the tumor and not the entire body.

THE TECHNOLOGY

IMRT - uses computer generated images to plan and then deliver even more tightly focused radiation beams to cancerous tumors than is possible with conventional radiotherapy. With this capability clinicians can exquisitely "paint" a precise radiation dose to the shape and depth of the tumor, while significantly reducing the adverse effects of dose on healthy tissues .IMRT ,an advanced form of 3-D conformal therapy enables radiation oncologists to deliver substantially more cancer-killing energy (generally X rays) to tumor while potentially decreasing harmful doses to surrounding healthy tissues.

Clinical studies indicate that higher dose rates delivered with IMRT techniques are improving the rate of local tumor control. IMRT helps radiation oncologists to achieve an increased precision through a combination of computerized machines (called medical linear accelerators) that produce and deliver the radiation ,advanced planning and control soft ware, and specialized mechanical devices used to shape " sculpt " the radiation beam.

IMRT TREATEMENT ELEMENTS INCLUDE

- image acquisition soft ware - for linking diagnostic images such as CT- scans into the treatment planning process
- Treatment planning software- for calculating the number of beam angles, beam shapes exposure time and the treatment schedule needed to deliver the prescribed dose to the tumor while minimizing the exposure to surrounding healthy tissues
- Treatment simulators - for generating low energy x-ray images that can be used to locate the tumors, position of the patient on the treatment couch and review the IMRT treatment plans.
- Medical linear accelerators - used for delivering therapeutic x-rays (photons) or electron radiation doses to the desired sites.
- Dynamic multi leaf collimators - computer - controlled mechanical devices that use upto 120 movable tungsten "leaves" that can confirm the shape of the radiation beam to the shape of the tumor from any angle.
- Treatment verification system - imaging hardware and software that capture the processed image beams exiting from the patient's body to review the treatment delivery and verify that the prescription was delivered properly.
- Radiotherapy treatment information systems- for managing a patient's complete cancer care regimes - from scheduling ,through treatment, through billing.

TREATMENT DELIVERY

A Radiation Oncologist prescribes the type and amount of treatment that best suits a particular patients needs. He closely works with other doctors and also heads a highly trained team. This team includes 1) a radiation physicist who participates in the planning process and ensures that the machine delivers the right amount of radiation,2) a dosimetrist ,who plans the treatment with the oncologist and the physicist,3) a radiation therapy nurse, who provides nursing care and helps patient learn more about treatment and how to manage any side effects and4) a radiation therapist, who sets the patients up for treatment and runs the equipment that delivers radiation.

In the treatment room the radiotherapist uses the marks on the patient's skin to locate

treatment area. the patient is positioned on the treatment table. Some times, special model devices are used to help with positioning. The radiation therapist leaves the treatment room before the machine is turned on .The machine is controlled by the control panel situated outside the room. The patient can be seen on a television screen or through a window in the control room and can talk to the patient through an intercom. If the patient feels uncomfortable the machine can be stopped at any time. The first IMRT treatment session is sometimes longer than the subsequent ones so that additional X-rays films and checks can be done. A typical IMRT Treatment session lasts for about 15-30 minutes for 2 - 3 weeks, only 5 days in a week. Rarely there are chances for some radiation reaction to occour,but can be managed with drugs. Usually there will not be any reactions.

THE PROCESS OF IMRT

The IMRT process is similar to a typical radiation treatment, and it depends, to some extent, on a particular hospital's approach to radiation oncology. Typically, after conducting a physical examination and a medical history review, the radiation oncologist determines an individualized course for each patient.

The following are the steps in the process

Patient setup, Immobilization and Image Acquisition

The minimization of patient setup uncertainty is more important in 3D-CRT relative to conventional radiotherapy due to the improved conformality of the dose distribution and this is even more critical in IMRT. Immobilization devices and precise positioning procedure should be used throughout the process of image acquisition, simulation and treatment.

The use of simulators may be decreasing in 3D-CRT and IMRT, relative to its use in conventional radiotherapy. If a simulator is used, an initial (pre-CT) simulation may be carried out in conjunction with patient immobilization to establish fiduciary skin marks, define the tentative treatment isocenter, and obtain anteroposterior and lateral radiographic films. A second simulation may be performed, after the treatment plan has been accepted, to position the patient at the established isocenter and acquire reference radiographs of each field for comparison with the corresponding BEV digitally reconstructed radiographs and subsequently, the verification portals films.

A complete 3D CT image set is usually obtained with the patient in the treatment position on a flat couch. The number of CT slices and the interesting spacing are according to protocols, depending on the size, shape and location of the target and the treatment technique. However to produce high-resolution digitally reconstructed radiographs, the slices are contiguous with interslice spacing of 3 to 5mm. For certain sites, magnetic resonance imaging and positron emission tomography are complimentary to CT and helpful in defining the extent of disease, especially when used with image-correlation software.

The advent of CT simulation may make the process more efficient, by combining the simulation and CT imaging sessions into one. The CT simulator combines the capabilities of spiral (or helical) scanners for volumetric data acquisition and high-speed workstation for rapid image reconstruction and display. This permits the so-called virtual simulation, carried out on a computer

workstation using the 3D CT data set (the virtual patient) . In this process, a complete 3D data set is first obtained with the patient in the treatment position using 3- to 5-mm slice thickness. Then, corresponding to the specific treatment set-up parameters, patient anatomy is reconstructed from the CT data and displayed in beams eye view(BEV), and simulation fluoroscopic and radiographic images are digitally generated for viewing, decision-making, and documentation.

Aside from the improved efficiency, CT simulation eliminates or minimizes systematic uncertainties in the registration of simulation films to CT data sets, and in set-up errors when transferring the patient from one mechanical coordinate system to another. The disadvantage is the degraded image quality of reconstructed radiographs as compared with a conventional simulation film.

Delineation of Treatment Volume and Selection of Treatment Beams

Treatment volumes are derived from the CT images according to the ICRU report 50 nomenclature with clinical target volume (CVT) being the visualized tumor plus the regions at risk and planning target volume (PVT) to include setup and other uncertainties. The manual delineation of these and their adjacent critical organs are time consuming and would benefit from new and improved tools. . Other contours needed for treatment planning and dose-distribution calculation such as the outer skin, bone, lungs, and air cavities can be outlined automatically with existing edge detection algorithms.

3D-CRT, display is probably less critical in IMRT than in 3D-CRT. We believe that as long as there are a sufficient number of radiation fields (five to seven or more), the number of beams and their orientations are less important. This is because the many degrees of freedom in intensity modulation can effectively compensate the less optimal beam direction. Also, fields size and shape can be completely defined by the assignment of intensity pattern in the optimization process

Computer-Aided Plan Optimization in Intensity-Modulated Radiotherapy

The most distinguishing feature in IMRT planning is the use of intensity modulation to improve the dose distribution. An iterative process is invariably used, alternately adjusting the intensity pattern of the beams and assessing the resulting dose distribution until an acceptable plan is devised. The iterative process could be computer automated or could involve significant manual intervention. In general, computer automation is needed in the so-called inverse planning, while user experience and involvement are more important in the forward planning of IMRT.

In forward planning the starting point is a number of open beams and their field shapes. Based on the resultant dose distribution, the user adjusts the intensities in parts of some of the beams and recalculates the dose distribution. This is repeated until an optimal dose distribution is obtained. This approach is sensitive to the experience of the planner and may be restrictive in terms of the number of intensity levels and the complexity of the intensity-modulation pattern.

In inverse method of treatment planning, the user specifies the number and orientations of the beams, and the desired objectives for the PTV and the critical organs. The computer algorithm divides each beam into individual rays and

iteratively alters the ray weights until the composite 3D dose distribution conforms to the specified objectives. The beams derived by this method are intensity modulated (i.e., the pattern of radiation varies within each beam).

Central to the success of computer optimization is the specification of some quantitative measure of the goodness of a treatment plan. In inverse planning, the criteria are stated mathematically as objective functions that the optimization algorithm attempts to minimize. At present, most algorithms are dose or dose-volume based (i.e., constraints are defined as doses to the target and normal tissues, or to volumes of interest). The use of biologically weighted objective functions is in principle more relevant, but is currently limited by the lack of validated biophysical models on tumor control and organs toxicity.

The starting point is the specification of the number of beams and their direction, and then the algorithm optimizes the intensities in the different parts of each beam.

TREATMENT PLAN EVALUATION

The process of treatment plan evaluation is continuing to evolve, particularly for IMRT with inverse planning. The tools by which plans are evaluated are largely the same for 3D-CRT and IMRT : two-dimensional (2D) dose distributions superimposed on CT images, 3D volumetric rendering of dose distribution and the PTV (and critical organs), structure-specific dose volume histograms (DVH), and biologic indices as a rough guide. Additionally, for IMRT plans, inspection of the intensity profiles either from an observer view (like a relief map) or by the projection of iso intensity lines on a BEV is often useful in evaluating an individual beam's contributions to the dose distribution. Even though the evaluation tools for 3D-CRT and IMRT plans are quite similar, the approach and action are quite different.

In the forward process of 3D-CRT or IMRT, the planner and the physician evaluate the initial treatment design(s) with a view to improve on it by altering some of the beam parameters (e.g., directions, weights, shapes, wedges, and so forth). The identifications of deficiencies and the devising of remedies rely on the experience and intuition of the planner. The effort-intensive nature of this approach limits the practical number of iterations of evaluation and alteration. On the other hand, the application of class solutions to some disease sites minimizes the alternations needed and facilitates convergence to an acceptable solution within a few cycles.

In the inverse process of IMRT, to exploit the benefits of the increased degrees of freedom with modulated individual ray weights, a significant number of iterations (typically five to ten) are both needed and carried out. As the goal of the iterative adjustment of beam modulation by the optimization algorithm is to minimize the user-specified function, the resultant plan should satisfy the clinical criteria of acceptance. Thus, ideally the evaluation process should be primarily checking and approval.

DOSE DISTRIBUTION CALCULATION

To fully account for the intricacies of IMRT dose delivery, the calculation model must accurately predict the incident energy fluence and use a dose kernel that describe the transport of photons and electrons and energy spread in an inhomogeneous medium. One accurate method of dose calculation involves the convolution of pencil beams. In our current dose model, in homogeneity is accounted for by the traditional

equivalent path length method, and pencil beam convolution is used only as a correction factor to account for variation of intensity as opposed to a flat, uniform field. However, in a highly heterogeneous media such as the lung, the accuracy of this calculation method needs improvement, for which development is in progress.

Beam-on time calculation for IMRT treatment is a method in which the dose D at a point (x,y,z) is given as D(x,y,z)=MU x F, where MU is the monitor unit of radiation beam, and F is a product of several factors, including the field size or output factor, and so forth. For IMRT, we modify the equation using a factor that accounts for the intensity modulation.

In delivering the intensity-modulated field with an MLC, only a portion of the entire field is exposed at a given time. As a result, the total MU required is usually longer than that normally required. There is no simple relationship between the maximum beam intensity and MU. In practice, the total beam-on time MU is calculated by a computer program, taking into account effects such as leaf transmission, rounded leaf ends, tongue and groove, distributed source, maximum leaf speed, and dose rate. In general, the more complex the intensity modulation, the larger the MU.

Commissioning and Quality Assurance Program

Both mechanical and dosimetric measurements are required in the commissioning of IMRT delivery using DMLC. Because the dose delivered using DLMC is directly related to the gap widths between pairs of opposing leaves, precise leaf position is much more important in the approach than in treatments using static MLC. Mechanical calibration of the leaf positions can be accomplished using the recommended procedure and software supplied by the manufacture. We have determined that a precision of 0.2 mm in leaf position is necessary.

Dosimetric characterization of the MLC, using film, ion chambers, or both, includes measurements of radiation transmission through the leaves and their rounded ends, and the determination of head scatter. The dosimetric contributions of these factors, which can amount to as much as 15% of the dose, are accounted for in the leaf sequencer algorithm of the treatment planning system.

Another important consideration of DLMC is the monitoring of the performance of the MLC during treatment. While the beam is on, the MLC control computer check all leaf positions every 55 msec, compared them to the planned leaf positions in the DMLC file, and records them in a DMLC log file. If any leaf deviates from its planned position beyond a present tolerance the control computer invokes a beam hold off, and radiation delivery is withheld until all the leaves are within tolerance again. Deviation that invoke beam holdover should occur infrequently as the leaf sequencer algorithm, in generating the DLMC file, has duly considered the maximum MLC leaf speed and the nominal dose rate. Test using clinical fields indicate that deviations of greater than 1mm occur less than 1% of the time. For the initial group of patients, we tested the delivery of each field before treatment.

In addition, a comprehensive program to evaluate the routine performance of DMLC needs to be in place. This includes a quality assurance procedure and periodic dosimetric verification of intensity-modulated fields. Image patterns of predesigned fields are produced on radiographic

films twice a week by radiotherapists to provide a quick visual assessment that the DMLC is functioning properly. Ion chambers and diode array measurements at different gantry constancy of the DMLC output and to track long-terms stability. Ion chamber measurements in solid phantom for patient fields provide a straightforward check on the MU calculations. Films dosimetry, with sufficient spatial resolution for the intensity-modulated patterns, efficiently compares the delivered and the planned dose distribution. It is also used intensity during the testing of new software and new treatment sites for IMRT, as well as for a periodic spot checks. The general procedure is to irradiate the film in a homogeneous plastic phantom and to digitize the exposed film with a laser scanner.

Treatment Delivery with Dynamic Multileaf Collimator

The intensity-modulated field can be delivered with an MLC. The 2D intensity distribution is divided into one-dimensional intensity profiles, with each profile delivered by one pair of leaves. In the dynamic MLC (DMLC or sliding-window) method, the leaves are in continuous motion during radiation delivery.

The MLC can also be used to deliver intensity-modulated beams in the multiple static segment (MSS, or the so-called step-and shoot) mode. In this mode, the MLC travels in a stepwise manner to discrete positions, with the beam turned off during the stepwise movement.

For treatment implementation, the DMLC files of the intensity-modulated fields are transferred either via a floppy disk or electronically to the MLC control computer of the treatment machine. Also transmitted for each intensity-modulated beam is its fluence aperture, defined as the area with greater than 1% of the maximum intensity, or approximately the MLC aperture with the leading leaves and the trailing leaves at their respective final and initial positions. This fluences aperture is used for acquiring portal image and for recording and verification purposes by that system.

Before the first treatment, portal localization electronic portal imaging device (EPID) images are taken of each intensity-modulated field with its fluence aperture. The portal localization films are than compared with the corresponding digitally reconstructed radiographs overlaid with the maximal DMLC apertures. This verifies that the radiation is directed properly, relative to the bony anatomy of the patients. During the treatment course, weekly portals are obtained for each field using the EPID. Using the EPID in combination with the MLC allows machine setup and image acquisition to occur without the therapist reentering the room between fields, thereby improving efficiency.

Before the dose delivery in patient treatment, the record and verify computer checks the initial leaf setting and the other machine parameters: the gantry angle, collimator angle, jaw position, and MU setting. During radiation delivery, the MLC control computer monitors the leaf positions every 55 msec, compares them with the planned positions, and records the results in a DMLC log-file. On completion of the first DMLC field, the record and verify system records the MU delivered and the final positions of the MLC. Without reentering the room, the radiotherapists rotate the gantry to the next orientation, program the accelerator and the MLC for the next intensity-modulated field, and then deliver the radiation. The process is repeated for each of the fields.

Consideration of Treatment Uncertainties

To account for treatment uncertainties, arising from patients set-up variation and organ motion, a margin is added to the target in radiotherapy planning. As the dose distributions become more conformal in 3D-CRT and even more so in IMRT, the outcome of the treatment may become more sensitive to these uncertainties. Thus, it is important to quantify them, develop corrective methods to minimize them, and account for the residual components in treatment planning.

Ideally, patient-specific treatment uncertainty data should be used, but practically it would be difficult. Thus, approaches have been developed to quantify treatment uncertainties of a patient population. Set-up errors have been measured for several disease sites using serial portal images and interfraction organ motion by repeated CT scans. Based on such data, analysis can be performed to assess the potential effect of treatment uncertainties on treatment outcome using statistical sampling technique.

The previously mentioned population average uncertainty data can also be useful for the calculation of the dose distribution in treatment planning. Specifically, the frequently distribution of treatment uncertainties can be incorporated into the pencil beam kernel, which is equivalent to a convolution of the idealized dose distribution with the frequency distribution of treatment uncertainties. This yields an average dose distribution representing the effects of random, or daily, errors occurring over a treatment course.

Working of a Medical Liner Accelerator

Medical linear accelerators are key system used for delivering radiotherapy treatments. Standing approximately nine feet tall by nearly 15 feet long and weighing as much as 18,700 pounds, the accelerator consists of four major components: an electronics cabinet called a "stand," housing a microwave energy generating source, a rotating gantry containing the accelerator structure that rotates around the patients, an adjustable treatment couch, and operating electronics. Accelerators are located within specially constructed concrete treatment rooms to provide X-ray shielding.

In operation, microwave energy, similar to that used in satellite television transmission, is used to accelerate electrons to nearly the speed of light (186,000 miles per second). They attain this velocity in a short distance, typically one meter or less. As they reach maximum speed they collide with a tungsten target, which in turn releases photons, or X-ray, with such energy they are measured in millions of volts (MV).Certain models can be switched so that the electrons bypass the target for direction electron therapy, This energy is measured in millions of electron volts (MeV).Very small beams in varying intensities can be aimed at a tumor from various angles to attack the target in a complete three-dimensional manner. In fact, IMRT can be delivered with beams of the size of 2.5 X 5-millimeter pixels-the size of a pencil tip-each with varying intensity. The idea is to deliver the lowest dose possible to the surrounding tissue, reducing the chance of causing a radiation side effect, while still delivering the maximum dose to the tumor. Radiation oncologist and physicists use electron or photon therapies for different types cancer treatments. As the radiation strikes human tissue it produces (largely from naturally occurring water in the body) highly energized ions which are lethal to both normal and malignant cells. While both good and

bad cells suffer from radiation, healthy cells can adapt over successive regenerative cycles. Malignant cells do not possess this adaptation mechanism and thus do not survive, a fact which generally dictates the practice o administering repeated radiation treatments rather than a single blockbuster dosage.

In conclusion,IMRT is a powerful technique that provides extra degrees of freedom in customizing the dose distribution for photons radiotherapy. With the development of computer-controlled treatment machines equipped with DMLC,it is now possible to deliver these treatments reliably.It is likely that this new modality will become widely accepted applied in future.

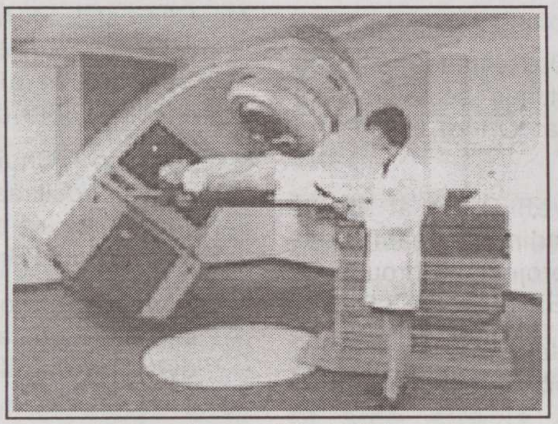

Figure 1 : A patient is positioned for treatment IMRT

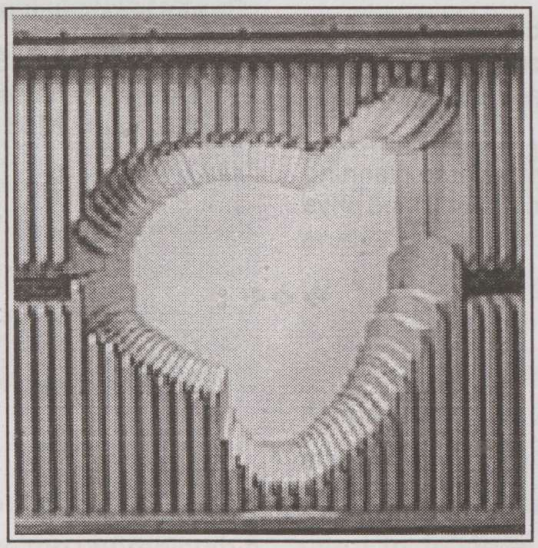

Figure 2 : An image of the Multileaf Collimator which shapes the radiation beam in accordance with the most optimized treatment plan.

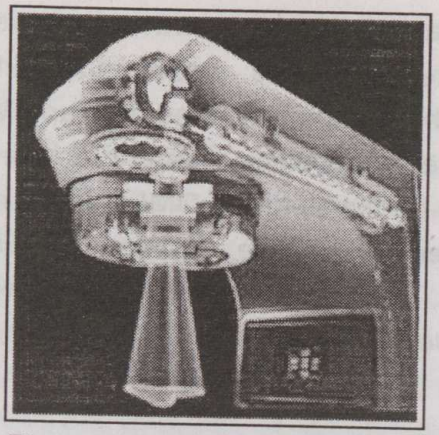

Figure 3 : Emission of photons

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THE INHIBITION OF GASTRIC MUCOSAL INJURY INDUCED BY ASPIRIN AND ETHANOL BY PUNICA GRANATUM LINN. (POMEGRANATE) METHANOLIC EXTRACT.

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ABSTRACT

Punica granatum Linn, commonly called as Pome granate is used in folklore medicine for the treatment of various diseases such as ulcer, hepatic damage, snakebite etc. Administration of 70% of methanolic extract of Punica granatum Linn. fruit rind (250mg/kg b.wt and 500mg/kg b.wt) inhibited the Aspirin and Ethanol induced gastric ulceration in a dose dependent manner as compared to the control group. The in vivo antioxidant levels such as SOD, Catalase, Glutathione (GSH) and Glutathione peroxidase (GPx) levels were increased in treated groups of animals as compared to the control group and these levels also coming closer to the normal level in treated groups of animals. The tissue lipid peroxidation level was found to be decreasing in treated groups of animals as compared to the control group. The histopathological studies of the ulcerated animals also confirmed the potent cytoprotective activity of the extract. The results were compared to that of ranitidine a well known Antiulcer drug.

INTRODUCTION

Gastric ulcer therapy faces a major drawback in modern days due to the unpredictable side effects of the long-term uses of commercially available drugs. As it affects 5% of the global population (Debashis B et al; 2002), the treatment of this painful disease and its prevention become one of the challenging problems today. Hence, the search is still on to find out a drug possessing antioxidant and antiulcer properties which will serve as a powerful therapeutic agent to cure gastric ulceration, and the search is extends to the systematic development of natural products. From ancient times plants have been proved to be powerful therapeutic agents for the treatment of various human sufferings, including atherosclerosis, cancer, ulcer etc. Due to the lack of side effects than the synthetic drugs approximately 60% of the world's population relies almost entirely on plants for medication and natural products have long been recognized as an important source of therapeutically effective medicines. In traditional Indian medicine several plants and herbs have been used to treat gastrointestinal disorders, including gastric ulcer (Sattyavathi et al; 1987) and the phytochemical analysis of these plants have yielded a number of compounds with gastro protective activity (Meena K et al, 1997).

Punica granatum Linn, commonly called as Pome granate is a large deciduous shrub or small tree used medicinally in Europe, Indo China, The Philippine Islands and South Africa. The plant is used in folklore medicine for the treatment of various diseases such as ulcer, hepatic damage, snakebite etc. The unripe fruit is a good appetizer

and tonic, useful in vomiting, causes biliousness. The ripe fruit is a tonic, astringent to the bowels, aphrodisiac cures biliousness, fever, heart diseases, sore throat, stomatitis etc. The rind of the fruit is antihelmintic; useful in diarrhea, dysentery and ulcer (ayurvedha) (Kirthikar, 1935).

The present study was aimed at evaluating the In vivo antioxidant and antiulcer activity of the 70% methanolic extract of *Punica granatum* fruit rind.

MATERIALS AND METHODS:

Chemicals:

Nitroblue tetrazolium (NBT), 5-5' dithiobis (2-notrobenzoic acid) (DTNB) were purchased from Sisco Research Laboratories, Mumbai, India. Deoxyribose and Riboflavin were obtained from Merck, India. All other chemicals used were of analytical reagent grade.

Animals:

Male Wistar Rats (180- 200) were purchased from the Small Animal Breeding Station, Kerala Agricultural University, Mannuthy, Kerala, India. The animals were maintained under standardized environmental conditions (22-28^{0}C, 60-70% relative humidity, 12hr dark/light cycle) and fed with standard rat feed (Lipton India) and water ad libitum.

Preparation of the *Punica granatum* 70% methanolic Extract:

Punica granatum fruits were collected locally and identified by Prof. Regi, Taxonomist, St: Mary's college, Thrissur, Kerala, India. The air-dried, powered material was extracted with 70% methanol by stirring at room temperature for 24hrs. The extract was filtered, concentrated and evaporated to dryness. The dried extract suspended in distilled water and used for further studies.

Ethanol (80%) induction of gastric ulcer in rats and its prevention by the extracts of *P.granatum* :-

The animals were divided into 5 groups of 6 animals in each group. Group I was kept as normal without any treatment and all other groups were fasted for 36hrs and administered with 80% ethanol (1ml). Group II animals were received 1ml of 80% ethanol alone. Group III, IV, V were treated with Ranitidin (50mg/kg b. wt), *P.granatum* extract (250mg/kg b. wt, 500 mg/kg b wt) respectively one hour prior to the administration of 80% ethanol. The animals were sacrificed after 4hr of the administration of ethanol (Paiva et al; 1990) and measured the ulcer index.

Effect of 70% methanolic extract of *P.granatum* on gastric ulcer induced by aspirin:

The animals were divided into 5 groups of 6 animals in each group. Group I was kept as normal without any treatment and all other animals were administered with aspiin (400mg/kg b.wt) alone and group III, VI, V, is pretreated with ranitidine (50mg/kg b.wt), seed extract (250mg/kg b.wt, 500mg/kg b.wt) respectively. After 4hr the animals were sacrificed, stomachs were removed, opened along the greater curvature to determine the ulcer index (Parmar and Desai; 1993).

Determination of Ulcer Index (U.I):

The ulcerative index was calculated by severity of gastric mucosal lesions graded as follows.

Erosions	Score
1mm or less	1
1-2mm	2
More than 2mm	3

Then the U.I. was calculated by using the formula

U.I = 1X (number of lesions of grade 1) + 2X (number of lesions in grade 2) + 3X (number of lesions in grade 3)

Then the overall score was divided by a factor 10, which was designated as ulcer index (Main and Whittle, 1975).

Biochemical analysis:

After the measurement of gastric lesions, the mucosa of the glandular stomach was removed by scrapping with a blunt knife and 25% homogenate was prepared and subjected to biochemical analysis by the following methods. The Superoxide Dismutase (SOD) was estimated by the Riboflavin photoreduction method (Mc Card and Fridovich, 1969) and Catalase by measuring the rate of decomposition of H_2O_2 at 240nm (Aebi, 1983) . The tissue lipid peroxidation was estimated by the TBA method (Ohkawa et al , 1979), Glutathione (GSH) by measuring its reduction with DTNB (Moron et al, 1979) and Glutathione peroxidase (GPx) was estimated by the method based on the degradation of H_2O_2 in the presence of GSH (Hafeman DG et al, 1974). The protein content of the enzyme was determined by Lowry's (Lowry, 1951) method.

STATISTICAL ANALYSIS:

The values were expressed as mean ± S.D. statistical significance for ulcerogenic and in vivo antioxidant activities were calculated using the Student's t-test.

RESULTS

The present study reports for the first time the in vivo antioxidant and antiulcer activity of 70% methanolic extract of *P.granatum* fruit rind against Aspirin and Ethanol induced gastric ulceration. The extract shows significant decrease in rat mucosal injury induced by Aspirin and Ethanol in a dose dependent manner. Administration of 80% ethanol (1ml) and Aspirin (400mg/kg b.wt) to 36hr fasted animals resulted in severe gastric damage visible from the outside of the stomach as thick reddish- black lines. After opening, the stomach lesions were found in the mucosa and consisted of elongated bands, 1-10mm long, usually parallel to the long axis of the stomach. They were located mostly in the corpus (the portion of the stomach secreting acid and pepsin) whereas no gross lesions were developed in the fore stomach (the non-secretary part of the stomach).

Administration of 80% ethanol in rats produced severe gastric erosions with an ulcer index of 4.1±0.89 whereas the ranitidine and P.granatum 70% methanolic extract treated groups shows a significant reduction in ulcer index with a percentage of inhibition of 51.22, 21.95, 63.41 in Ranitidine, 250mg.kg b.wt, 500mg/kg b.wt groups of animals respectively (Table No.1). All animals treated with Ethanol alone (control group), 3 animals from ranitidine treated group and 4 animals from *P.granatum* treated group (250mg/kg b.wt) shows intraluminal bleeding in the glandular portion of the stomach, while all animals in the 500mg/kg b.wt treated group were protected from intraluminal bleeding. The biochemical parameters such as SOD, Catalse, Tissue lipid peroxidation, GSH, GPx and total protein levels were found coming closer to the normal level in treated groups of animals compared to the control group (table No.2).

Aspirin administration to rats produced severe gastric damage in control group of animals whereas the treated groups of animals showed a significant decrease in ulcer index values in a dose dependent manner (Table NO.3). All animals in the control group and 3 animals in ranitidine

treated group and 3 animals in the *P.granatum* (250 mg/kg b.wt) treated group showed intraluminal bleeding whereas no intraluminal bleeding is found in other animals. The oral administration of P.granatum extract increased the values of SOD, Catalse, GSH, GPx and total protein levels while the tissue lipid peroxidation level was found to be decreased in treated groups of animals compared to the control group.

DISCUSSION

Reactive Oxygen Species (ROS) generated in the cells of aerobically respiring organisms due to many factors, have been implicated in the pathogenesis of many human sufferings like Parkinson's, Alzheimers, Huntington's diseases, liver cirrhosis, ulcer, atherosclerosis, cancer. The roles of ROS as a causative factor in certain ischemic cardiovascular and pulmonary diseases, cataratogenesis and reproductive disorders have also been studied extensively (Halliwell B and Gutteridge, 1989).

Involvement of ROS in pathogenesis of gastric ulceration was first evident from the studies on ischemia-reoxygenation-induced gastric mucosal injury (Perry et al, 1996, Yoshikawa et al, 1989, Yuda T, 1993). A growing body of experimental and clinical evidence suggests that gastric mucosal damage by ethanol (Szelenyl et al, 1988, Pihan et al 1987), non-steroidal anti-inflammatory drugs (Vaananann et al, 1991, Yoshikawa et al, 1993) and Helicobactor pylori (Davies et al, 1994) is mediated through reactive oxygen species (Phull et al, 1995, Yoshikawa et al, 1987). Moreover ROS may play an important role in gastric ulceration induced by several kinds of stress (Phull et al, 1995, Yoshikawa et al, 1987). ROS also decreases the levels of endogenous antioxidants such as GSH, α-tocopherol and ascorbate and make the mucosa more prone to oxidative damage (Phull et al, 1995). Supplementation with non-toxic antioxidants may have a chemoprotective role in these conditions.

Ingestion of ethanol is the predisposing cause of acute and hemorrhagic gastric erosions in human (Jeffries, 1978). Ethanol lowers the concentration of non- protein sulphydryls especially by glutathione (Szabo et al, 1981) thereby exerting ulcerogenic effect by increasing ROS formation (Szelenyl, 1988 and Pihan, 1987). In ethanol induced gastric ulceration the administration of the extract shows an increase in SOD, Catalase, GSH and GPx activities revealing the potent activity of the extract to scavenge the ROS formation. Studies focusing on the pathogenesis of ethanol induced gastric mucosal injury suggest that an initial event is disruption of the vascular endothelium resulting in increased vascular permeability edema formation and epithelial lifting. In treated group of animals a significant decrease in the ulcer index values are obtained as compared to control group and the protection of ethanol induced gastric ulceration in the present investigation suggest the involvement of cytoprotection by the *Punica granatum*.

Non- steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, indomethacin, ibuprofen etc, which are commonly used as pain killers in the treatment of rheumatoid arthritis and many other acute and chronic inflammatory conditions cause gastric mucosal damage (Ivey, 1988). The best-studied drug, aspirin, by inhibiting prostaglandin synthesis, interferes with protective mechanism such as mucus and bicarbonate secretion, surface epithelial hydrophobicity and mucosal blood flow (Langman et al, 1991). These changes permit back diffusion of acid through the breached surfaces to destroy cells, capillaries and

vein causing hemorrhagic ulcer. Enhancement of leukotriene synthesis by NSAIDs exhibits damaging effect. Aspirin also decreases mucosal ATP synthesis and cell turnover process. The changes brought about by NSAIDs, as described above, in totality can induce gastric damage through the generation of ROS (Vaananann et al, 1991) and inhibiting cell proliferation (Vaananann et al, 1991, Yoshikawa et al, 1993). NSAIDs also inhibit gastric peroxidase and increase mucosal H_2O_2 and OH level to cause oxidative mucosal damage (Banerjee, 1990). This OH causes lipid peroxidation and increases gastric lesions induced by Aspirin (Pihan et al, 1987). These lipid peroxidation causes decrease in levels of GSH in the gastric mucosa. In treated groups of animals a significant decrease in ulcer index values and a significant increase in SOD, Catalase, GSH, GPx values are obtained which revealed the potent cytoprotective activity of the extract. The significant inhibition in ulcer index values in the present investigation also shows the potent activity of the extract against the inhibition of prostaglandin synthesis, enhancement of leukotriene synthesis, decrease in the mucosal ATP synthesis and cell turnover process and inhibition of GPx by NSAIDs.

The histopathological studies of ethanol and aspirin induced ulceration models shows severe erosion of gastric mucosa, with necrotic patches, sub-mucosal edema and neutrophils infiltration in control animals. The control group also shows the presence of necrotic debri in the laminar proper of the mucosa infiltered with polymorpho nuclear leukocytes. The depth of the injury extends up to the muscularis with RBC extravasations. All of these symptoms were found to be normal in treated groups of animals. The protection against ulcerogenesis as manifested in significant reduction in ulcer index as well as protection to the gastric mucosal GSH, GPx and antioxidant status of ulcerated animals was clearly confirmed the cytoprotective effect of P.granatum 70% methanolic extract against aspirin and ethanol induced gastric ulceration.

Plants, which contain alkaloids, flavonoids and polyphenols are reported to have several biological properties including protective effects through several mechanisms such as antioxidant effects. *Punica granatum* is reported to contain alkaloids such as pelletierine, pseudopelletierine (Kirthikar, 2000) and the preliminary phyto-chemical screening of the plant also showed the presence of flavonoids and terpenes. These compounds have several biological properties including protective effects through several mechanisms such as antioxidant effects. In the present study it is evident that the in vivo antioxidant and antiulcer activity of *Punica granatum* may be due to the presence of flavonoids and terpenes.

Table 1 : Stabilization of gastric mucosa by *P.granatum* in EtOH induced gastric ulcer

	Normal	Ethanol 80% (1ml)	EtOH 80% (1ml)+ Ranitidine 50mg/k.g.b.wt	EtOH 80% (1ml)+ *P.granatum* 250mg/kg.b.wt.	EtOH 80% (1ml)+ *P.granatum* 500mg/kg.b.wt
Ulcerindex	-	4.1 ± 0.89	2.0 ± 0.64*	3.2 ± 0.82	1.5 ± 0.4**
% of inhibition	100	0	51.22	21.95	63.41
Intraluminal bleeding	-	6	3	4	0

P<*0.05, **0.01 values are mean ± S.D. of 6 animals in each group.

Table2 : *P.granatum* mediated cytoprotection In EtOH induced gastric ulcer

	Normal	Ethanol 80% (1ml)	EtOH 80% (1ml)+ Ranitidine 50mg/k.g.b.wt	EtOH 80% (1ml)+ *P.granatum* 250mg/kg.b.wt.	EtOH 80% (1ml)+ *P.granatum* 500mg/kg.b.wt
SOD U/mg protein	9.8 ± 1.36	4.94 ± 0.98**	8.43 ± 1.25	7.9 ± 1.53	9.67 ± 1.27
CatalaseU/mg protein	26.01 ± 1.43	14.2 ± 2.32**	23.48 ± 2.89	19.5 ± 2.08**	24.81 ± 3.26
Tissue lipid peroxidation nmol/mg	2.41 ± 0.45	5.1 ± 0.82**	3.28 ± 0.57	3.14 ± 0.64	2.58 ± 0.51
GSH nmol/mg	41.0 ± 4.27	24.2 ± 3.1**	39.0 ± 2.12	33.1 ± 2.3	44.9 ± 3.4
GPX protein U/mg	38.9 ± 3.32	17.64 ± 2.63**	27.08 ± 3.28**	29.37 ± 2.48**	33.47 ± 3.06
Total protein nmol/mg	2.25 ± 0.53	2.06 ± 0.38	2.5 ± 0.32	2.2 ± 0.21	2.7 ± 0.61

P<*0.05, **0.01 values are mean ± S.D. of 6 animals in each group.

Table 3 : Stabilization of gastric mucosa by *P.granatum* in Aspirin induced gastric Ulcer.

	Normal	Aspirin 400mg/kg.b.wt	Aspirin+ Ranitidine 400mg/kg.b.wt	Aspirin+ *P.granatum* 250mg/kg.b.wt	Aspirin+ *P.granatum* 500mg/kg.b.wt.
Ulcer index	-	3.8 ± 0.63	2.1 ± 0.4**	2.95 ± 0.61	0.98 ± 0.5*
% of inhibition	100	0	44.74	22.37	74.21
Intraluminal bleeding	-	6	3	3	0

P<*0.05, **0.01 values are mean ± S.D. of 6 animals in each group.

Table 4 : *P.granatum* mediated cytoprotection in Aspirin induced gastric ulcer.

	Normal	Aspirin 400mg/kg.b.wt	Aspirin+ Ranitidine 400mg/kg.b.wt	Aspirin+ *P.granatum* 250mg/kg.b.wt	Aspirin+ *P.granatum* 500mg/kg.b.wt.
SOD U/mg protein	9.8 ± 1.36	5.46 ± 0.87**	8.74 ± 1.46	7.05 ± 0.79**	9.46 ± 1.78
CatalaseU/mg protein	26.01 ± 1.43	17.84 ± 2.16**	22.69 ± 1.28**	19.63 ± 1.46**	27.36 ± 2.42
Tissue lipid peroxidation nmol/mg	2.41 ± 0.45	5.29 ± 0.69**	3.43 ± 0.54**	3.96 ± 0.85**	2.67 ± 0.32
GSH nmol/mg	41.0 ± 4.2	26.3 ± 1.96**	37.4 ± 2.05	32.9 ± 2.14**	44.0 ± 2.92
GPX protein U/mg	38.9 ± 3.32	19.31 ± 2.63**	29.45 ± 3.18**	26.86 ± 3.28**	34.58 ± 3.21*
Total protein nmol/mg	2.25 ± 0.5	0.90 ± 0.18**	2.3 ± 0.35	1.75 ± 0.39	3.14 ± 0.41**

P< *0.05, **0.01. values are mean ± S.D. of 6 animals in each group.

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INFLUENCE OF HORMONE COMBINATIONS ON CAMPTOTHECIN PRODUCTION FROM *OPHIORRHIZA MUNGOS* CALLUS

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ABSTRACT

Ophiorrhiza mungos is identified as a source of camptothecin - an antitumour alkaloid. The callus cultures of the plant can be used for many biotechnological applications to enhance the production of alkaloid. Callus culture was established from leaf segments in MS medium supplemented with auxins. B5 medium has a better response over MS medium in callus cultures of camptothecin. Similarly cytokinin combination - BA (2 mg/ml) + Kin (1 mg/ml) gave a very good amount of camptothecin yield.

INTRODUCTION

The camptothecin analogues are a promising family of anticancer agents with a unique mechanism of action- the inhibition of DNA unwinding enzyme topoisomerase I. Camptothecin is a naturally occurring alkaloid found in the bark and wood of Chinese tree, *Camptotheca accuminata*, isolated by Wall *et al* in 1966. It is used in the treatment of gastro-intestinal cancer, malignant melanoma etc. The presence of camptothecin is identified in *Ophiorrhiza mungos*, which is an annual herb indigenous to south India(Tafur *et al*,1975). Ethnolic extract of the leaves, root and stem of this plant showed potential inhibition of Herpes virus.

Production of alkaloids in nature is subjected to many uncontrollable parameters; both biotic and abiotic. Large scale harvesting of these plants may lead to termination the species on earth. In order to produce the alkaloids commercially, preserving the population of concerned species, modern biotechnological techniques can be applied. *In vitro* manipulation of the plant can be exercised to produce camptothecin in large scale by using callus culture, multiple shoot culture, suspension culture etc.

One of the most common techniques used is media manipulation- mainly hormonal manipulation. Plant growth regulators or phytohormones affect growth and differentiation and thus affect secondary metabolism of cultured cells. The effect varies with the type and the quantity of phytohormones applied. Some important phytohormones are auxins, cytokinins, abscisic acid, gibberellins and ethylene. Present study attempts the enhancement of production of camptothecin by modifying the auxin, cytokinin combinations of the medium.

MATERIALS AND METHODS

Plant material

Ophiorrhiza mungos was collected from Konni, a southern region in Kerala and authentically identified by Dr.Sasidharan, Taxonomist, Kerala Forest Research Institute, Peechi, Kerala. A voucher specimen was kept in the herbarium of our institute,ACRH.No.98.

The mother plants were maintained in a

green house, with proper care and sprayed with fungicides at weekly intervals.

Culture conditions

Different plant parts, including inflorescence axis, leaf lamina, nodal segments etc. were used as explants. These were surface sterilized by treating with Tween 20 (0.01%v/v) for 2 minutes, followed by 0.1% mercuric chloride for 3-5 minutes. After 4-6 washings with sterile distilled water, explants were inoculated to various culture media.

Culture media

Murashige and Skoog medium, Gamborg's B5 medium etc. were used together with various phytohormones. PH of the medium was adjusted to 5.8 before adding 0.7% agar. Culture tubes containing aliquots (15ml) were autoclaved at 121^0 C for 20 minutes. After inoculation cultures were incubated at complete darkness for callus induction.

Culture initiation

Callus induction was carried out in auxin rich medium.0.5-1.5 mg/ml Naphthalene acetic acid (NAA), 0.1-1.5 mg/ml 2,4-Dichlorophenoxy acetic acid (2,4-D),0.5-2.5 mg/ml Indole acetic acid(IAA), 0.5-2.5 mg/ml Indole-3-butyric acid (IBA) alone or in combination with 0.1-0.5 mg/ml Benzyl adenine(BA) and 0.1-0.5 mg/ml Kinetin (KIN) were supplemented with MS or B5 media for callus induction.

Each treatment consisted of 10-18 replicates of one explant per culture tube and the experiment was repeated at least once.

The calli formed were subcultured at 3 weeks interval till the required amount of dry weight (for analysis) is obtained.

Camptothecin extraction and quantification

The calli (3 weeks old) were harvested, washed thoroughly with distilled water to remove agar particles and the fresh weight was determined. After that they were freeze dried and the dry weight was determined. The calli was then powdered and defatted with petroleum ether 3-4 times followed by extraction with chloroform (3-5 times). The chloroform extract was evaporated to dryness. This partially purified extract was used for HPLC analysis.

The chloroform extract was dissolved in acetonitrile:Water mixture (40:60) and centrifuged to remove undissolved contaminants. The clear supernatant was then injected to reverse phased C-18 column of Shimadzu HPLC system at the flow rate of 1ml/minute using the solvent system Acetonitrile: Water (40:60) .The wave length of detection was 256 nm.

The yield of camptothecin in each sample was determined and compared with that of control culture in basal MS medium.

RESULTS AND DISCUSSION

Of the various plant parts used, only the leaf petioles and lamina gave callus. Other plant parts like inflorescence axis and internodal segments produced phenol exudates in the medium and dried later.

1.Influence of auxin /cytokinin combinations on cpt production from callus(Fig :1)

In this experiment the highest amount of cpt was obtained in the combination MS+2,4-D(1) mg/ml + Kin 1 mg/ml. The friability of the callus was very high. But higher concentrations of 2, 4-D further increased friability resulting in spongy callus and early cell death due to cell lysis. Combination of BA and 2,4-D showed faster

growth and formed compact yellow callus which produced reasonable amounts of cpt. But in this case also increase in 2,4-D concentrations resulted in cell death . NAA/BA combinations showed moderate growth rate. Here the callus was highly compact in nature ,hence cannot be subjected to scale up. In general , an increase of auxin levels such as 2,4-D in the medium stimulates differentiation of the cells and consequently diminishes the level of secondary metabolites. In suspension cultured cells of *C.roseus*, the addition of 2,4-dichlorophenoxy acetic acid (2,4-D) and other auxins caused a suppression of transcription of the genes encoding the enzyme tryptophan decarboxylase(tdc) and strictosidine synthase (sss) (Goddijn *et al*, 1992; Pasquali *et al*,1992). Here eventhough 2,4-D produced higher amounts of cpt, this is very low compared to cytokinin alone combinations.

2.Influence of basal media on cpt production from callus (Fig:2)

Influence of various basal medium -MS and B5- on cpt production was checked. It was found that With the same hormone combination, B5 medium produced higher amount of camptothecin compared to MS. The highest amount obtained was 0.0066%. The growth rate of the callus was higher in B5 medium.

3.Influence of cytokinin on cpt production from callus (Table 1)

The cytokinins BA and KIN differ in their influence. In general increasing the concentration of BA increase the growth rate. The increase in growth rate some times decreases the secondary metabolite production. This may be the reason for the decrease in cpt content with increased concentrations of BA. Kinetin is not capable of increasing the growth rate as BA. The increase in concentration of Kinetin resulted in increase in cpt production slightly.

Cytokinins were found to stimulate alkaloid biosynthesis in some tumourous cell lines of *C.roseus* also. (Kodja *et al*, 1989).The effects observed were dependent on the cell line, the nature and the concentration of the cytokinin and the growth phase at which the cells were treated (Decendit *et al*, 1992). The response to cytokinin is dependent on the plasma membrane calcium influx with the involvement of calmodulins for alkaloid accumulation in suspension cultures of *C.roseus* (Liu *et al* 1990)

Table 1

Basal medium	Hormone combinatioin(mg/ml)	Yield(% of Dry weight)
MS	----	0.1×10-5
MS	3 BA+ 1 KIN	0.0006
MS	2 BA+1 KIN	0.008
MS	1 BA+2KIN	0.002
MS	1BA+3KIN	0.004

Figure 1
Influence of auxins/ cytokinin combinations on cpt production from callus on cpt production from callus

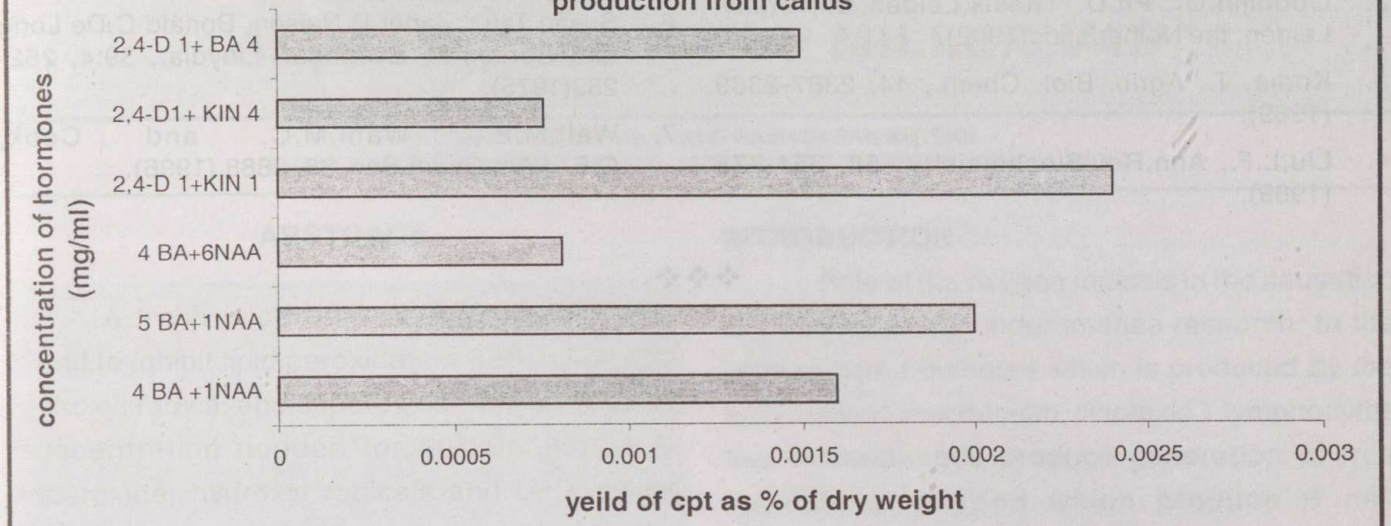
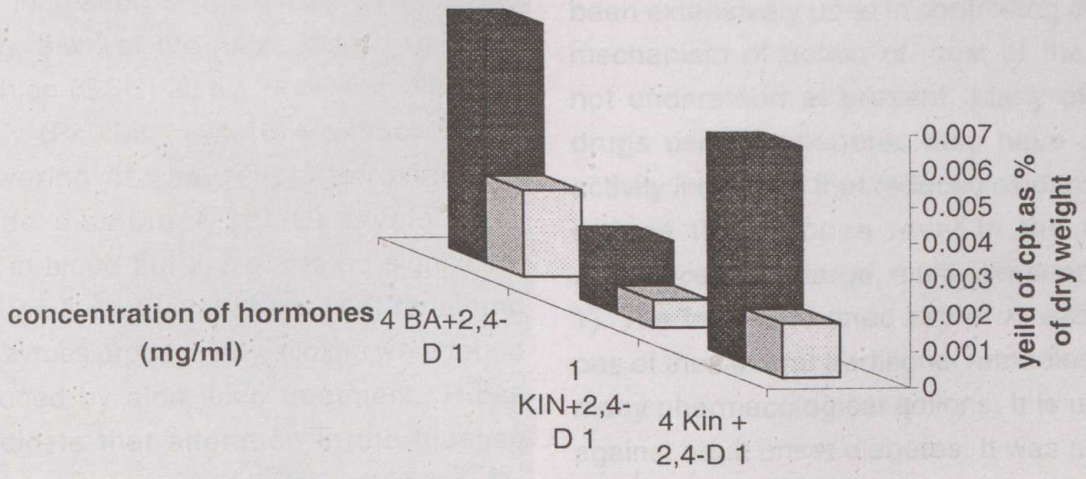


Figure 2. Influence of basal medium on cpt production from callus



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ANTIDIABETIC ACTIVITY OF *ALOE ARBORESCENS* MILLER *VAR*

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ABSTRACT

A fresh juice of *Aloe arborescens* was found to inhibit lipid peroxidation (LP), scavenge hydroxyl radical and superoxide radicals *in vitro*. Concentration needed for 50% inhibition of superoxide, hydroxyl radicals and LP radicals were 54, 70.5 and 52.2 μg/ml, respectively. Administration of *A. arborescens* (200 and 1000 mg/kg, b.wt) to normal rats increased glucose tolerance significantly ($P<0.001$). Continued administration (15 days) of the *A. arborescens* 200 and 1000 mg/kg produced 26.9 and 42.3 % reduction respectively in the elevated serum glucose level produced by alloxan administration. *A. arborescens* juice also produced significant increase in antioxidant status in rat after alloxan administration. Blood catalase was significantly ($P<0.005$) increased in the group treated with 1000mg/kg, b.wt. of the juice. Tissue calalase and glutathion (GSH) levels were also elevated significantly ($P<0.001$) with this extract. There was a lowering of tissue lipid peroxidation. Superoxide dismutase (SOD) levels were increased in blood but there was no significant change in the liver. Elevated levels of renal and hepatic enzymes produced by alloxan were found to be reduced by aloe juice treatment. These results indicate that alteration in the glucose utilizing system and oxidation status in rats increased by alloxan were partially reversed by the administration of *A. arborescens* juice.

INTRODUCTION

Role of the oxygen radicals in the causation of diabetes is still under intense research. In the case of type I diabetes which is produced by the auto immune mechanism, cytotoxic T lymphocytes and macrophages produce generation of free radicals of oxygen which produce ß cell destruction. Free oxygen radicals have a role in the insulin resistance also in type II diabetes as seen in a recent report on a positive relationship between tumor necrosis factor and insulin resistance (1).

Many indigenous Indian medicinal plants have been found to be useful to successfully manage diabetes (2-6). Despite the introduction of hypoglycemic agents from natural and synthetic sources diabetes and complications continue to be a major problem. Several herbal drugs have been extensively used in controlling diabetes; the mechanism of action of most of these drugs is not understood at present. Many of the herbal drugs used in diabetes also have anti-oxidant activity indicating that reduced oxidant stress may reduce the glucose level in the body. *Aloe arborescens* is a large, multi-stemmed shrub (Fig. 1). The fresh and dried sap of *A. arborescens* is one of the several traditional remedies, which has many pharmacological actions. It is used in tribal against adult onset diabetes. It was also found to inhibit pancreatic cancer induced by carcinogenesis. A related species, *Aloe vera* has been reported to have antidiabetic property (7).

In the present study we had selected the *A. arborescens*, to establish its role as antidiabetic and antioxidant drug and its role to reduce hepatic and renal toxicity was evaluated.

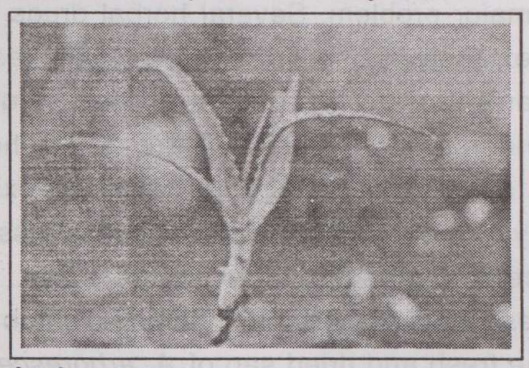
MATERIALS AND METHODS

Animals

Male Wistar albino rats (250-300g) were obtained from the Veterinary College, Mannuthy, Kerala. They were housed in ventilated cages and fed with a pelleted diet (Lipton, India Ltd) and water *ad libitam.*

Preparation of *Aloe arborescens* juice

The leaves of Aloe arborescens Miller var (Family-Liliaceae) were collected from Wyanad and it was identified by Dr. Ansari, Botanist, Malabar Botanical Garden, Kozhikkode. Fresh pulp was taken out with blunted knife. 20g of fresh pulp was crushed in the mortar and suspended in 40 ml of distilled water. This juice was passed through a muslin cloth, and used for the experiments. *Zingiber officinale* (Ginger) was collected from Thrissur and was extracted twice with water and evaporated to dryness.



Chemicals

Alloxan monohydrate was obtained from Sigma, St. Louis, M.O. 1-chloro-2,4-dinitrobenzene, glutathione and 5-5-dithiobis (2-nitrobenzoic acid) were purchased from Sisco Research Laboratory, Mumbay, India. Thiobarbituric acid was obtained from E-Merck, Germany. All other chemicals used were of analytical reagent grade.

Determination of in vitro antioxidant activity

Aloe arborescens juice was diluted to 1000 mg/ml and various concentration of the juice used for the *in vitro* experiments and IC_{50} (50% inhibition concentration) were calculated. The antioxidant levels of *Aloe arborescens* was compared with a known antioxidant 'ginger' (*Zingiber officinale*).

Superoxide radicals scavenging activity

Superoxide scavenging was determined by the Nitroblue tetrazolum (NBT) reduction method (8). The reaction mixture contained EDTA (6 μM) containing NaCN (3 μg), riboflavin (2 μM), NBT (50 μM), various concentrations (200 and 1000 mg/kg) of the aloe juice (10-100 μg/ml) and phosphate buffer (67 mM, pH 7.8) in a final volume of 3 ml. The tubes were uniformly illuminated with an incandescent lamp (Philips, 40W) for 15 min, and the optical density was measured at 530 nm before and after the illumination. The percentage inhibition of superoxide generation was evaluated by comparing the absorbance values of the control and experimental tubes.

Hydroxyl radical-scavenging activity

Hydroxyl radical scavenging was measured by studying the competition between deoxyribose and the test compounds for hydroxyl radicals generated from the Fe3+/ ascorbate / EDTA / H_2O_2 system. The hydroxyl radical attack deoxyribose, which eventually results in thiobarbituric acid reacting substance (TBARS) formation (9). The reaction mixture contained deoxyribose (2.8 mM), $FeCl_3$ (0.1 mM), EDTA (0.1

mM), H_2O_2 (1 mM), ascorbic acid (0.1 mM), potassium phosphate buffer (20 mM, pH 7.4), and various concentrations of the extract (50-200 µg/ml) in a final volume of 1 ml. The reaction mixture was incubated for 1 hr at 37^{0}C. Deoxyribose degradation was measured as TBARS and percentage inhibition was calculated.

Lipid peroxide scavenging activity

Reaction mixture (0.5 ml) containing rat liver homogenate (0.1 ml, 25% w/v) in tris-HCl buffer (40 mM, pH 7.0), KCl (30 mM), ferrous iron (0.16 mM) and ascorbic acid (0.06 mM) was incubated for 1 hr at 37^{0}C in the presence and absence of the extract (10-100 µg/ml). The lipid peroxide formed was measured by TBARS formation (10). For this, 0.4 ml of incubation mixture was treated with sodium dodecyl sulphate (SDS-8.1%, 0.2 ml), thiobarbituric acid (TBA-0.8%, 1.5 ml) and acetic acid (20%, 1.5 ml, pH 3.5). The total volume was then made upto 4 ml by adding distilled water and kept in a water bath at 100^{0}C for 1 hr. After cooling, 1 ml of distilled water and 5 ml of a mixture of n-butanol and pyridine (15:1 v/v) were added and shaken vigorously. After centrifugation, the absorbance of the organic layer was measured at 532 nm. The percentage inhibition of lipid peroxidation was determined by comparing the results of the test compounds with those of controls not treated with the extracts.

Determination of Glucose tolerance test (GTT)

Male Wistar rats (150 - 200 g) were divided into four groups. Glucose (2 g/kg b. wt.) was given orally to all groups. Control rats (Group 1) were given 1 ml distilled water immediately after glucose administration. Fresh juice of *Aloe arborescence* (200 and 1000 mg/ kg b.wt.) was given orally to second and third groups. Insulin (2 IU/animals) was administered to group 4 by intraperitoneal injection. Blood samples were collected from the tail vein just prior to drug administration and 30 min, 1, 2 and 4 hour after the glucose loading and blood glucose levels were measured by GOD/POD enzymatic method (11).

Determination of Antidiabetic activity

Single and multidose Study

Diabetes was induced in male rats by injecting (ip.) a single of alloxan monohydrate (120 mg/kg b.wt) (12). Serum glucose level was checked after 72 hour. Animal with serum glucose level greater than 250 mg/dl were considered diabetic and used for the study (13). The rats were divided into five groups of 6 rats each. Group I were normal rats and Group II diabetic animals were given distilled water. Group III and IV were given aqueous suspension of *Aloe arborescens* orally at a dose level of 200 and 1000 mg/kg b. wt. respectively on third day after alloxan treatment. Group V was given insulin (2 IU/ animal) as intraperitoneal injection. Fasting blood sample were collected from the tail vein third day after alloxan treatment prior to the extract administration and at 1, 2, 4 and 6 hour intervals. In the multidose study, animals were continued with the same dose of the extract once daily for 15 days. Blood was collected one hour after drug administration and serum glucose levels were measured on 3, 6, 9, 12 and 15th day. The body weight of the animals was monitored in one-week interval.

Determination of *in vivo* antioxidant activity

The diabetic and drug treated animals were sacrificed on 18th day after alloxan administration. Blood was collected from the heart and serum was separated. The liver was separated, washed with normal saline and kept in the freezer (-20°C) till the experiment carried out. The liver samples were

homogenized with Tris HCl buffer.

Erythrocytes were prepared by the method of Minami and Yoshikawa (14) and superoxide dismutase was estimated by the modified method of McCord and Fridovich (8). The assay is based on the ability of the enzyme to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide (O_2), which is generated by the photo reduction of riboflavin. Enzyme activity was calculated from the inhibition of reduction using a standard curve constricted by varying amounts of homogenate. One unit of enzyme activity is defined as amount of enzyme giving 50% inhibition of the reduction of NBT and expressed as Units/mg protein.

Catalase was estimated in the erythrocytes and liver tissue by the method of Aebi (15) by measuring the rate of decomposition of hydrogen peroxide (H_2O_2) at 240 nm. A decrease in absorbance was observed after the addition of H_2O_2 to the reaction mixture containing either the tissue homogenate or the erythrocyte sediment. Lipid peroxidation (LPO) levels in liver was estimated using thiobarbituric acid (TBA) method of Ohkawa (10) by using 1,1,3,3 tetramethoxy propane as standard and the serum lipid peroxidation was estimated by the method of Satoh (16). Glutathione were estimated both in blood and liver tissue by the method of Moron (17) based on the reaction with DTNB and values were calculated from the standard of GSH treated with the same reagent.

Determination of liver and kidney function test

Normal and diabetic animals were treated with aloe juice for 15 days at dose levels of 200 and 1000 mg/kg body weight. They were sacrificed on 18th day after alloxan treatment. Blood was collected and serum was separated and alkaline phosphatase (ALP; 18), glutamate pyruvate transaminase (GPT; 19), blood urea nitrogen (BUN; 20) and creatinine (21) were estimated. Protein was determined by the method of Lowry et al., (22). Differential count and total leukocyte count (23) were estimated initially and on 7th and 14th day after alloxan administration. Haemoglobin was estimated with Drabkin's reagent.

The liver glycogen was estimated by Hassid and Abraham, (24) and modified by Good et al. consists in the digestion of the tissue in hot concentrated KOH, precipitation of the glycogen with ethanol, hydrolysis of the glycogen with acid, and determination of the glucose in the hydrolysate as reducing sugar.

STATISTICAL ANALYSIS

The values were expressed as mean ± Standard error of mean (SEM). Statistical comparisons between of all groups were performed by using student 't' test (25).

RESULT

The in vitro antioxidant activity of *Aloe arborescens* was compared with a known antioxidant 'ginger'. *A. arborescens* was found to scavenge the superoxide generated by photoreduction of riboflavin in a concentration dependent manner. The concentration needed for 50% scavenging of superoxides was 54 mg/ml. The concentration needed for 50 % inhibition of hydroxyl radical generation was 70.5 mg/ml. Lipid peroxidation induced by Fe^{3}+/ ascorbate in rat liver homogenate was found to be inhibited and the concentration needed for 50 % inhibition was 52.2 mg/ml. Whereas the concentration needed for 50% scavenging of superoxides, hydroxyl radical and lipid peroxidation of ginger were 20.0, 150.0 and 30.0 mg/ml respectively (Table 1)

Administration of 2 g glucose/kg b.wt. to the normal rats increased the serum glucose level 77.8± 5.1 to 147.5 ±6.4 at 60 minutes and further reduced to normal at 240 minutes. *A. arborescens* extract (200 and 1000 mg/kg b.wt.) administration suppressed the elevation of serum glucose level significantly (Table 2).

Continuous administration of the *A. arborescens* (200 mg/kgb.wt.) produced 26.9 % reduction in the elevated glucose level on 18th day after alloxan administration. At dose level of 1000 mg/kg b.wt. There was 42.3% reduction in blood glucose level on 18th day ($P<0.005$) as compared to the glucose level of untreated animals on the same day (Table 3).

The body weights of normal and diabetic animals were recorded before treatment, and 1st day, 7th day and 14th day after drug treatment. In normal animals there was a slight increase in the body weight was shown. Alloxan diabetic animals showed significant decrease in the body weight from 7th day. Administration of *A. arborescens* juice reduced the weight loss induced by alloxan administration in a dose dependent manner and 1000 mg/kg drug treated group, the body weight of the animals were increased when compared to initial weight of the same group (Table 4).

In vivo antioxidant activity of A. arborescens after administration for 18 days is shown in table 5. SOD levels in blood were significantly ($p<0.005$) increased after A. arborescens treatment. The SOD values in blood were found to be increased from 422.5± 18.9 to 520.2 ± 20.1 at a dose level of 1000 mg/kg b.wt. and there was no significant increase in the liver tissue. There was only marginal difference in the values of blood and liver. Catalase values, were not significantly changed when compared with normal and treated group (Table 5).

Glutathione levels were significantly decreased in alloxan-induced group when compared with normal animals. The GSH levels were increased ($p<0.005$) in the liver tissue after treatment with the drug and no significant increase was shown in blood GSH after the *A. arborescens* and insulin treatment. Tissue lipid peroxidation was decreased from 4.2 ± 0.3 to 3.5 ± 0.17 by the administration of 1000 mg/kg b.wt. of A. arborescens ($P<0.005$) and there was no significant decrease in the serum lipid peroxidation (Table 6).

There was significant elevation in ALP and GPT in liver and serum ($P<0.001$) in alloxan-induced diabetes when compared with normal aniamls. Animals treated with 1000 mg/kg of the aloe juice showed significant ($P<0.001$) reduction in the elevated level of ALP and GPT. The liver glycogen was significantly ($P<0.001$) reduced from 84.8 ±2.22 to 57.4±1.97 when compared with normal and diabetic group. Administration of aloe juice (1000 mg/kg, b.wt.) and insulin treatment significantly ($P<0.001$) increased the glycogen level when compared with diabetic group (Table 7).

Renal function indicators like creatinine and BUN were also elevated significantly ($P<0.001$) in the alloxan diabetic rats when compared with normal rats. The elevated creatinine level of 4.16mg/dl by alloxan administration was found to be reduced to 3.4 mg/dl ($P<0.001$) by the administration of 1000 mg/kg b.wt. of the *A. arborescens* juice. Elevated levels of BUN were also found to be reduced significantly ($P<0.001$) the in animals treated with 1000mg/kg dose of *A. arborescens* and in insulin treated group (Table 8).

The effect of *A. arborescens* on total WBC in alloxan diabetic animals is shown in Table 9.

Total and differential count were calculated before treatment and on day 1st day, 7th day, and 14th day of the experiment. The total count was found to be 9340 in normal animal rats and in alloxan diabetic rats it was 5830, on 14th day of the experiment. Administration of *A. arborescens* (1000 mg/kg) considerably reversed alloxan-induced cellular damage as seen from the increased number of total WBC ($P<0.001$) when compared with diabetic control group. Lower dose had also a comparable effect. The differential count was found to be increased by the *A. arborescens* juice treatment (data not shown).

DISCUSSION

The fresh juice of the Aloe plant (aloes) is one of the several traditional remedies and has several pharmacological actions. Aloe arborescens is being used by the tribal people, as anti diabetic drug however there is no experimental evidence for its activity. Furukawa et al (26) have reported its use in pancreatic carcinogenesis induced in hamsters. Umano et al (27) isolated and identified some phytochemicals from the leaves of *A. arborescens* leaf extract. All et al, (28) studied the antifungal activity of the plant. In the present study, we have checked the antidiabetic activity of *A. arborescens* in detail using alloxan diabetic rats. We have also looked in to the antioxidant activity of the extract, by *in vitro* and *in vivo* method. We have extended our study to find out the activity of the extract against hepatic and renal toxicity induced by the alloxan on rats.

Under *in vivo* condition, glutathione (GSH) acts as an antioxidant and its decrease was reported in diabetes mellitus (29). The increased GSH content in the liver of the rats treated with *A. arborescens* may be one of the factors responsible for the inhibition of lipid peroxidation. Superoxide dismutase and catalase are the two major scavenging enzymes that remove the toxic free radicals *in vivo*. Vucic et al (30) reported that the activity of SOD is low in diabetes mellitus. The *A. arborescens* treated rats showed increased activity of SOD and GSH.

Alloxan produces free oxygen radicals in the body, which cause pancreatic injury (31) and could be responsible for increased blood sugar seen in the animals. However, action is not specific to pancreas as other organs such as liver, kidney and haemopoietic system also affected by alloxan administration as seen from the elevation of marker enzymes and reduction of hematological parameters. This was reversed by the continued administration of *A. arborescens* extract.

The present study showed that the antioxidant plant extract may protect the pancreas in alloxan induced oxidant stress in rats and thereby reduces the diabetes. It is probable that ROS may induce LP and modify SOD and catalase in presence of alloxan and the treatment with *A. arborescens* mitigates this action. It is suggested that *A. arborescens* may function as an antidiabetic agent and lower alloxan induced peroxidative damage and oxidative stress in diabetes. In conclusion, oral administration of the fresh juice was found to reduce the serum glucose tolerance in alloxan diabetic rats. It could also increase the antioxidant potential and reduces the liver and kidney toxicity and normalize the hematological changes induced by prooxidants.

Table 1. Effect of *Aloe arborescens* juice on *in vitro* antioxidant activity

Treatment	Concentration needed for 50% inhibition of oxygen radicals (μg/ml)		
	Superoxide	Hydroxyl radical	Lipid peroxidation
A. arborescens	54.0±1.5	70.5±1.8	52.2±1.9
Z. officinale	22.0±1.2	150.0±2.5	30.0±1.1

Values are mean ± SD, n=3

Table 2. Effect of *Aloe arborescens* on glucose tolerance test (GTT) in normal rats

Treatment	Serum glucose level (mg/dl)				
	Initial	30 min	60 min	120 min	240 min
Glucose (2g/kg b.wt.)	77.8 ± 5.1	93.8 ± 6.6	147.5 ± 6.4*	118.0 ± 6.3	88.1 ± 7.1
Glucose + *A. arborescens* (200mg/kg b.wt.)	78.4 ± 5.4	88.1 ± 5.7	132.1 ± 4.45*	108.5 ± 4.46	81.9 ± 6.0
Glucose + *A. arborescens* (1000mg/kg b.wt.)	83.9 ± 3.56	94.0 ± 6.5	114.1 ± 5.5*	100.2 ± 7.4	83.5 ± 7.0
Glucose + Insulin (2 IU/animal)	79.7 ± 4.3	87.1 ± 6.1	101.0 ± 7.7	85.9 ± 5.3	79.2 ± 6.7

* P<0.001 (compared to same group of initial value) Values are mean ± SD, n=6

Table 3. Effect of *Aloe arborescens* in alloxan induced diabetic rats (Multidose study)

Treatment Group	Serum glucose level (mg/dl)					
	3rd Day	6th Day	9th Day	12th Day	15th Day	18th Day
Alloxan	342.5±22.4	410.2±10.4	428.8±13.5**	459.6±22.1**	467.1±22.8**	381.3±23.6
Alloxan + *A.arboescens* (200mg/kg b.wt.)	361.1±23.2	326.2±19.3	307.4±13.5	294.5±16.6	272.9±8.3**	263.9±5.0*
Alloxan + *A.arborescens* (1000 mg/kg b.wt.)	415.1±30.14	379.0±32.6	345.7±28.6	312.1±20.2	270.0±23.5**	239.6±24.3*
Alloxan + Insulin (2 IU/animal)	444.9±26.2	430.6±20.3	360.3±13.9	305.0±15.4*	251.7±12.7**	208.3±8.7*

Values are mean ± SD, n=6

** P<0.005, *p<0.001 (Compared to value on 3rd day after alloxan treatment of the same group)

Table 4. Effect of *Aloe arborescens* body weight changes in normal and drug treated alloxan diabetic animals

Treatment	Body weight (gms)			
	Initial	1st day	7th day	14th day
Normal	285.7 ± 14.4	289.8 ± 14.6	294.1 ± 14.9	300.1 ± 16.9
Alloxan	287.4 ± 11.5	236.7 ± 14.6	197.7 ± 9.3*	223.2 ± 8.0*
Alloxan + *A.arboescens* (200mg/kg b.wt)	269.6 ± 22.9.	246.5 ± 19.9	248.7 ± 20.3	251.7 ± 19.8
Alloxan + *A.arborescens* (1000 mg/kg b.wt.)	260.1 ± 24.4	248.0 ± 24.4	258.3 ± 21.4	266.3 ± 23.0
Alloxan + Insulin (2 IU/animal)	280.8 ± 14.9	261.5 ± 33.6	272.1 ± 36.1	285.8 ± 38.7

* P<0.001 (values are compared to alloxan diabetic group)

Values are mean ± SD, n=6

Table 5. Effect of *Aloe arborescens* on superoxide dismutase and catalase activity in alloxan induced diabetic rats

Treatment	CATALASE		SUPEROXIDE DISMUTASE	
	Blood[A]	Liver Tissue[B]	Blood[A]	Liver Tissue[B]
Normal	8.7 ± 1.21	7.5 ± 0.79	675.2 ± 10.6	12.7 ± 0.95
Alloxan	5.5 ± 1.44	5.2 ± 0.82	422.5 ± 18.9	7.5 ± 0.18
Alloxan + *A.arboescens* (200mg/kg b.wt.)	6.2 ± 1.01	6.1 ± 0.92	455.7 ± 16.7	8.0 ± 0.71
Alloxan + *A.arborescens* (1000 mg/kg b.wt.)	6.9 ± 0.99**	6.8 ± 0.74*	520.2± 20.1**	9.0 ± 0.62
Alloxan + Insulin (2 IU/animal)	6.8 ± 0.91**	6.9 ± 0.65*	525.1± 20.7**	9.1± 0.95

** p<0.005 (Values are compared to Alloxan diabetic group)
Values are mean ± SD, n=6 A-U/g Hb, B- U/mg protein/min

Table 6. Effect of *Aloe arborescens* on lipid peroxidation and glutathione levels in alloxan induced diabetic rats

Group	Lipid peroxidation		Glutathione	
	Serum[A]	Liver[B]	Blood[C]	Liver[D]
Normal	2.1 ± 0.14	3.0 ± 0.32	26.7 ± 1.53	38.0 ± 2.0
Alloxan	2.6 ± 0.08	4.2 ± 0.30	20.3 ± 1.53	29.3 ± 1.15
Alloxan+ *A. arboescens* (200mg/kg b.wt.)	2.1 ± 0.17	3.5 ± 0.15	22.0 ± 1.0	33.2 ± 1.26
Alloxan+ *A.arborescens* (1000 mg/kg b.wt.)	2.3 ± 0.06	3.6 ± 0.17**	25.7 ± 1.53	34.8 ± 1.04**
Alloxan+Insulin (2 IU/animal)	2.3 ± 0.03	7.2 ± 0.29*	26.5 ± 1.32	36.2± 1.44**

** p<0.005 (Values are compared to Alloxan diabetic group)
Values are mean ± SD, n=6
A-n moles/ml, B-mM/100g wet tissue, C-n moles/g Hb, D-n moles/mg protein

Table 7. Effect of *Aloe arborescens* in the alteration of hepatic function induced by alloxan

Group	ALKALINE PHOSPHATASE		GLUTAMYL PYRUVATE TRANSFERASE		GLYCOGEN
	Liver tissue[A]	Serum[A]	Liver[B]	Serum[C]	Liver[D]
Normal	33.9 ± 2.2	18.6 ± 1.1	228.6 ± 18.6	75.1 ± 6.6	84.8 ± 2.22
Alloxan	51.8 ± 5.5	36.6 ± 3.2	456.1 ± 12.3	215.2 ± 8.6	57.4 ± 1.97
Alloxan + *A. arboescens* (200mg/kg b.wt.)	48.6 ± 2.2	32.1 ± 2.1	401.6 ± 18.5	180.1 ± 9.8	66.1 ± 1.43**
Alloxan + *A. arborescens* (1000 mg/kg b.wt.)	40.1 ± 4.3	25.6 ± 1.4	342.2 ± 14.1*	138.6 ± 12.6*	61.2 ± 2.86
Alloxan + Insulin (2 IU/animal)	39.8 ± 4.0	23.3 ± 2.1**	320.1 ± 16.8*	145.1 ± 13.7*	68.3 ± 1.55*

** p<0.005 (Values are compared to Alloxan diabetic group)
Values are mean ± SD, n=6
A- KA/dl, B- U/mg protein, C-U/ml, D-mg/g tissue

Table 8. Effect of *Aloe arborescens* on Blood urea nitrogen and Creatinine in alloxan induced diabetic rats

Group	Blood urea nitrogen (BUN)	Creatinine
Normal	15.3 ± 0.52	2.7 ± 0.11
Alloxan	25.5 ± 0.82	4.2 ± 0.15
Alloxan + *A.arboescens* (200mg/kg b.wt.)	24.7 ± 0.90	3.9 ± 0.16
Alloxan + *A.arborescens* (1000 mg/kg b.wt.)	21.7 ± 1.61	3.4 ± 0.18*
Alloxan +Insulin (2 IU/animal)	20.7 ± 1.23	3.2 ± 0.22**

** p<0.005 * p< 0.001 (Values are compared to Alloxan induced diabetic group)
Values are mean ± SD, n=6

Table 9. Effect of *Aloe arborescens* Total count on normal and alloxan diabetic animals

Group	Before treatment	1st day	7th day	14th day
Normal	9340 ± 495.2	9305 ± 570.3	9309 ± 477.1	9257 ± 564.5
Alloxan	9072 ± 275.0	6247 ± 281.0	5830 ± 385.1	7143 ± 155.7
Alloxan + *A.arboescens* (200mg/kg b.wt).	9384 ± 442.0	7047 ± 115.7	7115 ± 92.6	7534 ± 125.1
Alloxan + *A.arborescens* (1000 mg/kg b.wt.)	9841 ± 231.9	7249 ± 695.3	7843 ± 376.2	8108 ± 188.7
Alloxan +Insulin (2 IU/animal)	9560 ± 210.4	8217 ± 128.3	8650 ± 203.5	9031 ± 90.1*

* P<0.001 (values are compared to alloxan diabetic group)
Values are mean ± SD, n=6

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ANTI-TUMOUR ACTIVITY OF 2-ETHYL-3-[(4-AMINO-5-(3,4,5 - TRIMETHOXY BENZYL) PYRIMIDIN - 2YL)] QUINAZOLIN - 4 -ONE (SPA- II)

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ABSTRACT

The anti-tumour activity of 2-Ethyl-3-[(4-amino-5-(3,4,5-trimethoxy benzyl)Pyrimidin-2-yl)]quinazolin-4-one has been evaluated against solid tumour sarcoma 180 in swiss albino mice. Sarcoma 180 was maintained and propagated intraperitonially by serial transplantation in adult female mice. The SPA-II treated group (oral and intraperitoneal) has significantly increased the volume doubling time and growth delay when compared with the respective control. The survival time have also been increased in the drug treated groups.

Key words : Quinozonil-4-one, trimethoprim, sarcoma-180. anti-cancer activity.

INTRODUCTION:

Quinazolin-4-one is a versatile lead molecule for designing potential bio-active agents. Methaqualone an approved quinazolin-4-one derivative is used as sedative. Quinazolin-4-one derivatives were tested for the broad spectrum biological activities like anti-cancer (anti-proliferative) and anti-HIV2. Our present work is to synthesis some novel 2, 3-disubstituted quinazolin-4-one by condensation of 2-ethyl benzoxazin-4-one with Trimethoprim and the product was evaluated for anti-cancer activity in sarcoma 180 in Swiss albino mice.

MATERIALS AND METHODS:

Chemicals Used :

Anthranilic acid, acetic anhydride, ethanol, Trimethoprim and glacial acetic acid.

Synthesis of 2-Ethyl-3,1-Benzoxazin-4-one:(SP-II):

A mixture of anthranilic acid (0.01mol) and propionic anhydride (0.2 mol) was refluxed under anhydrous condition for 4 hours, then the reaction mixture was cooled to room temperature and poured into crushed ice and set aside for 60min. then the residue was filtered with suction. The residue was washed several times with petroleum ether. The dried product was then recrystallised from ethanol. The percentage yield of synthesized products was depicted in Table No.1

Synthesis of Ethyl-3-[94-amino-5(3,4,5-trimethxoy benzyl) pyrimidin-2-yl)] quonazolin-4-one:(SPA-II):

An equimolar (0.01mol) mixture of 2-Ethyl-1, 3- benzoxazin-4(3H)-one and Trimethoprim was refluxed for six hours in the presence of 10 ml glacial acetic acid. Then the mixture was cooled at room temperature and poured into crushed ice and the solid thus obtained was recrytallised from ethanol. The percentage yield was given in Table No.1.

Purity Checking:

Purity was checked by TLC technique

using silica gel G as stationary phase and chloroform : methanol (9:1) as mobile phase and iodine vapours as the visualising agent. Melting point was checked by using open ended capillary tube method and are uncorrected. The value are given in Table No.1.

Acute Toxicity Study:

The acute toxicity study of SPA-II in terms of LD50 was determined as described in the healthy adult albino mice of either sex weighing between 20-28gms were divided into 7 groups of animals each.

Group I, II, III, IV, V, VI, VII received 25, 30, 45, 50, 65, 95mg/kg of SPA-II. Group VII served as solvent control (0.5% CMC). The drug was then observed for every 2 hours upto 24 hours and later at 72 hours, 14 days and 30 days for any mortality and the number of animals. Dead and survived were noted and the LD50 was calculated according to the method described by the read et al. Results are tabulated in Table No.2. A plot was drawn by taking log dose on X-axis and probit on Y-axis and the LD50 was found to be 49.20mg/kg. The graph was represented in fig. No.1.

Animals:

Swiss albino mice (20-25 gm) were used throughout the study.

They were housed in Tarson's polypropylene cages with metal grill tops and were given standard rat pellet diet (Lipton India Ltd., Bangalore) and water ad libitum.

Cells:

Solid tumour sarcoma 180 (S-180) cells were originally produced from the cancer research institute, Parel, Bombay, India, was maintained and propagated intraperitoneally by serial transplantation in adult female mice.

Method:

Solid tumours were obtained by intradermal inoculation of 5×10^5 viable tumour cells on the dorsal side of mice. Tumour diameter was measured in three planes with a vernier caliper.

Tumour volume (V) was calculated by using the formula, $V = \pi/6$ (D1, D2, D3)
where, D1,D2,D3 are the three dimensions.

Animals were divided into 12 per group. The drugs were administered to different groups were SPA-II 1 mg/kg in 5% CMC orally and SPA-II 1 mg/kg in 0.2ml ground nut oil intraperitoneally. Vehicle treated control group receiving equal volume of CMC orally and 0.2 ml of ground nut oil were included for comparison. Treatment had been started after the tumour volume reaches the size of 100 mm^3. The tumour bearing mice were weighed and checked daily and any mortality was recorded.

Anti-cancer activity was assessed by measuring the parameters like tumour regression, volume doubling time and growth delay as quantitative measure. Tumour volume doubling time (VDT) was estimated according to the formula,

$$Td = \frac{\text{Log}_2 \times (T_1 - T_0)}{\text{Log } V_1 - \text{Log } V_0}$$

Where,

V_0 - The volume when the tumour was first measured.

V_1 - The volume at the termination of the experiment.

Tumour growth delay (GD) is the difference in number of days for the treated tumour (T) to reach 200 mm^3 compared to untreated control.

GD = T - C

The students "t" test was employed to analyse the result statistically. The result of anti-cancer activity was shown in Table No.3.

RESULT AND DISCUSSION:

The acute toxicity study LD50 of SPA-II was calculated 1/5th or 1/10th of LD50 is the effective dose (ED50) of SPA-II.

The SPA-II (1mg/kg) treated group (oral and intraperitoneal) has significantly increased the volume doubling time and growth delay when compared with the respective control ($P < 0.001$). The survival time have also been increased in the SPA-II treated groups.

Table No.1
Physical parameters of the Synthesised Compound

S.No.	Code	Mol.Wt	% Yield	m..p(C)	Rf. Value
1	SP - II	C10H9O2N5	68	114	0.72
2	SPA - II	C23H25O4N5	98	124	0.81

Table No.2
Acute toxicity study of Compound SPA - II

S.No	Dose mg/ml	Log.Dose	Dead/Total	% Dead	Corrected	Probit
1	15	1.1760	0/10	0	2.5	3.04
2	30	1.4471	1/10	10	10	3.72
3	45	1.6532	4/10	40	40	4.75
4	60	1.7781	7/10	70	70	5.52
5	75	1.8750	9/10	90	90	6.28
6	90	1.95542	10/10	100	100	6.96

Correction Formula,

For the 0% Dead = 100 (0.25/n)
For the 100 % Dead = 100 (n-0.25/n)

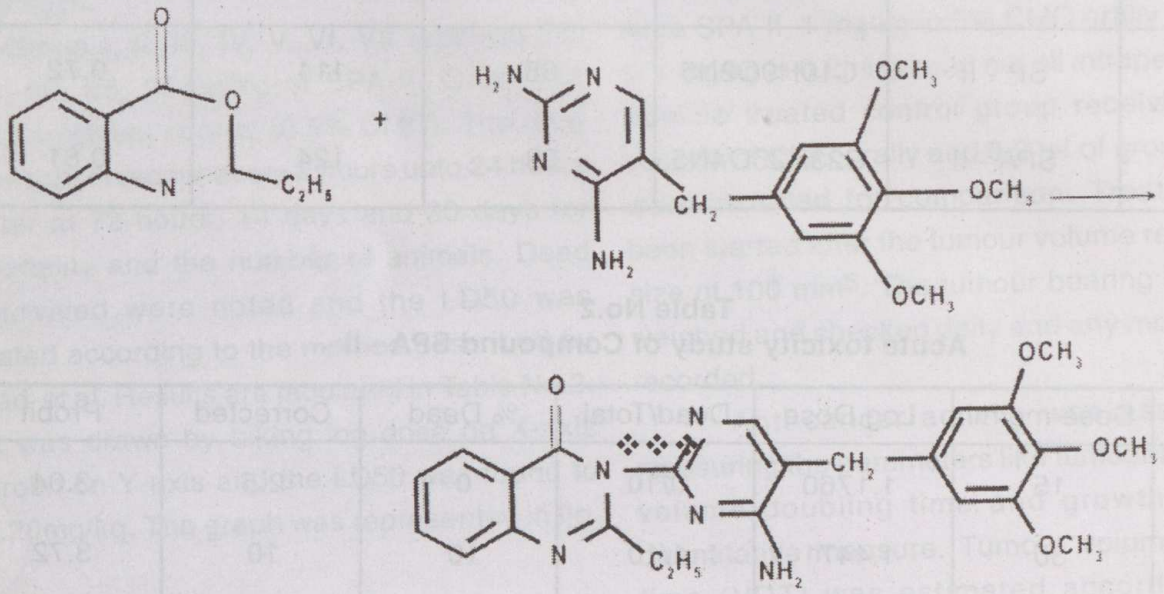
where ,

n = the no. of animals in the group.

Table No.3
Anti Tumor activity of Synthesised Compund - SPA - II

S.No	Treatment	VDT (Days)	GD (Days)
1	Control (5ml CMC, P.O)	3.07	=
2	Control (0.2ml Groundnut Oil, I.P)	3.10	=
3	SPA - II (1mg/kg,P.O)	8.1*	6.3*
4	SPA - II (1 mg /kg, I.P)	7.3*	5.4*

* P < 0.001 Vs respective control by student "t" tests.



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ANTI-CARCINOGENIC ACTIVITY OF *PHYLLANTHUS* AMARUS EXTRACT

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ABSTRACT

Cancer chemoprevention of chemically induced tumors by *P. Amarus* extract was studied on 7, 12 dimethyl benz(a)anthracene (DMBA) initiated papilloma formation in Balb/c mice and MNNG induced stomach cancer in Wistar rats. Number of papillomas produced by the application of DMBA as initiator and croton oil as promoter in mice were considerably inhibited (45.2% and 58.1%) by the prior application of 1mg and 5mg of *P. Amarus* respectively. Moreover there was considerable decrease in the number of tumor bearing animals and their onset. γ-glutamyl trans peptidase activity which was elevated to 20.3± 6.7 mmol/min/mg protein by MNNG application was lowered to 2.8± 0.9 by administration of 750mg/ kg of *P. Amarus* extract. Similarly elevated glutathione S- transferase activity (1317± 211 n mol/min/mg protein) and glutathione (368± 66) levels in the MNNG treated group were found to be lowered to 494.8± 76 and 192± 45 respectively. *P. Amarus* administration increased GSH from 4.6± 0.9 to 8.5±1.4 n mol/ min/mg protein. In our study, it was observed that AgNOR dots and clusters were more nearer to normals in *P. Amarus* treated animals.

INTRODUCTION

Prevention of carcinogenesis is one of major strategies for cancer control. Carcinogenesis is a multistage process that encompasses prolonged accumulation of injuries at several different biological levels and producing both biochemical and genetic changes in cells. At each of the levels there is an opportunity for intervention, a chance to prevent, slow or even halt the gradual change of healthy cells towards malignancy (Peter.,1996). Inspite of the immense efforts to improve treatment and find cures for advance disease, overall mortality rates for most forms of epithelial cancer have not declined in the past 25 years (Hong and Sporn., 1997). Efforts to prevent the disease would be a more desirable as well as practical approach for cancer control.

Stomach cancer is the third commonest malignancy in south India. Exposure to environmental nitrite and nitrosation of smoked foods has been associated with an increased risk of stomach cancer. Although the key nitrosating agent is nitrite, the situation with regard to the formation of carcinogenic nitrosocompounds is greatly complicated by the presence of other chemicals in the environment.

In our center several phytochemicals have been isolated and identified and have been demonstrated to block or suppress the different stages of carcinogenesis (Sukumaran et al., 1994, Rajesh Kumar and Kuttan., 2001, Jose et al., 1997, Joy et al., 2000, Jose et al., 1999).

Simultaneous administration of *P. Amarus* extract along with carcinogen has been reported to inhibit the hepatocellular carcinoma development induced by NDEA (Joy and Kuttan.,

1998). *P. Amarus* extract could inhibit the hepatocarcinogenesis and increased life span in tumor bearing animals (Rajesh Kumar and Kuttan., 2000). *P. Amarus* extract administration increased the life span of ascites tumor harboring mice and inhibited the sarcoma development induced by 20MC (Rajesh Kumar and Kuttan., 2002).

The present study was designed to assess its anti-carcinogenic activity of *P. Amarus* against DMBA induced papilloma formation in Balb/c mice as well as its effect on MNNG induced gastric cancer in Wistar Rats.

MATERIALS AND METHODS

Extraction of *P. Amarus*

Leaves and stems of *P. Amarus* were collected from Thrissur district of Kerala and were dried at 50^0C. The plant was identified with a voucher specimen that has been kept at Amala Ayurvedic Hospital and Research Centre.

Preparation of alcoholic extract

Dried parts of *P. Amarus* were powdered and this powder was extracted twice in 5 volumes of 75 % methanol by stirring overnight and centrifuged at room temperature. This supernatant was evaporated to dryness at 50^0C under reduced pressure using a rotary evaporator. The yield of the extract was 8%.

Determination of the effect of *P. Amarus* treatment on papilloma formation initiated by DMBA and croton oil

The concept of two stage carcinogenesis consists of initiation and promotion was first proposed by Berenblum., 1941. The former stage is an irreversible process while the latter is associated with reversible and irreversible changes, 12-0-Tetradecanoyl phorbol 13-acetate (TPA), present in croton oil is a typical tumour promoter having various biological and biochemical effects on susceptible tissues (Harris., 1991)

Male Balb/c mice were used for the studies. They were kept as groups of 10 animals / group. Aggressive males were removed and kept separately. The dorsal region (2 cm diameter) of mice were shaved with a razor at least two days before treatment with DMBA. Only mice which did not show signs of hair regrowth were used for the experiments. Animals were divided into different groups;

Group I- DMBA + croton oil, twice weekly for 8 weeks (Positive control).

Group II- DMBA alone (Initiation only).

Group III- DMBA + methanol (10μl/ mouse, topical) 30 minutes before croton oil application, twice weekly for 8 weeks (Vehicle control).

Group IV- *P. Amarus* (1mg/ mouse, topical), 10 continuous days prior to the application of DMBA followed by croton oil, twice weekly for 8 weeks (Prior to initiation).

Group V- *P. Amarus* (5mg / mouse, topical), 10 continuous days prior to the application of DMBA followed by croton oil application, twice weekly for 8 weeks (Prior to initiation)

Group VI- DMBA + *P. Amarus* (1mg/ mouse, topical) 30 minutes before each croton oil application, twice weekly for 8 weeks (Drug promotion).

Group VII- DMBA + *P. Amarus* (5mg/ mouse, topical) 30 minutes before each croton oil application, twice weekly for 8 weeks (Drug promotion).

Single dose of DMBA (470nmol/ mouse in 200μl acetone) was used in this study (George & Kuttan, 1997). Two weeks after DMBA application animals in group I, III, IV, V, VI, and VII were

applied with 10% croton oil (in 200μl acetone). *P. Amarus* was administered topically dissolved in methanol. The animals in all groups were watched for food intake as well as any apparent toxicity such as weight loss or mortality during entire period of the study. Skin tumor formation was recorded weekly, and the tumors greater than 1mm in diameter were included in the cumulative total if they persisted for 2 weeks or more. Delays in the onset of tumors in various groups were recorded.

Determination of the effect of *P. Amarus* on N-Methyl- N-nitro, N- nitrosoguanidine (MNNG) induced stomach cancer

Treating the animals with MNNG results in the formation of preneoplastic cells. Further treatment of MNNG causes conversion of preneoplastic cells to neoplastic cells. Anti-neoplastic activity of extract is evaluated by administering *P. Amarus* extract with MNNG which may prevent the development of neoplastic cells.

Male Wistar rats (10 animals / group) were used for the study. They were divided into four groups:

Group I : Normal, untreated
Group II : MNNG treated
Group III : MNNG + *P. Amarus* 150mg/kg. b. wt
Group IV : MNNG + *P. Amarus* 750 mg/kg.b. wt.

MNNG was given orally as a solution at a concentration of 1mg/ ml for the first 28 weeks. *P. Amarus* was given orally starting from the day of MNNG administration and continued for 20 weeks. The control group (Group II) was given distilled water. All the rats were killed at the 44th week of the experiment. The oesophagus, forestomach, glandular stomach and duodenum were removed. The stomach was opened along the greater curvature and examined the gastric mucosa grossly. The location and size of tumors were recorded.

The following biochemical parameters were done in the homogenized stomach mucosa to assess the anti-carcinogenic activity of *P. Amarus*, γ- glutamyl trans peptidase (Tate and Meister; 1974), cytosolic glutathione- s-transferase (Haibig; 1974), tissue glutathione (Moron et al; 1979) and cytosolic glutathione reductase activity (Racker; 1955).

Tumor and normal appearing stomach sections were stained with haematoxylin and eosin and were examined under microscope (10x). Nucleolar organizer region associated proteins (AgNORs) were studied in paraffin sections of stomach mucosa.

STATISTICAL ANALYSIS

The values are expressed as mean ± standard deviations. The results were analysed statistically by Student's unpaired t-test.

RESULT

Effect of *P. Amarus* extract on papilloma formation

Topical application of *P. Amarus* prior to croton oil administration in DMBA initiated mice resulted in a significant protection against skin tumor promotion in a dose dependent manner. *P. Amarus* administration substantially lowered the percent of mice with tumors and decreased the total number of tumors per mice (Table.1)

P. Amarus administration prior to DMBA application (Group IV & V) showed an inhibition in papilloma development in mice, indicating that *P. Amarus* had an effect on the tumor initiation process. These inhibitory effect were also depend on the dose of *P. Amarus*. In the control animals the first tumor appeared at 4 weeks after DMBA application where as in *P. Amarus* treated groups the first tumour appeared at 6 weeks (Fig.1).

Topical application of *P. Amarus* was found to be effective in delaying tumor appearance. *P. Amarus* also lowered the percentage of tumor bearing mice (Fig. .2). All the control animals developed tumors by 20 weeks while only 56% of the 5mg *P. Amarus* treated group developed tumors at week 20. The average number of tumors per mouse at week 20 were 6.2± 2.6 in the control group, and in 1 and 5 mg *P. Amarus* treated groups were 3.4± 1.5 and 2.6±1.8 respectively.

Application of DMBA (470nmol/mouse) alone (group II) did not produce any tumors, suggesting that this dose level was ineffective to elicit carcinogenic potential without further promotion. Moreover animals topically treated with DMBA, croton oil and methanol (Group III) were also found to produce tumors like control group suggesting that methanol itself could not inhibit papilloma formation.

Effect of *P. Amarus* extract on MNNG induced carcinogenesis

P. Amarus extract administration inhibited the stomach cancer induced by MNNG in a dose dependent way. All the untreated rats in MNNG group developed tumors. Administration of *P. Amarus* inhibited stomach tumor development in the rats on the 44th week of the experiment. Only 66% animals developed tumors in the 150mg/ kg P. amarus treated group and only 44% in the animals treated with 750mg/ kg body weight of P. amarus extract.

A typical morphological picture of stomach of rats treated with MNNG as well as MNNG and P. amarus is given in Fig 3. All the untreated animals had significant number of tumors in the non- glandular region of stomach. Number of tumors developed in P. amarus treated group was found to be significantly less.

In MNNG treated group, the stomach weight was increased to 1.06±0.22g/100g b.wt as compared to normal rat stomach weight 0.52± 0.14g/100g b.wt. The rats which were treated with 150 and 750mg/ kg of P. amarus extract showed 0.89± 0.17 and 0.75± 0.26g/ 100g b.wt of stomach weight (Table. 2).

The treatment with P. amarus extract effectively lowered the elevated γ-GT, a marker of neoplasm (Hanigan and Pitot., 1985) from 20.3± 6.7 to 2.8± 0.9μmol/min/mg protein (Table.3). This result indicate that P. amarus could reduce the proliferation of tumor cells. MNNG administration increased mucosal GST to 1317±211 n mol/min/ mg protein as compared to normal value, which was 344.9± 22 n mol/min/mg protein. Administration of P. amarus 150 and 750mg/ kg b.wt significantly reduced these elevated levels to 779.8±144 and 494.8±76 n mol/min/mg protein respectively. Similarly stomach mucosal GR was increased to 36.8±66 n mol/min/mg protein as compared to normal value of 129±124 n mol/min/ mg protein, which was significantly reduced by 150 and 750mg of P. amarus administration to 286± 41 and 192± 145 n mol/min/mg protein respectively. MNNG administration decreased GSH from the normal value of 9.8±22 n mol/min/ mg protein to 4.6± 0.9 n mol/min/mg protein. 150 and 750 mg/ kg b. wt of P. amarus increased these levels to 5.5± 1.3 and 8.5± 1.4 n mol/min/mg protein respectively.

During the experiment, the rats in the control, MNNG treated group and P. amarus treated group did not show any significant difference in the body weight.

Effect of P. amarus on AgNOR counts

The normal rats showed very low AgNOR values. This is to be expected since in the normal

cells, hypertrascriptional activity of rDNA genes is not necessary. AgNOR dots and clusters of MNNG administered arts were increased (Table 4) as compared to normal rats. AgNOR dots and clusters of normal rats were found to be 1.5±0.29 and 0.4±0.11 which were raised to 3.9± 0.98 and 1.5± 0.45 respectively by MNNG administration. AgNOR dots and clusters were reduced by P. amarus treatment. P. amarus treatment 150 and 750mg/ kg reduced dot value to 2.9±0.30 and 1.8±0.51 and cluster value to 1.8± o.51 and 0.7±0.37 respectively.

Histopathology of rat stomach treated with MNNG with or without P. amarus indicated that there was marked necrosis of the stomach tumor in animals treated with P. amarus and tumor burden was much less compared to untreated animals.

DISCUSSION

In this chapter we have shown the anti-carcinogenic activity of P. amarus extract. P. amarus administration was found to inhibit papilloma formation by the two stage carcinogenesis induced by DMBA as initiator and croton oil as promoter in mice and MNNG induced stomach cancer in rats. Administration of P. amarus was found effective indicating a direct role of P. amarus in modulating carcinogenesis. P. amarus was found to have anti- oxidant activity and was shown to scavenge the oxygen free radicals generated invitro. Cancer promotion involves increased oxygen radical production that produces DNA strand breaks. This may be the reason for increased effectiveness when the drug administration was continued during promotion period.

The involments of oxidative stress and lipid peroxide formation has found in the process of DMBA carcinogenesis (Baoxu et al., 1998). Sasaki et al., 1995 reported that transversion of adenine to thiamin occurs in H- ras oncogene in mouse treated with DMBA/ TPA. The free radical hypothesis also supported by the fact that the anti-oxidants can inhibit the DMBA/ TPA carcinogensis (Slaga et al., 1995).

Several anti-oxidant and anti- inflammatory compounds isolated from plants such as resveratol, quercetin, kaemphenol, rutin, curcumin etc effectively inhibit the DMBA/ croton oil induced carcinogenesis in experimental animals (Meishiang et al., 1997, Kato et al., 1993, Soudamini and Kuttan., 1989).

P. amarus extract was also found to have significant activity against MNNG induced stomach cancer. γ-GT, GST and GR which were elevated very high in MNNG treated animals were almost normalized by co- treatment with P. amarus extract. MNNG treatment was found to suppress the level of GSH and this was found to be significantly increased by P. amarus treatment.

Nucleolar organizer regions (NORs) are loops of DNA located on acrocentric chromosomes in the nuclei of normal and abnormal cells (Sirsat and Khanolkar., 1962, Massimo et al., 1990). They encode for ribosomal RNA (rRNA) and the associated proteins are argyrophilic (hence the term AgNOR). Since RNA are the sites of protein synthesis, the number of AgNORs per nucleus has a direct relationship to the cellular activity (Newbold., 1990). It has been shown recently that the increase in the number of AgNORS in a proliferating cell, is due to a wider dispersal of otherwise compact clusters of NOR associated proteins (Crocker et al., 1988).

AgNOR staining has now been recommended as a prognostic (Simha et al., 1996) and diagnostic tool in human (Anon., 1987) and

canine (Bostock et al., 1989) histopathology. Statistical significance of AgNORs, showed that tumors having less than 4 AgNOR counts and less proliferative index will be of a benign lesion, while more counts than that will indicate tumors of a malignant nature (Mehrotra and Chandra., 1998). P. amarus extract also produced significant decrease in AgNOR counts indicating that the extract has anti- carcinogenic activity.

Histological studies of MNNG induced gastric tumorigenesis indicated that P. amarus prevents gastric mucosal development or stops its progression.

The novel hydrolysable tannin, named amariin and geraniin, corilagin, 1, 6-digalloylucopyranoside, rutin and quercetin -3-0-glucopyranoside, were isolated from the polar fraction of the methanolic extract of aerial parts of P. amarus (Foo., 1993). Some of the hydrolysable tannins isolated from P. amarus were found to be potent inhibitors of wheat embryo Ca2+ dependent protein kinase (CDPK), rat brain Ca2+ protein kinase and phospholipid dependent protein kinase (PKC) and Ca2+- calmodulin dependent myosin light chain kinase (Polya et al., 1995). How this activity is related with the anti-carcinogenic activity of the extract is not known at present. The extract was also found to inhibit P450 enzymes, which are needed in the activation of carcinogen (Rajesh Kumar and Kuttan., 2002). Inhibition of cell cycle regulation, topoisomerase II (Rajesh Kumar and Kuttan., 2002), P450 enzymes as well as anti-oxidant activity may contribute to the overall activity of the extract against carcinogenesis induced in animals.

Table 1
Effect of P. amarus extract on papilloma induction initiated by DMBA and promoted by Croton oil

Group	Animal status	Number of mice developed papillomas by 20 weeks	Number of papillomas per tumour bearing mice	% reduction in papillomas per tumour bearing mice
I	DMBA+ Croton oil	9/9	6.2±2.6	-
II	DMBA alone	0/7	-	-
III	DMBA+ Croton oil+ Methanol	8/8	6.1±2.5	-
IV	DMBA+ Croton oil+ *P. amarus* (1mg/mouse, Prior treatment, Topical)	6/9	3.4±1.5*	45.2
V	DMBA+ Croton oil+ *P. amarus* (5mg/mouse, Prior Topical)	5/9	2.6±1.8**	58.1
VI	DMBA+ Croton oil+ *P. amarus* (1mg/mouse, Topical)	7/9	3.8±1.9*	38.7
VII	DMBA+ Croton oil+ *P. amarus* (5mg/mouse, Topical)	5/9	3.2±2.2**	48.4

*P< 0.05; **P< 0.01as compared with group I

Table 2
Effect of P. amarus in incidence of tumours and stomach weight in MNNG induced rat gastric carcinogenesis

Group	Treatment(Dose/kg b. wt)	Number of tumor bearing rats	Relative stomach weight g/ 100gb. wt
I	Normal	Nil	0.53±0.05
II	Control(MNNG)	9/9	1.06±0.10
III	P.amarus150mg	6/9	0.89±0.08***
IV	P.amarus750mg	4/9	0.71±0.06**

***P< 0.001as compared with group II

Table 3
Effect of P.amarus on γ-GT, GST, GSH and glutathione reductase levels in the stomach mucosa of rats treated with MNNG

Group	Treatment (Dose/kg b. wt)	γ-GT (umol/min/ mg protein)	GST (nmol/min/ mg protein)	GSH (nmol/min/ mg protein)	GR (nmol/min/ mg protein)
I	Normal	1.6±4.6	344.9±22	9.8±1.2	129±24
II	Control(MNNG)	20.3±6.7	1317.6±211	4.6±0.9	368±66
III	P.amarus150mg	10.5±1.4	779.8±144**	5.5±1.3	286±41*
IV	P.amarus750mg	2.8±0.9***	494.8±76***	8.5±1.4**	192±45***

* P< 0.05; **P< 0.01; ***P< 0.001as compared with group II

Table 4
Mean frequency of AgNOR dots and clusters with different doses of P. amarus

Group	Treatment (Dose/kg b.wt)	AgNOR dots	AgNOR clusters
I	Normal	1.5±0.29	0.4±0.11
II	Control(MNNG)	3.9±0.98	1.5±0.45
II	P.amarus150mg	2.9±0.30*	1.0±0.47
IIV	P.amarus750mg	1.8±0.51**	0.7±0.37*

*P< 0.05; **P< 0.01as compared with group II

Fig 1 Effect of P. amarus extract on DMBA/Croton oil induced papilloma

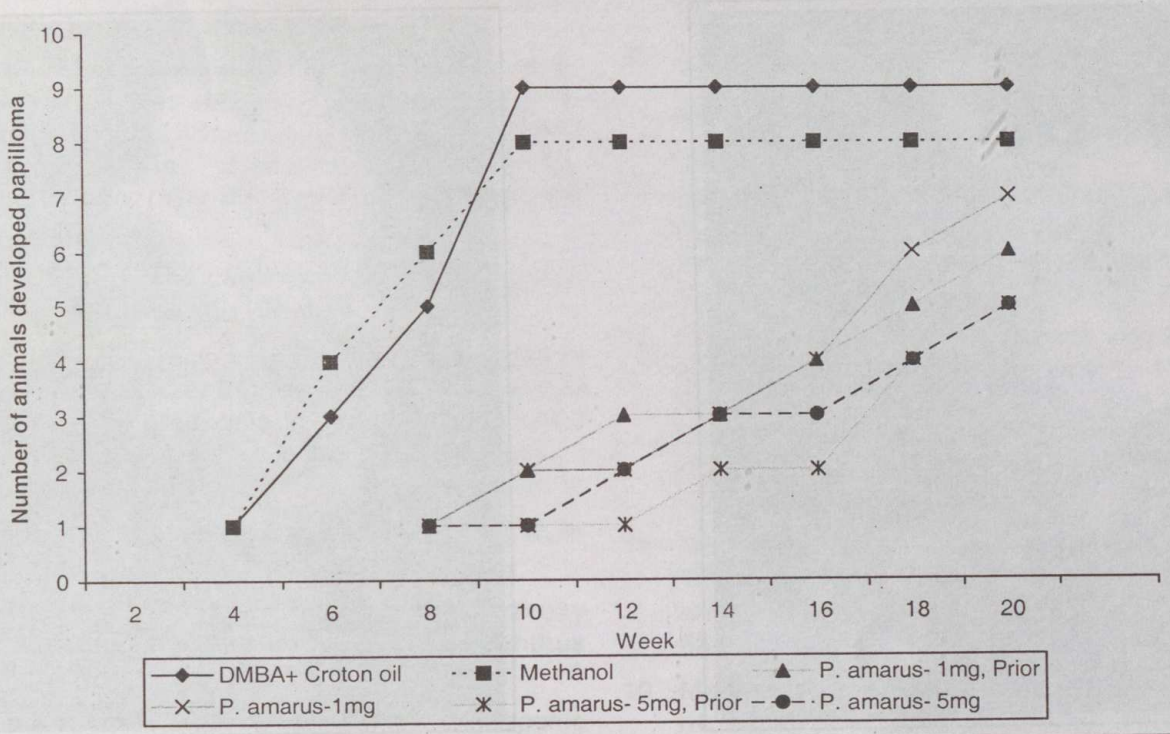


Fig 2 Inhibitory effect of P. amarus extract on the number of papilloma/ mice developed by DMBA/ Croton

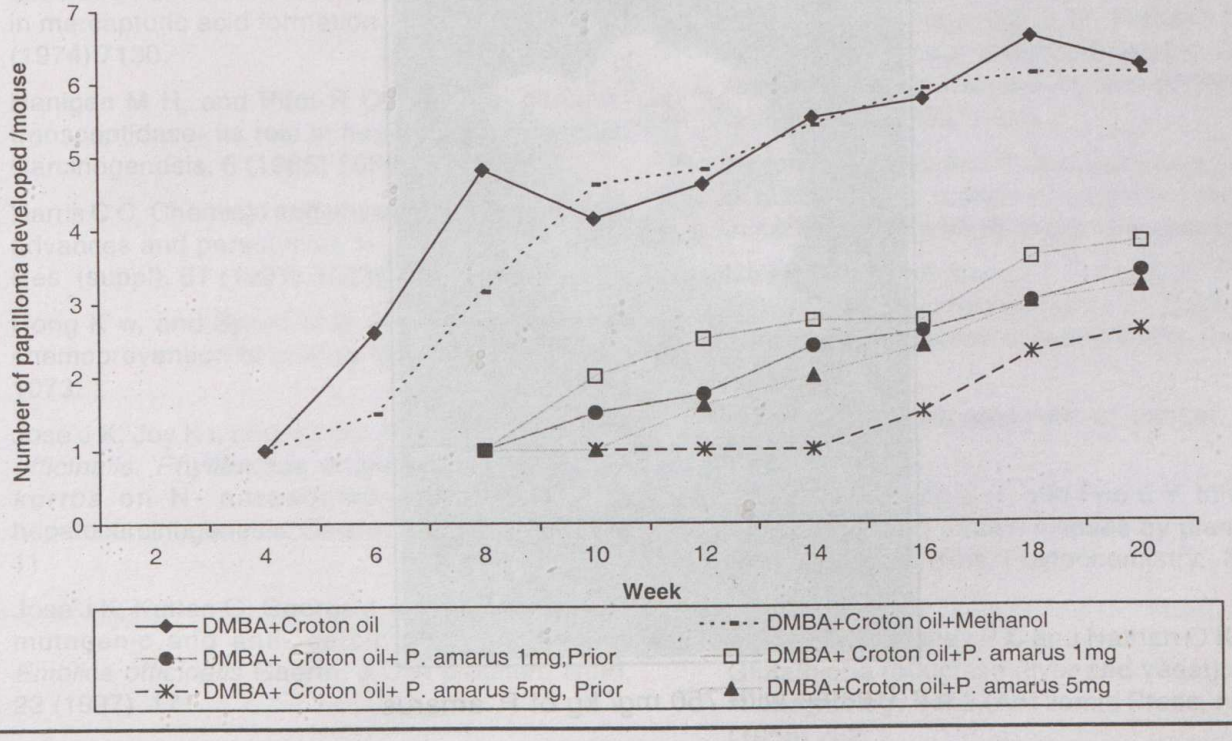
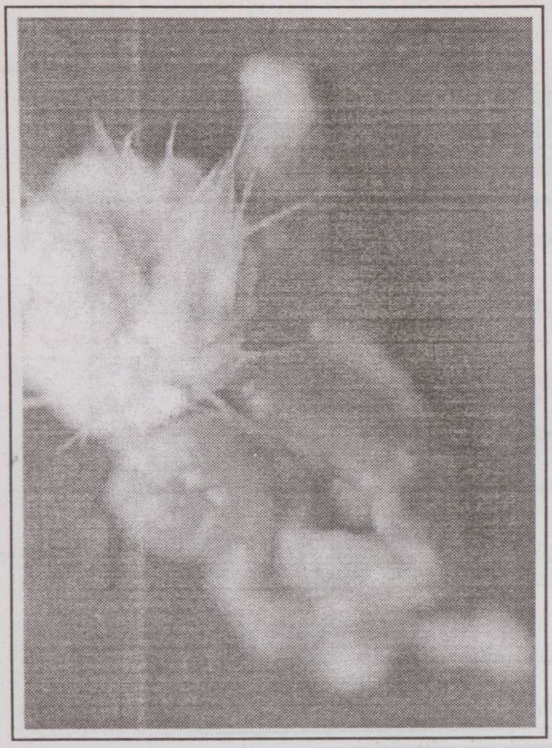
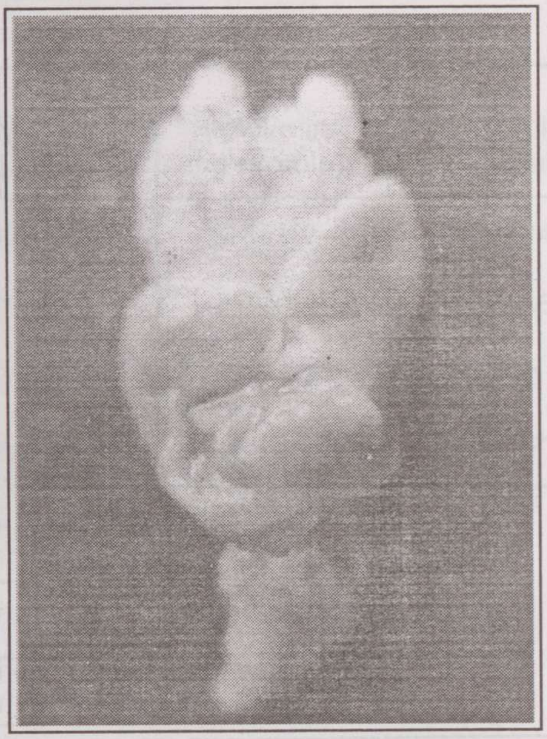


Figure 3

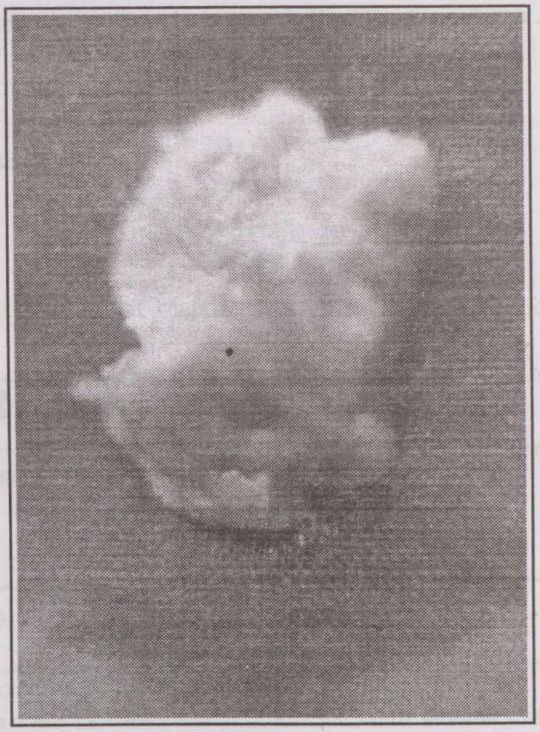
Morphology of stomach of MNNG administered rats



3.a MNNG (positive control)



3.b Treated with 15o mg/ kg of P. amarus



3.c Treated with 750 mg/ kg of P. amarus

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ROLE OF BRAHMA RASAYANA ON THE IMMUNE STATUS IN IRRADIATED MICE

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ABSTRACT

Oral administration of Brahma Rasayana (BR-50mg/dose/animal for 10 days) insignificantly increased the [^{3}H]-thymidine uptake of PHA stimulated and non-stimulated cells of thymus, spleen and bonemarrow cells of irradiated mice. Whole body radiation was found to suppress the antibody titre and antibody forming cells in normal animals. In BR treated irradiated mice, maximum antibody titre was obtained on 18th and 21st day (512) and maximum number of plaque forming cells were found to be 293±20.7 on 4th day.

INTRODUCTION

Cancer treatment with chemotherapeutic agents and ionizing radiation has considerable effect on haemopoietic system. In fact, profound myelosuppression and the resulting leukocytopenia is a major side effect of ionizing radiations. A variety of immunopotentiating agents administered to mice before irradiation increased the survival of animals after lethal irradiation (Thatte and Dhanukar, 1989; Sakagami *et al*, 1991). The radioprotective effect conferred by immunomodulators could reduce these side effects (Mihich, 1982). Immunotherapy is costly and may not be immediately useful to the patients in several developing countries.

Traditional use of herbal medicine is usually an integral part of culture, which was developed within an ethnic group before the development and spread of modern science. There are several herbal preparations used in indigenous medical therapy, which can enhance the body's immune status. Rasayanas are a group of drug preparations made of several plant products used in Ayurvedic system of medicine to improve body's immune system (Singh, 1990) .

Brahma Rasayana (BR) is a non-toxic polyherbal preparation. According to Ayurvedic texts Rasayana therapy arrests aging, increases intelligence, vigour and resistance to diseases. However no concrete studies have done to substantiate these claims. Mahsarishi Amruth Kalash (MAK), a modified indigenous herbal preparation, possesses many of activities produced by BR (Sharma *et al*,1992).The biological products obtained from plant sources such as polysaccharides, lectins, peptides etc. have been shown to stimulate the immune system (Hajto *et al*, 1989, Kuttan and Kuttan, 1992). Immunomodulatory and immunorestorative properties of Rasayanas find their use in diseases like cancer, AIDS, tuberculosis etc. Chemoprotective effects of Rasayanas have been shown to stimulate stem cell proliferation and possibly its differentiation (Praveenkumar *et al*, 1994) Present study has been designed to establish the effect of Brahma Rasayana on immune response in normal and irradiated mice.

MATERIALS AND METHODS

Brahma Rasayana (BR) was purchased from

Vaidyaratnam Oushadhasala, Ollur, Kerala, India. Water suspension of BR was used for all *in vivo* studies.Tissue culture medium- RPM1 1640 (Rose Well Park Memorial Institute), Hank's balanced salt solution (HBSS), Bovine serum albumin (BSA) and Minimum essential medium (MEM) were obtained from Hi-media Laboratories, Mumbai. Foetal calf serum (FCS) was obtained from Biological Industries, Kibbutz Beit Haemek, Israel. Phytohaemagglutinin (PHA-M) was obtained from Difio, USA. [^{3}H] -thymidine was obtained from BRIT, Babha Atomic Research Centre (BARC), Mumbai. Dimethyl sulfoxide (DMSO) and perchloric acid were obtained from Sisco Research Laboratories, Mumbai. Trypan blue was obtained from E-Merck (India). All other reagents were of analytical grade. Sheep red blood cells (SRBC) were collected in Alsever's solution from local slaughterhouse.

Animals

Inbred strains of Balb/c mice (4-5 weeks old, 20-25g) were reared from our animal house and were housed in ventilated cages in air-controlled rooms and fed with normal mouse chow and water *ad libitum*. All experiments were carried out only after ethical clearance from Institutional Animal Ethics Committee.

Radiation treatment

Whole body radiation (400 rads) was given using Cobalt -60 teletherapy unit (Theratron 780, Canada). Animals were kept in capacity of holding ten mice and irradiated using gamma rays at a dose level of 100 rads per minute.

Effect of BR on [^{3}H] thymidine uptake of thymus, spleen and bonemarrow cells of normal and irradiated mice (*in vivo*)

Inbred strains of Balb/c mice (4-5 weeks old) were used for this study. All mice were divided into four groups. Group I kept as normal control, group II was treated with BR (50mg/dose/mouse,po) for five days, group III was kept as irradiated control and group IV was treated with radiation + BR (50mg/dose/mouse,po) for 5 days. In the case of group III and IV, animals were treated with single exposure of whole body radiation (400 rads/mouse) on 4th day.

All mice were sacrificed on 5th day. Thymus, spleen and bone marrow were collected from each animal. Single cell suspension of thymus or spleen or bonemarrow cells was prepared under sterile, cold conditions (0^{0}C). Mice were killed by cervical dislocation, bone marrow from both femurs were collected into a sterile tube containing PBS (pH7.2) and 2% heat inactivated goat serum, centrifuged washed thrice, made up to single cell suspension. After checking viability using typan blue solution and the cells number was adjusted to 1x10^{6}cells/0.1mL.Spleen and thymus were removed from mice, thoroughly cleaned and cut into small pieces for smashing the tissue. The smashed cells (single cells) were collected into sterile tubes containing PBS and 2% goat serum. The tubes were kept at 4^{0}C for 3 minutes. After removing the debris, the tubes were centrifuged, washed thrice, checked viability and adjusted the cells number to 1x10^6 cells/0.1mL. Processed cells (1x10^{6}cells) were cultured in 1ml RPMI -1640 medium containing 10% FCS in presence and absence of mitogen (PHA-M, 6μg/mL) different concentrations of extract of BR (0, 0.5, 1,2,5,10 and 50μg/mL) and antibiotics. Culture vials were incubated in a humidified atmosphere of 5% CO_2 at 37^{0}C. After 48 hour incubation, [^{3}H]-thymidine (1μCi/vial) was added and was continued for incubation (16-18 hour) at 37^{0}C.

After incubation, vials were kept in ice buckets and centrifuged to remove the supernatant. To each vial, ice cold 2mL Perchloric acid (0.8M PCA) and 100μL of 1% BSA were added and kept at -20^{0}C for 20 minute. After incubation, each vial was vortex mixed, centrifuged and supernatant was removed. The above process was again repeated for once. Each vial containing the cell pellet was dissolved by adding 0.5mL of 0.5N sodium hydroxide. The dissolved solution (0.5ml) from each vial was taken into a 5mL scintillation fluid and kept overnight in dark and the radioactivity was measured in β-counter (Mustafa, 1992) Cell proliferation was measured by stimulation index (SI= cpm test/cpm control).

Effect of BR on circulating antibody titre in irradiated mice.

Inbred strains of Balb/c mice (4-5weeks old) were used for this experiment. All mice were immunized with 0.1ml of 20% SRBC intraperitoneally (ip) on 5th day. Group I (6 mice/ group) was kept as normal. Group II and III received single exposure of whole body radiation (400 rads/mouse) on 4th day. Group II was kept as irradiated control. Group III was treated with BR (50mg/dose/mouse, po) for five days. Blood was collected from caudal vein every 3rd day after 5th dose of drug administration and continued for a period of thirty days. Antibody titre was estimated by the method, using SRBC as antigen. Sera samples of each group were pooled and heat inactivated at 56^{0}C for 30 minutes. Two fold dilutions of sera samples were made in PBS (p^H 7.2) in microtitre plates and mixed (1:1) with 1% trypsinized suspension of SRBC in PBS. Agglutination was noted after incubation for 3 hour at room temperature (Nelson and Davey, 1992).

Effect of BR on antibody forming cells in normal and irradiated mice.

Jerne's plaque assay (Jerne and Nordia, 1963) using a modified slide technique was employed (Mehrotra, 1992). Inbred strains of Balb/c mice (4-5 weeks old, 20-25g) were divided into 4 groups (8mice/group). Group I was kept as normal. Group II was treated with BR (50mg/ dose/ mouse, po) for five days. Group III and IV were treated with single exposure of whole body radiation (400 rads/mouse) on 4th day. Group III was kept as irradiated control. Group IV was treated with BR (50mg/dose/mouse, po) for five days. All mice were immunized with SRBC (2.5x10^{8}cells/mouse) intrapertioneally (ip) on 5th day. The animals were sacrificed on various days (3 to 9) and spleens were processed into single cell suspension (8x10^6 cells/mL) in Hank's balanced salt solution (HBSS). To 0.5mL of 5% agarose prepared in HBSS, 50μL of 7% SRBC and 50 mL of spleen cell suspension were added, mixed well and poured over a glass slide. The slides were allowed to solidify and incubated with fresh rabbit serum as a source of complement for one hour at 37^{0}C. The plaques formed were counted using a colony counter and represented as plaque forming cells (PFCs) per million spleen cells.

STATISTICAL ANALYSIS

Transformation of the variances was done and homogeneity of variances was observed. Two-way classifications with multiple (unequal) observations per cell were done. Data was expressed as mean ± standard deviation (SD).

RESULTS

Effect of BR on [^{3}H] thymidine uptake of thymus, spleen and bonemarrow cells of

normal and irradiated mice, *in vivo*

Figure 1 represents the effect of oral administration of BR on [^{3}H]-thymidine uptake of cells of normal mice (*in vivo*). In non-stimulated condition, tritiated thymidine uptake in spleen and thymus cells were found to be increased two times in BR treated mice (717±155cpm, 814±371cpm respectively) when compared to normal mice (352±55.6cpm, 432±36cpm respectively). When the cells were stimulated with PHA, [^{3}H]-thymidine uptake was found to be increased in treated and untreated cells. [^{3}H]-thymidine uptake in PHA stimulated thymus, spleen and bonemarrow cells of BR treated animals were 1617±91, 1627±197 and 1600±92 respectively. [^{3}H]-thymidine uptake in PHA stimulated thymus, spleen and bonemarrow cells of normal animals were 1438±798, 965±249 and 1177±120 respectively. There was a decrease in [^{3}H]-thymidine uptake of PHA stimulated and non-stimulated cells of thymus, spleen and bonemarrow cells of irradiated mice when compared to normal mice. Irradiation suppressed the mitogenic activity of PHA. In PHA stimulated and non-stimulated conditions, BR treatment showed only insignificant increase in [^{3}H]-thymidine uptake when compared to irradiated control mice.

Variances were homogenous for [^{3}H]-thymidine uptake of thymus, spleen and bonemarrow cells of normal and irradiated mice. From the analysis, it was found that, there were significant variations ($p<0.05$) between the treatment groups(without and with PHA) and within the treatment groups (normal, BR treated normal, irradiated and BR treated irradiated animals). In the case of thymus, each treatment was significantly different from each other. In the case of spleen and bonemarrow cells, normal and BR treated normal animals were significantly different from irradiated control and BR treated irradiated animals .

Effect of BR on circulating antibody titre in irradiated mice.

The effect of BR on circulating antibody titre is shown in figure 2. Normal mice were showed a maximum titre value 128 on 15th to 24th day. Radiation (400 rads/mouse, single dose) treated normal mice were showed no titre value on 3rd day titre value increased gradually on 12th to 21st day and on 24th to 30th day, the value reached to 256. In BR treated irradiated mice, on 3rd day titre value was 32 and increased on following days, the maximum titre was obtained on 18th to 21st day (512) and on 24th to 30th day, value reached to 256. From this experiment, it was concluded that BR treatment is effective to the enhance of antibody production in irradiated animals.

Effect of BR on antibody forming cells in normal and irradiated mice.

Figure 3 represents the effect of BR on antibody forming cells. Administration of BR was found to increase antibody-producing cells in both normal and irradiated mice. Irradiated control mice had decreased the number of antibody producing cells in spleen. Maximum number of plaques per 10^6 spleen cells was 80±10.6 on 4th day and reached to 68 ± 11.8 on 9th day. BR treated normal mice were showed an increase in number of plaque forming cells on 5th day and the value was 442±20.9. and on 9th day the number of plaques were reached to 108 ± 3.8. In BR treated irradiated mice, the maximum number of plaque forming cells per 10^6 spleen cells were found to be 293±20.7 on 4th day and on day 9, the maximum number of plaque forming cells per 10^6 spleen

cells were 122 ±15.6 and on day 9, the maximum number of plaque forming cells per 10^6 spleen cells were 122 ±15.6.

DISCUSSION

Present study highlights the effect of BR on cell proliferation in normal cells and cells treated with radiation as well as on antibody forming cells and antibody titre. When accidential exposure to radiation occurs in the normal body, one of the first observable effects of radiation on cells both *in vitro* and *in vivo* is the prevention of cells from entering mitosis, often referred to as G_2 block or mitotic delay (Whitmore et al, 1967). Bonemarrow and thymus are the primary lymphoid organs. Bonemarrow is the major site of haemopoiesis, provides antigen independent differentiation of B-cells and antigen processing environment.Thymus is responsible for antigen-independent maturation and development of T-lymphocytes that effect cell-mediated immunity and regulate most humoral and cell-mediated immune responses. Spleen is the secondary lymphoid organs. It acts as temporary reserve site for lymphocytes, provides antigen-processing environment and is auxiliary site of hemopoiesis in extraordinary circumstances (Robert *et al*, 1989). Earlier studies reveals that BR enhanced both humoral and cell-mediated immunity (Praveen kumar *et al*, 1999).

BR treated normal mice (in vivo) showed an increase in the proliferation of thymus, spleen and bonemarrow cells in stimulated and non-stimulated conditions. Irradiation was found to suppress the proliferation level in PHA treated irradiated cells of normal mice. There was no change in the PHA non-stimulated irradiated cells when compared to PHA non-stimulated cells of normal mice. This effect may be due to cells which are in mitosis are not prevented from completing the division, but no new prophases are formed for a time that seems to be dependent upon the dose of radiation administered and the cell cycle time of un-irradiated cells (Doida and Okada, 1969). It was observed that, in vitro BR treatment enhanced proliferation of thymus, spleen and bonemarrow cells in mitogen stimulated cells and non-stimulated conditions and it may be due to the ability of BR to enhance the stimulation of mitosis. Administration of BR showed an increase in antibody titre on 18^{th} and 21^{st} day and plaque forming cells on 4^{th} day in mice treated with radiation. Antibody titre is based on soluble antigen (Ag) is passively absorbed to red blood cells. Plaque forming cells is based on the ability of antibodies (Abs), secreted by a single plasma cell (mature B-lymphocyte), to bind to an antigenic determinant on an erythrocyte. These assays showing the stimulation of humoral immune responses during BR treated irradiated mice when compared to irradiated control mice.

Figure1

Effect of Brahma Rasayana (BR) on [^{3}H] - Thymidine Uptake of Thymus, Spleen, and Bonemarrow Cells of Normal and Irradiated Mice - *in vivo*

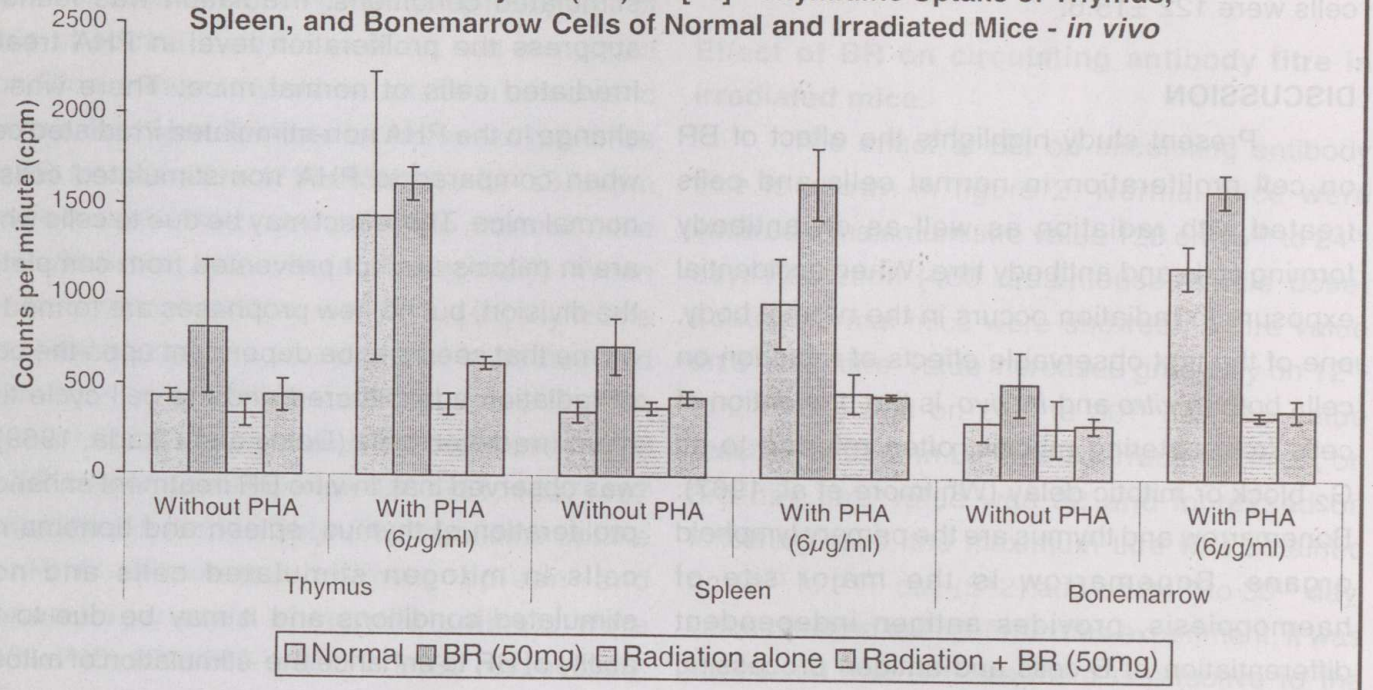


Figure 2

Effect of Brahma Rasayana (BR) on Circulating Antibody Titre in Irradiated Mice

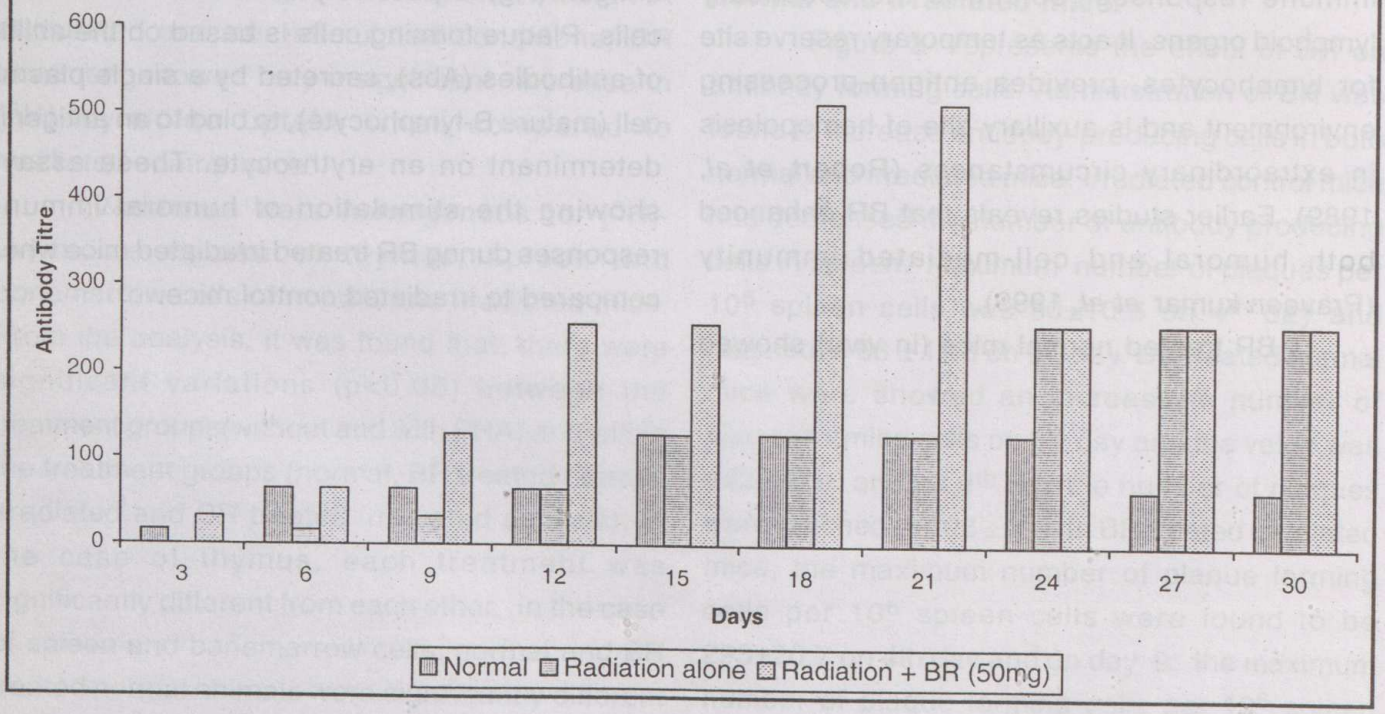
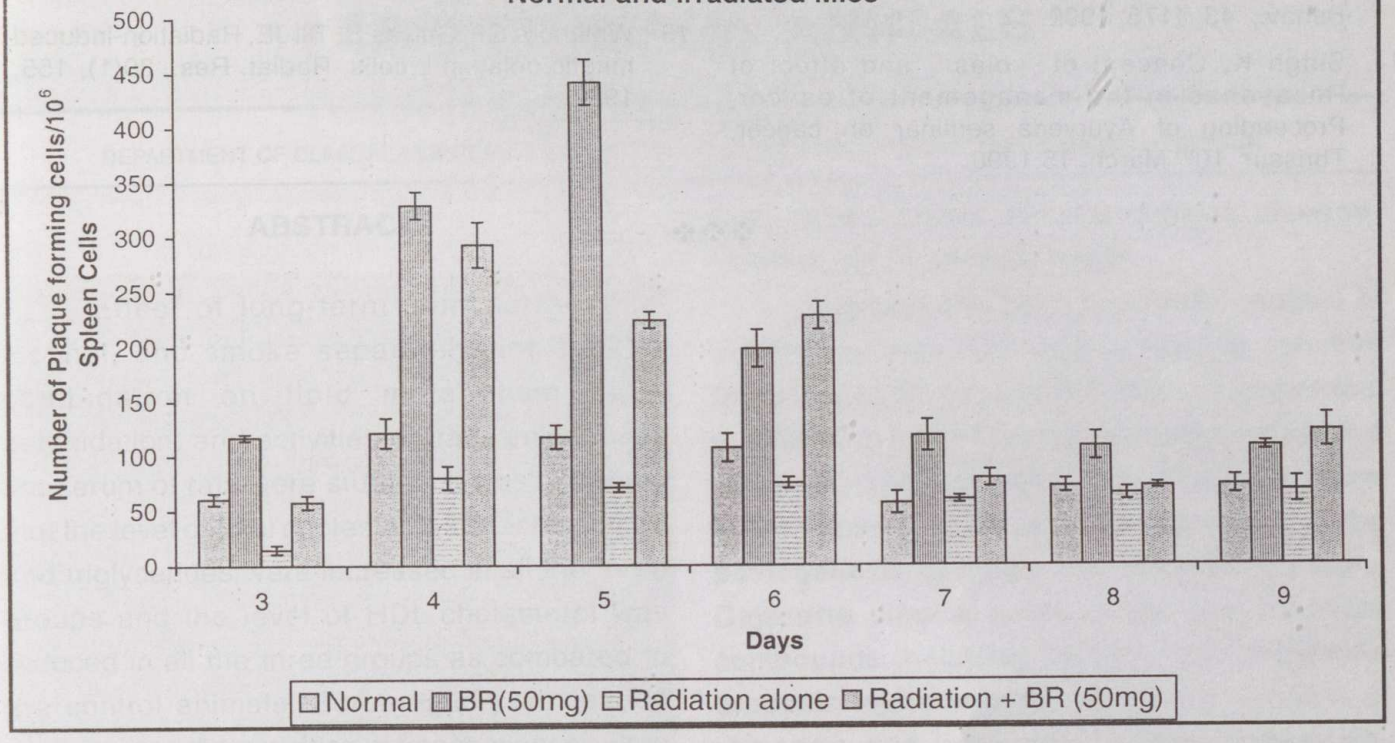


Figure 3
Effect of Brahma Rasayana (BR) on Jernes Plaque Forming Cells Assay in Normal and Irradiated Mice



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COMBINED EFFECT OF ALCOHOL AND SMOKE IN EXPERIMENTAL ANIMALS

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ABSTRACT

Effect of long-term administration of alcohol, and smoke separately and both in combination on lipid metabolism, lipid peroxidation, and activities of transaminases in the serum of rats were studied. It was observed that the level of total cholesterol, LDL-cholesterol and triglycerides were increased in all the three groups and the level of HDL-cholesterol was reduced in all the three groups as compared to the control animals. Serum lipid peroxidation products and the activities of transaminases were also found to be increased in all the three groups.
Key words : Alcohol, smoke, lipid profiles, Lipid peroxidation, transaminases.

INTRODUCTION

Ethanol is currently recognized as the most prevalent known cause of abnormal human development. Alcohol abuse and alcoholism represents one of the major health, social and economic issues facing the world. Liver is the most susceptible organ to the toxic effects of alcohol (1). Ethanol can also alter the normal function and composition of membranes, which may ultimately lead to serious cellular impairment. In man excessive alcohol consumption is associated with the appearance of excess fat in the parenchymal cells with a more complex acute change in which there is liver cell death, the appearance of intracellular hyaline and an acute inflammation with degenerative, fibrotic hepatic disease, cirrhosis, which leads to death.

Cigarette smoke is potentially capable of generating high free radical load or reactive oxygen species, in-vivo from various biochemical reactions and also from the respiratory chain as a result of occasional leakage (2). Cigarette smoke is the known source of oxidants involved in the pathogenesis of lungs and vascular diseases. Cigarette smoke contains various chemical compounds including biphenyl and polycyclic aromatic hydrocarbons, which are capable of initiating and promoting oxidative damage. Cigarette smoking is the one of the most important exogenous factor which causes higher incidence of myocardial infarction(3). Death from coronary heart disease (CHD) is five times greater in smokers than in nonsmokers. Hypertension, hyperlipidemia and cigarette smoking are the important co-risk factors for coronary heart disease. ROS formed in the body as result of normal metabolic reactions, exposure to ionizing radiation cigarette smoke, and environmental pollution and by the influence of several xenobiotics are implicated in the causation of several diseases including cancer (4).

In the present study an attempt has been made to examine the combined effect of alcohol and smoking on lipid metabolism and lipid peroxidation in albino rats; as large number of people have the habit of both drinking and smoking.

MATERIALS AND METHODS

Male albino rats, Sprague Dawley strain weighing (150-200gm) were used for the study. They were maintained under environmentally-controlled condition with free access to standard food (Lipton, India) and water.

The animals were grouped as follows

Group I. Normal diet.

Group II. Normal diet + 18% alcohol (4gm alcohol / kg body weight/day)

Group III. Normal diet + smoke exposed

Group IV. Normal diet + 18% alcohol (4gm alcohol/kg body wt/day) + smoke exposed

The dose of 18% ethanol was administered orally for a period of 30 days. Animals were exposed to cigarette smoke by keeping a bottomless rectangular container on the top of the polypropylene cage containing rats. The rectangular container had 2 holes of about 3 and 1.5 cm diameter, one on the either side of the container. A burning cigarette was introduced through one hole. Animals were exposed to cigarette smoke for 30 minutes daily for 30 days. At the end of the experimental period all the animals were decapitated and the blood was collected for various biochemical analysis

Serum cholesterol was determined by the CHOD- PAP method (5). One ml of reaction solution which contains 10ml of serum and standard (200mg/dl). One ml reaction solution which contains pipes buffer (pH 7.5,99mmol/l), 4 amino antipyrine (0.5mmol/l, peroxidase (≥100/l) was mixed well and incubated for 5minutes at 37°c. Then the absorbance was measured at 546nm.

Serum HDL cholesterol was determined by CHOD- PAP method (6). Triglycerides in serum were determined by GPO-PAP method(7). 10 ml of serum was mixed with 1ml reaction solution which contains Good's buffer (pH 7.2, 50mmol/l) 4-chlorophenol (4mmol/l), Mg (15mmol/l), glycerokinase (≥0.4kul), peroxidase (≥2 KU/l), lipoprotein lipase (^{3}2KU/l) , 4amino antipyrine (0.5mmol/l), glycerol-3-phophate oxidase (≥1.5 KU/l). 10ml of triglycerides was used as the standard, mixed and incubated at 37°c for 10minutes. Absorbance was measured at 546 nm. Lipid peroxidation in serum was estimated by the thiobarbituric acid (TBA) method as modified by Yoshioka et al (8), using trichloro aceticacid and TBA. Serum glutamate oxaloacetate transaminases was estimated by the method of Reitman et al (9) and serum pyruvate transaminases by the method of Bergmeyer et al (10). Statistical analysis was carried out using the Student's t test. Values are expressed as mean ±SD. Values having $p<0.001$ on comparison were considered as significant

RESULT AND DISCUSSION

From the present study it was observed that ethanol and smoke separately and both in combination significantly ($P<0.001$) increases the lipid profiles such as total cholesterol, triglycerides, LDL-cholesterol and it significantly ($P<0.001$) reduces the HDL-cholesterol (Table I). It was also observed that the alcohol, smoke and both in combination increases significantly ($P<0.001$) the activities of serum glutamate oxaloacetate transaminase and serum aspartate amino-transaminase (Table III). It was also observed that the alcohol, smoke and both in combination significantly ($P<0.001$) increases the concentration of serum lipid peroxidation products (Table II)

Ethanol feeding has been found to

enhance the endogenous synthesis of triglycerides and reduces the utilization of dietary lipids resulting in their accumulation in the liver and plasma. Alcohol enhances blood level of the liver enzymes aspartate aminotransferase (AST: serum-glutamate- oxaloacetate transaminases SGOT and alanine amino transferase ALT: serum glutamate- pyruvate transaminases SGPT). These enzymes are released into the blood because of liver injury caused by alcohol. Ethanol has been found to enhance the generation of oxygen free radicals during its oxidation in the liver and these free radicals are responsible for causing lipid peroxidation in the serum of alcohol treated rats. The prevalence of hypercholesterolemia and hypertriglyceridemia in heavy smokers were reported earlier (11) The elevated levels of cholesterol and triglycerides by smoke exposure may be due to cadmium a metallic element present in the cigarette smoke effect the metabolism of lipids. The increased cholesterol synthesis is due to the increased activity of HMGCoA reductase, which is the key enzyme involved in cholesterol synthesis. Chromaffin cells of adrenal medulla synthesize catecholamines by the stimulation of nicotine. Adipose tissue lipolysis is carried out by catacholamines, which in turn elevate the level of cholesterol and triglycerides Nicotine exposure may predispose the formation of atherosclerotic lesion and also cause endothelial injury.. Cigarette smoke itself contains oxidants and free radicals. These free radicals and oxidants in smoke are responsible for enhancing lipid peroxidation. Carbon monoxide and nicotine content present in cigarette smoke, which decreases the activity of lipoprotein lipase resulted in the elevated level of triglycerides. It was also reported earlier that erythrocytes of smokers have an increased tendency to oxidize the lipids compared with nonsmokers (12).

The combined treatment of alcohol and smoke enhances the lipid profiles and lipid peroxidation in the body. It could be concluded that the combined effect of alcohol and smoke is likely to be more harmful than the smoke or alcohol alone. These findings have very high significance in public health management.

Table I

Concentration of total cholesterol, triglycerides and HDL- cholesterol and LDL-cholesterol in the serum of control, alcohol treated and smoke exposed rats (Values expressed are ±SD of 6 rats in each group. Concentration is expressed as mg/dl)

Group	Cholesterol	Triglycerides	HDL cholesterol	LDL cholesterol
Control	60.02±3.92	11.28±0.30	36.8±1.02	20.92±0.38
Alcohol	82.7±0.82*	28.28±0.32*	30.8±0.57*	46.20±0.35*
Smoke	98.2±2.10*	33.12±0.06*	25.8±0.78*	65.80±0.38*
Alcohol+smoke	100.8±1.08*	40.12±0.12*	12.1±0.17*	80.70±0.16*

*P<0.001

Table II
Concentration of malondialdehyde in the serum of control, alcohol treated and smoke exposed rats. (Values expressed are ±SD of 6 rats in each group. Concentration is expressed as n mol/ml serum).

Group	MDA n moles/ml serum
Control	3.77±0.03
Alcohol	8.77±0.08*
Smoke	9.88±0.91*
Smoke+ alcohol	11.38±0.10*

*P<0.001

Table III
Activities of serum transaminases of control, alcohol and smoke exposed rats. (Values expressed are ±SD of 6 rats in each group. Activities are expressed as U/L of serum)

Group	SGOT	SGPT
Control	20.09±0.98	14.23±0.49
Alcohol	50.01±1.05*	35.03±0.39*
Smoke	23.02±0.85**	18.08±0.28**
Alcohol+smoke	65.09±0.76*	58.01±0.39*

*P<0.001 **P<0.05

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COMPARISION OF HEPATOPROTECTIVE ACTIVITY OF VARIOUS EXTRACTS OF *PLUMBAGO ZEYLANICA* AND *PLUMBAGO ROSEA*. LINN.

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ABSTRACT

Plumbagin, a naphthaquinone isolated from *Plumbago rosea* is compared with different solvent extracts for its hepatoprotective activity in carbon tetrachloride induced hepatotoxicity(1). The effect of detoxified *Plumbago* extract is also studied. The analysis of various biochemical parameters and histopatholagical studies shows that plumbagin and petroleum ether extract have significant hepatoprotective activity. Other extracts of *P.rosea* and *P.zeylanica* shows less activity compared to petroleum ether extract. Detoxified petroleum ether extract of *P.rosea* shows less activity compared to pet. ether extracts of *P.rosea*.

Key words: *Plumbago zeylanica*, *Plumbago rosea*, hepato toxicity, hepatoprotection, detoxification, carbon tetra chloride.

INTRODUCTION

Plumbagin, a napthaquinone (5-hydroxy-2methyl 1-4naphthaquinone) is present in Plumbago sp. (Plumbaginaceae). *Plumbago zeylanica* and *P.rosea* are used in a number of ayurvedic and unani formulations like chitrakadi gutika, chitarak naritaki, chitraghrit, sudarshan churna, aquaruva- 1-kabir, majun blaladur, majun reigmahi etc. for their hepato tonic activity.3

In ayurvedic preparation *Plumbago* is used only after a detoxification process. In the present study the hepatoprotective activity of Plumbagin and different solvent extracts and the detoxified extract of *P.rosea* and *P.zeylanica* is compared.

MATERIALS AND METHODS

Detoxification of *Plumbago rosea*

About 30 g of *P.rosea* roots coarsely powdered and soaked in limewater. A red colored extract comes out which is decanted, washed repeatedly with lime water until red coloured principle is completely removed from the plant material the plant material is then dried in hot air oven at 50^{0}C and then extracted with petroleum ether (60-80^{0}C)

EXTRACTION PROCESS

Roots of *P.rosea* and *P. zeylanica* are collected separately, washed dried and cut into small pieces. Then coarsely powdered. Extraction is carried out in a soxhlet apparatus-using pet. ether (60-80) for 16 hours. The extract is collected, concentrated in a vacuum rotary drier and stored in desiccators. Marc is dried at 50°C in a hot air oven and then extracted with chloroform, acetone, ethanol, and water successively in the same manner.

Hepatoprotective activity 1,2

Adult Swiss albino rats weighing 150-180g body weights were randomly selected. They were divided into 14 groups, each groups consists of 6 rats. The animals were housed in ventilated cages and fed with pellet diet and water *ad libitum*.

Group A -served as normal control group received 1% tween80.

Group B- served as control group received a dose of 0.1ml of CCl_4.in groundnut Oil (1:1v/v) 100g-body weight per orally twice a week.

Group C -PLUMBAGIN (2mg/kg) 4 +0.1ml of CCl_4 /100g body weight

Group D-Received pet. ether ext o f *P.rosea* (5mg/kg) +0.1ml of CCl_4 /100g body wt.

Group E-received chloroform ext. of P.rosea. (200mg/kg) +0.1ml of CCl_4 /100g body wt.

Group F-received acetone ext. of *P. rosea* (200mg/kg) +0.1ml of CCl_4 /100g body wt.

Group G -received alcohol ext. of *P.rosea* (200mg/kg) +0.1ml of CCl_4 /100g body wt

Group H -received water ext.of *P.rosea* (200mg/kg) +0.1ml of CCl_4 /100g body wt.

Group I -received pet. ether ext. of *P. zeylanica* (5mg/kg) +0.1ml of CCl_4 /100g body wt.

Group J -received chloroform ext of *P.zeylanica* (200mg/kg) +0.1ml of CCl_4 /100g body wt

Group K -received acetone ext. of *P. zeylanica* (200mg/kg) +0.1ml of CCl_4 /100g body wt.

Group L -received alcohol ext of *P. zeylanica* (200mg/kg) +0.1ml of CCl_4 /100g body wt.

Group M -received water ext of *P. zeylanica* (200mg/kg) +0.1ml of CCl_4 /100g body wt.

Group N -received p. ether ext of *P.rosea* detoxified (5mg/kg) +0.1ml of CCl_4 /100g body weight

All extracts were given orally using 1%tween 80. Group A was maintained as normal control received 1% tween 80. All other groups received CCl4 (1ml/kg) i.p. with an equal volume of groundnut oil for 2 days group B animals were maintained as control receiving only CCl_4.

The vehicle or drug treatment was carried out orally from first day to ninth day with concurrent administration of CCl_4 on second and 8th day. During the period of drug treatment the rats were maintained under normal diet and water. On the 10th day blood was collected by cardiac puncture under mild ether anesthesia, serum was separated by centrifugation (3000 rpm for 15 min.), and biochemical parameters determined. Livers were removed and kept in 10% formalin solution for histopathological studies.

BIO-CHEMICAL PARAMETERS

Estimation of SGPT (IFCC) method 5,6

Principle: SGPT catalyses the transfer of amino group from L-alanine to 2-oxoglutarate with the formation of pyruate and L-glutamate. The pyruate so formed is allowed to react with NADH to produce L- lactate. The rate of this reaction is monitored by an indicator reaction coupled with LDH in the presence of NADH. The oxidation of NADH in this redaction is measured as the decrease in the absorbance of NADH at 340 nm, which is proportional to SGPT activity.

Procedure: Pipette out 100μl of the sample into 1000μl of working reagent, mixed well and aspirate - measured absorbance at 340 nm. The experiment is done using SGPT kit supplied by Trans Asia Bio-medicals ltd.

Estimation of SGOT (IFCCmethod) 7

Principle: SGOT catalyses the transfer of amino group from L aspartate to 2-oxoglutarate forming oxaloacetate and L glutamate. The rate of this reaction is monitored by an indicator reaction coupled with malate dehydrogenase in the presence of reduced nicotinamide adenine dinucleotide (NADH). The oxidation of NADH in this reaction is measured as decrease in absorbance of NADH at 340nm, which is proportional to SGOT activity

Procedure: Allow the working reagent supplied

by Trans Asia Biomedical Ltd. To attain 37^0c before performing the test. Mix 1000ml of working reagent and 100ml of sample, measured absorbance at 340nm.

Estimation of serum alkaline phosphatase (ALP)

Principle: Serum alkaline phosphatase hydrolyses P -nitro phenyl phosphate into P. nitro phenol and phosphate in the presence of oxidizing agent Mg 2+. This reaction is measured, as absorbance is proportional to the ALP activity.

Procedure: Pipette out 1000ml of working reagent and 20ml of sample and mixed well and measured absorbance a 405 nm in star-21 auto analyzer.

Estimation of serum bilirubin (Malloy and Evelyn method) 8

Principle: Bilirubin reacts with diazotised sulphanilic acid in acidic medium to form pink coloured complex azobilirubin with its absorbance is directly proportional to bilirubin concentration. Direct bilirubin being water-soluble is directly reacts in acidic medium. However, indirect or unconjugated bilirubin is solubilised using surfactant and it reacts similar to direct bilirubin.

Procedure: Take two test tubes and into each place 0.2ml of serum and 1.8ml of distilled water. To the unknown add 0.5ml diazoreagent and to the blank 0.5%Hydrochloric acid, finally to each add 2.5ml of methanol. Stand for thirty minutes and read in the colorimeter using a yellow green filter or set at 540nm.

Estimation of total serum proteins 9

Principle: The peptide bonds of protein react with Cu^{2+} ions in alkaline solution to form a blue-violet complex (so called biuret reaction). Each copper ion complexing with 5 or 6 peptide bonds. Tartarate is added as stabilizer. Iodide is added to prevent auto reaction of the alkaline copper complex. The formed is proportional to the protein concentration and is measured at 546nm.

Procedure: set up separate tubes as follows.

1. Test: added 0.1ml serum to 5ml working biuret solution.
2. Serum blank: added 0.1ml serum to 0.5ml tartarate-iodide solution.
3. Standard: added 0.1ml standard to 5ml working biuret solution.
4. Standard blank: added 0.1mlstandard to 5ml working tartarate-iodide solution.
5. Reagent blank: add 0.1ml water to 5ml working biuret solution.

Estimation of serum albumin 10

Principle: Albumin binds with bromocresol green (BCG) at pH 4.2 causing a shift in absorbance of the yellow BCG dye. The blue green color developed is proportional to the concentration of albumin present, when measured spectrophotometrically absorbance at 625nm.

Procedure: Added 20mml serum to 4ml working dye solution. Mixed, stand at 25° C for 10 minutes and read absorbance at 625nm against a blank 20ml.

RESULTS AND DISCUSSION

Plumbagin and pet. ether extract of roots of *P.rosea* and *P.zeylanica* shows marked hepatoprotective activity compared to the control. Chloroform extracts had nearly equal enzyme level to that of control. Acetone, alcohol and water extract of both roots shows some activity compared to the control. Pet. ether extract (detoxified) shows reduced activity than p. ether extract of *P. rosea.*

HISTO-PATHOLOGICAL STUDIES

NORMAL (fig.5)

Scanner view of normal rat liver shows normal architecture of liver cells. Two to portal veins are seen. Low power view shows we arch cords of hepatocytes, sinusoid and central vein. High power view of hepatocyte cells shows moderate to abundant eosinophilic cytoplasm, and uniform round nucleus, kupffer cells are also seen in the sinusoids.

CONTROL (fig.9)

Low power view shows central vein with mononuclear inflammatory infiltrate around the central vein and portal triad. High power view shows intracytoplasmic vacuolation and anisonucleosis.

PLUMBAGIN (fig.6)

Low power view showing preserved liver architecture, portal triad and cords of hepatocytes, central vein with few inflammatory cells in filtrate, hepatocyte shows cytoplasmic vacuolation

PET.ETHER EXTRACT (fig.8)

Low power view showing cords of hepatocytes. Central vein with few inflammatory cells infiltrate hepatocytes shows cytoplasmic vacuolation.

CHLOROFORM EXTRACT (fig.4)

Scanner view showing portal triad, cords of hepatocytes, low power view showing cytoplasmic vacuolation, high power view showing anisonucleosis, intracytoplasmic vacuolation and dilated sinus. Acetone, alcohol (fig.3), water (fig.7) and detoxified pet. ether extracts (fig.10) of *P.rosea* were showing similar histopathological changes like intracytoplasmic vacuoles, dilated sinusoids, and anisonucleosis.

The histopathological studies showed plumbagin treated rat had much improvement compared to control, there is minimal intracytoplasmic vacuolation. Kupffer cells can be seen and maintained the normal liver architecture. Pet ether extracts also shows some improvement. Rest of the extracts shows most of the pathological signs of the control.

RESULTS:

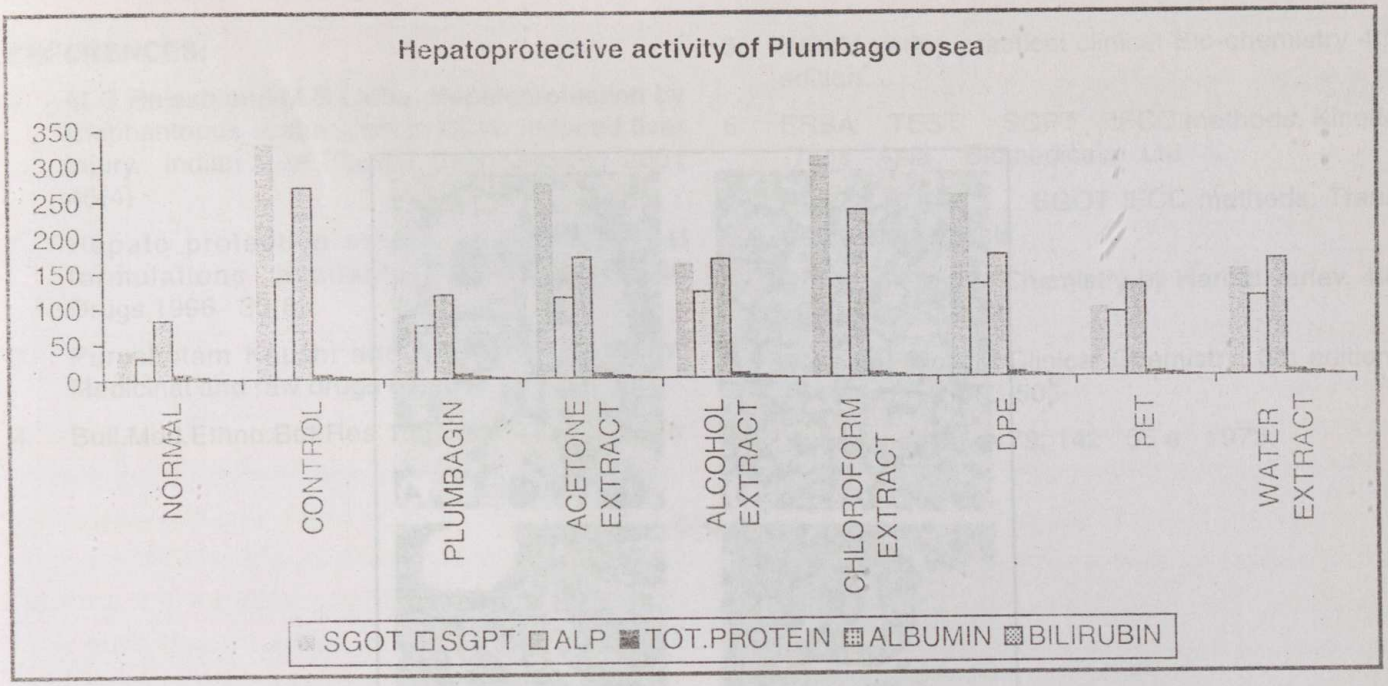
PLUMBAGO ROSEA

No.		SGOTIu/l	SGPTIu/l	ALPIu/l	Total Protein mg%	Albumin mg%	Bilirubin mg%
1	Normal	41.83±5.4	30.83±4.8	83.83±10.12	6.75±0.82	3.53±0.31	0.70±0.03
2	Control	330.5±24.5	142.2±13.2	269.1±27.8	6.30±0.71	3.03±0.28	0.78±0.05
3	Plumbagin	91.67±9.8	74.67±6.32	117.67±11.19	6.65±0.81	3.15±0.27	0.68±0.02
4	Acetone extract	274.50±28.12	113.75±9.67	170.08±21.84	6.22±0.70	2.93±0.09	0.68±0.03
5	Alcohol extract	162.00±22.10	120.83±1236	167.17±13.21	6.55±0.76	3.23±0.11	0.68±0.01
6	CHCl3 extract	312.12±37.56	138.3±11.46	235.51±32.58	6.32±0.71	3.07±0.21	0.70±0.03
7	DPE	258.70±20.71	130.21±18.22	171.36±18.76	6.36±0.68	3.10±0.18	0.73±0.04
8	PE extract	97.75±13.15	89.67±9.87	129.12±9.82	6.28±0.59	3.12±0.28	0.67±0.03
9	Water extract	151.14±18.73	111.64±14.32	164.07±16.70	6.48±0.67	3.32±0.34	0.70±0.02

*Values are mean±SD of 6 determinations.
* DPE: detoxified pet. ether extract, PE extract: pet.ether extract.

PLUMBAGO ZEYLANICA

No.	EXTRACT	SGOT iu/l	SGPT iu/l	Total protein mg%	Albumin mg%	Bilirubin mg%
1	acetone	249.58±24.6	119.50±14.24	6.57±0.64	3.17±0.28	0.72±0.03
2	alcohol	242.46±21.45	116.25±13.64	6.42±0.62	3.22±0.24	0.68±0.02
3	chloroform	320.83±39.25	138.17±15.78	6.32±0.56	3.26±0.26	0.77±0.04
4	pet.ether	116.25±12.82	66.25±6.8	6.52±0.60	3.48±0.32	0.70±0.03
5	water	185.56±17.48	112.58±12.74	6.50±0.58	3.38±0.30	0.67±0.03


Hepatoprotective activity of Plumbago rosea
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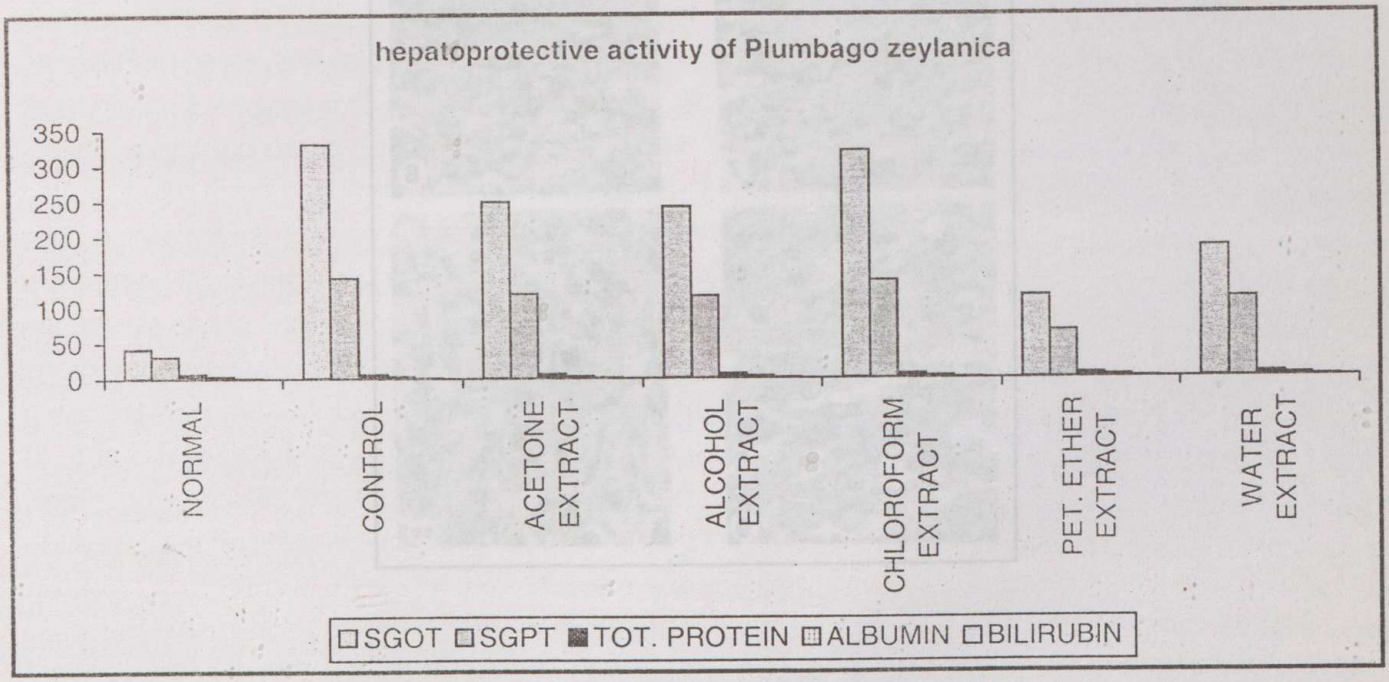
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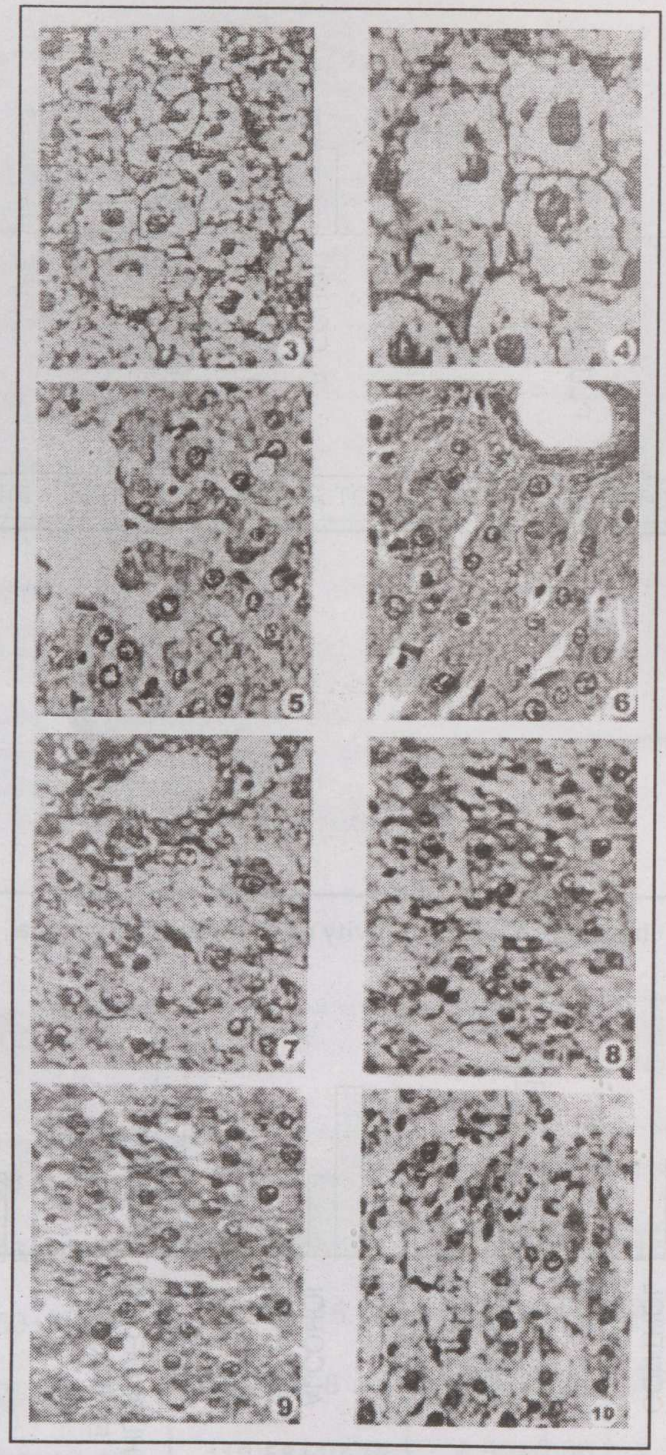
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ANTIOXIDANT VITAMINS AND TRACE ELEMENTS IN ACUTE MYOCARDIAL INFARCTION

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ABSTRACT

Acute myocardial infarction (AMI) continues to be a major public health problem in the developing world, despite impressive strides made in its diagnosis and management. Among the several causes of the disease alteration in the levels of micronutrients and trace elements are also important. The present study was focused on the alteration of these elements in AMI patients as compared to that of the healthy individuals and their changes depending up on different risk factors. The present study included 300 AMI patients and 100 healthy individuals as controls. We observed a decreased value of vitamin E, vitamin C, zinc and magnesium ($p<0.01$) in the serum of AMI patients when compared to that of the normal controls. However the values of iron, % transferrin saturation and ferritin were found to be significantly increased in these patients ($p<0.01$), but the value of total iron binding capacity was found to be significantly decreased ($p<0.01$) in AMI patients. The distributions of these parameters significantly differed among various risk factors studied.

Key Words: Acute myocardial infarction, micronutrients, Total iron binding capacity, Ferritin, %transferrin saturation.

INTRODUCTION

Acute myocardial infarction (AMI), an important manifestation of coronary artery disease (CAD) is emerging as one of the major cause of mortality worldwide. Several factors have been attributed for the cause of the disease. But the exact mechanism responsible for the cause and complication is still unknown. Micronutrients and trace elements are very essential for the normal functioning of the body. Even though they are required in very small amounts, an alteration in the level of these elements may lead to serious impairments which in turn lead to diseases like CAD. Prospective studies have demonstrated reduced risk of coronary artery disease in subjects with a greater intake of vitamin E[1] or vitamin C[2], because these antioxidant vitamins inhibit oxidation of low-density lipoprotein (LDL), a critical event in coronary artery disease. Further, vitamin E and vitamin C, the two essential micronutrients, is capable to remove the oxygen-derived free radicals by virtue of their antioxidant property.

The role of essential micronutrient metals in lipid metabolism is of recent investigation. The metabolism of zinc (Zn) is significantly altered in patients with cardiovascular disease as evidenced by abnormally low plasma or serum concentrations of zinc. Recent studies have shown a relationship between zinc status and the metabolism of cholesterol and lipoproteins[3]. Magnesium (Mg), another trace element is essential for the activation of a variety of enzymes involved in cellular metabolism, especially in neuro-chemical transmission and muscular activity. It has been

shown that magnesium depletion modifies coronary blood flow, blood clotting and atherogenesis[4]. There is a strong relationship between iron levels and cardiovascular disease. The amount of iron is found to be increased considerably in patients with acute myocardial infarction, which in turn can stimulate the lipid peroxidation[5].

The present study evaluates the role of thee trace elements such as iron, zinc and magnesium and micronutrients such as vitamin E and vitamin C along with ferritin, total iron binding capacity and % transferrin saturation in patients with acute myocardial infarction and their sequential variation in the complication of acute myocardial infarction associated with different risk factors taken into account.

MATERIALS AND METHODS

All patients admitted to the Intensive Coronary Care Unit (ICCU) of Amala Cancer Hospital with a diagnosis of acute myocardial infarction presenting within 24 hours of onset of chest pain were included in the study. Present study included 300 AMI patients and 100 sex and age matched control subjects. Myocardial infarction was diagnosed by at least 0.1-mv ST segment elevation in two or more contiguous limb leads or 0.2-mv ST segment elevation in two or more chest leads associated with typical chest pain. Patients with cardiogenic shock, cerebrovascular accident and significant hepatic or renal disease were excluded. Patients with clear evidence of infection anywhere in the body were also excluded.

In patients included in the study, detailed history was taken and a complete physical examination was carried out by the cardiologist. A 12 lead ECG with V3R and V4R is recorded immediately on admission and repeated after 2hrs, 6hrs, 12hrs, 24hrs, 48hrs and pre-discharge. 10 ml blood was withdrawn for laboratory analysis. Serial creatine kinase assays were also done to confirm the myocardial infarction and it was done at 2hrs, 6hrs, 12hrs, 24hrs, 48hrs after admission. Chest X- ray was done at the time of discharge from the ICCU. An echocardiographic examination was performed at the time of discharge or at the end of the first week or early second week after admission. All patients were seen in the cardiology out-patient department 4 to 6 weeks after discharge, when a detailed history was taken and complete physical examination was again carried out. A symptom limited treadmill test was done as per the Bruce protocol and maximum heart rate (HR), blood pressure (BP), double product, time to 1 mm ST depression, metabolic equivalences (METs) achieved, duration of exercise, angina, dyspnea and arrhythmias were recorded along with ST segment changes.

The normal volunteers had no past history or evidence of cardiovascular disease, hypertension or diabetes mellitus. The present study does not include control subjects with a history of neoplastic, hepatic, infectious or autoimmune disease or any surgical procedure in the preceeding 6 months.

The patients were allowed to relax and on the second day they were subjected to an oral questionnaire as described in our Performa, in order to collect the history of these patients. They were asked about the type of chest pain, the time of onset of the pain, radiation to other parts of the body and any previous history of chest pain. They were also asked about the symptoms associated with the chest pain, history of diabetes, history of hypertension, habits of smoking or alcohol or pan chewing, food habits (vegetarians or non

vegetarians) and any positive family history of AMI. Then these patients were categorized according to the following risk factors and based on these risk factors the study has been designed.

1) Age and sex
2) Time of onset of chest pain
3) History of diabetes and hypertension
4) A cholesterol value of <200 mg/dl and >200 mg/dl
5) Habits of smoking and alcohol intake
6) Food habits and a positive and negative family history of AMI

Plasma tocopherol is assayed using the Emmeric Engel reaction[6], Vitamin C was done by the photometric reduction method[7], Zinc in the serum was detected by the Nitro-PAPS method[8] and magnesium was done by the Xylidyl blue method[9]. Iron was estimated by the reaction with a, a'-dipyridyl[10], Total Iron Binding Capacity (TIBC) was done by the addition of magnesium carbonate, and then following the same reaction for iron [11]. Percentage transferrin saturation was done by using the formula: (iron/TIBC) X 100[12] and ferritin was estimated in the serum by a solid phase enzyme linked immunosorbent assay (ELISA)[13]. The statistical analysis was done by using the 'z-test' and the inter group comparison was done by the analysis of variance (ANOVA)[14].

RESULTS

Table 1 represents the values of micronutrients and trace elements in normal and in AMI patients. Here the values of vitamin E, vitamin C, Zn and Mg were found to be decreased significantly ($p<0.01$) in the serum of the AMI patients when compared to that of the normal controls.

Table 2 represents the values of micronutrients and trace elements in normal and in AMI patients according to the age and sex. Irrespective of the age and sex the values of all these parameters were significantly decreased ($p<0.01$) in all the groups when compared to that of their respective controls. Among the age groups the value of vitamin C was found to be significantly decreased in AMI patients with age <40 years when compared to the other two groups and the value Mg was found to be decreased significantly in AMI patients in the age group 40-60 years, when compared to that of the other two groups. Vitamin E and Zn did not show any significant variation among the groups. The values of vitamin C and Mg were found to be significantly decreased in AMI males when compared to that of AMI females. Here also the values of vitamin E and Zn did not show much alteration between the groups.

Table 3 represents the values of micronutrients and trace elements in normal and in AMI patients according to the time of onset of chest pain. Here the values of vitamin E, vitamin C, Zn and Mg were found to be significantly decreased ($p<0.01$) in all the AMI patients with different time of onset of chest pain. The values of vitamin E and vitamin C were found to be significantly altered in AMI patients with onset of chest pain from 12 midnight to 6 am when compared to the other three groups. The value of Mg was found to be significantly altered in AMI patients with onset of chest pain from 6 am to 12 noon when compared to the other three groups. However, the value of Zn did not differ significantly among the groups with different time of onset of chest pain.

Table 4 represents the values of micronutrients and trace elements in normal and

in AMI patients with the history of diabetes and hypertension. Here the values of these micronutrients and trace elements were found to be decreased significantly ($p<0.01$) in AMI patients with and without the history of diabetes and hypertension when compared to that of the normal healthy individuals. A comparison of AMI patients with and without the history of diabetes have shown significant values of vitamin E, vitamin C and Mg in AMI patients with the history of diabetes when compared to AMI patients without the history of diabetes. However, the values of Zn did not show any significant difference between these two groups. A comparison between the AMI patients with and without the history of hypertension has shown significant values of Zn and Mg in AMI patients with the history of hypertension when compared to that of the AMI patients without the history of hypertension. Vitamin E and vitamin C did not differ between these groups.

Table 5 gives the values of micronutrients and trace elements in normal and in AMI patients according to the values of cholesterol. Here the values of vitamin E, vitamin C, Zn and Mg were found to be significantly decreased ($p<0.01$) in AMI patients with cholesterol value >200 mg/dl and <200 mg/dl. A comparison between the values of these micronutrients and trace elements in AMI patients with cholesterol value >200 mg/dl and <200 mg/dl have shown significant values of vitamin E, Zn and Mg in AMI patients with cholesterol value >200 mg/dl when compared to AMI patients with cholesterol value <200 mg/dl. The value of vitamin C was found to be unaltered between these two groups.

Table 6 represents the values of micronutrients and trace elements in normal and in AMI patients according to the habit of smoking and alcohol intake. Here the values of vitamin E, vitamin C, Zn and Mg were found to be significantly decreased ($p<0.01$) in AMI patients with and without the habits of smoking and alcohol intake when compared to their respective controls. A comparison of AMI patients with and without the habit of smoking has shown significant values of vitamin C and Mg in AMI patients with the habit of smoking when compared to that of the AMI patients without the habit of smoking. Vitamin E and Zn did not show any significant alteration between the groups. Comparisons between the AMI patients with and without the habit of alcohol intake have shown significant value of Mg in AMI patients with the habit of alcohol intake when compared to AMI patients without the habit of alcohol intake. The values of vitamin E, vitamin C and Zn were unaltered between these two groups.

Table 7 represents the values of micronutrients and trace elements in normal and in AMI patients according to the food habits and family history of AMI. The values of all these micronutrients and trace elements were found to be significantly decreased ($p<0.01$) in AMI patients with the habit of both vegetarian and non vegetarian food intake and with and without the family history of AMI. A comparison between the AMI patients with vegetarian and non-vegetarian food intake have shown significant value of vitamin E and Mg in AMI patients with the habit of non-vegetarian food intake when compared to that of the AMI patients with the habit of vegetarian food intake. The values of vitamin C and Zn were found to be unaltered between these groups. A comparison between the AMI patients with and without the family history of AMI have shown the significant value of vitamin E in AMI patients without the family history of AMI when compared to AMI patients with the family history of AMI. The values of vitamin C, Zn and Mg were found to be

unaltered between these groups.

Table 8 represents the values of iron, TIBC, %transferrin saturation and ferritin in normal and in AMI patients. The values of iron, %transferrin saturation and ferritin were found to be significantly increased ($p<0.01$) in AMI patients when compared to that of the normal controls. However the value of TIBC was found to be significantly decreased ($p<0.01$) in AMI patients when compared to that of the normal controls.

Table 9 represents the values of iron, TIBC, %transferrin saturation and ferritin in normal and in AMI patients according to the age and sex. Here the values of iron, %transferrin saturation and ferritin were found to be significantly elevated ($p<0.01$) in AMI patients with different age groups and sex when compared to that of the normal controls. The value of TIBC was found to be significantly decreased ($p<0.01$) in AMI patients with different age groups and sex when compared to that of the normal controls.

Table 10 represents the values of iron, TIBC, %transferrin saturation and ferritin in normal and in AMI patients according to the time of onset of chest pain. The values of iron, %transferrin saturation and Ferritin were found to be significantly increased ($p<0.01$) in AMI patients with different time of onset of chest pain when compared to that of the normal controls. The value of TIBC was found to be significantly decreased ($p<0.01$) in AMI patients with different time onset of chest pain when compared to that of the normal controls. A comparison between AMI patients with different time of onset of chest pain have shown significant value of ferritin in AMI patients with time of onset of chest pain from 12 midnight to 6 am when compared to the other three groups. The values of iron, TIBC and %transferrin saturation were remained unaltered between the groups.

Table 11 represents the values of iron, TIBC, %transferrin saturation and ferritin in normal and in AMI patients with and without the history of diabetes and hypertension. Here the values of iron, %transferrin saturation and ferritin were found to be significantly increased ($p<0.01$) in AMI patients with and without the history of diabetes and hypertension when compared to that of the normal controls. The value of TIBC was found to be significantly decreased ($p<0.01$) in AMI patients with and without the history of diabetics and hypertension when compared to that of the normal controls.

Table 12 represents the values of iron, TIBC, %transferrin saturation and ferritin in normal and in AMI patients according to the values of cholesterol. The values of iron, %transferrin saturation and ferritin were found to be significantly increased ($p<0.01$) in AMI patients with cholesterol values >200 mg/dl and <200 mg/dl when compared to that of the normal controls. The value of TIBC was found to be significantly decreased ($p<0.01$) in AMI patients with cholesterol values >200 mg/dl and <200 mg/dl when compared to that of the normal controls.

Table 13 represents the values of iron, TIBC, %transferrin saturation and ferritin in normal and in AMI patients according to the habit of smoking and alcohol intake. The values of iron, %transferrin saturation and ferritin were found to be significantly increased ($p<0.01$) in AMI patients with and without the habits of smoking and alcohol intake when compared to that of the normal controls. However, the value of TIBC was found to be significantly decreased ($p<0.01$) in AMI patients with and without the habits of smoking and alcohol intake when compared to that of the normal controls.

Table 14 represents the values of iron,

TIBC, %transferrin saturation and ferritin in normal and in AMI patients according to the food habits and family history of AMI. The values of iron, %transferrin saturation and ferritin were found to be significantly increased ($p<0.01$) in AMI patients with the habits of vegetarian and non-vegetarian food intake and with and without the family history of AMI when compared to that of the normal controls. The value of TIBC was found to be significantly decreased ($p<0.01$) in AMI patients with the habits of vegetarian and non-vegetarian food intake and with and without the family history of AMI when compared to that of the normal controls. The comparison between the AMI patients with and without the family history of AMI have shown significant value of %transferrin saturation in AMI patients without the family history of AMI when compared to that of the AMI patients with the family history of AMI. However the other parameters did not show significant alteration between the groups.

DISCUSSION

Studies related to the role of micronutrients and trace elements in the pathogenesis of CAD are very rare. However, the significant role of antioxidant vitamins such as vitamin E and vitamin C, the two very important micronutrients in the pathogenesis of AMI have been described. Vitamin E that provides protection against endothelial injury have been associated with atherosclerosis by preserving endothelium derived nitric oxide (NO) activity[15]. Being a chain breaking antioxidant, vitamin E inhibits or delays arterial thrombogenesis[16]. Vitamin C, a water soluble antioxidant vitamin, have been reported to improve the endothelial function, thus reducing the risk of CAD. The antioxidant activity of vitamin C is not restricted to extracellular fluids. It is actively transported into cells and may play a role in the regulation of intracellular redox state and antioxidant defenses[17], possibly via regulation of intracellular thiol species such as glutathione.

In the present study the values of vitamin E and vitamin C were found to be decreased in AMI patients when compared to that of the normal healthy individuals, indicating the low values of these vitamins in the serum of AMI patients. Irrespective of the risk factors the values of these vitamins were found to be decreased in all the AMI patients either due to a low supplementation or due to an impaired metabolism. Further the value of vitamin C was found to be less in AMI patients with age <40mg/dl when compared to the other age groups. In AMI males the value of vitamin C was found to be less than that in AMI females. According to the time of onset of chest pain the values of vitamin E and vitamin C were found to be less in patients with time of onset of chest pain from 12 midnight to 6 am when compared to that of the other groups. The values of vitamin E and vitamin C were found to be less in AMI patients with the history of diabetes and hypertension, indicating that the values of these vitamins may be less in diabetic and hypertensive patients. Further, the value of vitamin E was found to be less in AMI patients with cholesterol value >200 mg/dl, in patients with non-vegetarian food intake and in patients without the family history of AMI. Vitamin C was found to be less in patients with the habit of smoking. All these results together indicate the impairment of the endothelial function in these AMI patients due to a reduction in the amount of these antioxidant vitamins in the body.

Zn and Mg, the two essential trace elements require for the normal functioning of the body is thought to impair the oxidative injury by free radicals or ROS[18]. Zn is an integral

component of various metallo-enzymes and along with other metals can activate a wide variety of enzymes. Further Zn functions to stabilize the membranes, perhaps by decreasing the lipid peroxidation of these structures. The salutary effect of Zn in wound healing may be related to the membrane stabilizing effect. Thus cell damage would be decreased[19]. Hypomagnesaemia has been reported in patients with coronary artery disease[20]. Magnesium is an obligatory cofactor in the enzyme reactions of GSH synthesis and in all biosynthetic enzyme reactions involving ATP and Mg deficiency has been reported to inhibit biosynthesis of GSH[21]. Further more, Mg deficiency also leads to reduced levels of endogenous antioxidant defenses (vitamin E and vitamin C), which may limit free radical detoxification[21].

In the present study we have observed the decreased value of Zn and Mg in all the AMI patients when compared to that of the normal controls, indicating the deficiency of these trace elements in AMI thus leading to the more complications of the disease. The value of Mg was found to be less in AMI patients with age ranges from 40-60 years and in AMI males when compared to that of the AMI females. The value was found to be even less in patients with the onset of chest pain from 6 am to 12 noon, in patients with the history of diabetes and hypertension, patients with a cholesterol value >200 mg/dl and in patients with the habit of non vegetarian food intake. The value of Zn was found to be less in patients with history of hypertension and a cholesterol value of >200 mg/dl. These results indicate the impaired values of Zn and Mg in AMI patients and it could potentiate the oxidative injury to myocardium by free radicals.

The presence of iron in cytochromes, catalse, hydroxylase, peroxidases, saturases, lipoxygenases and cyclooxygenases suggest that iron has an important role in various metabolic events related to lipids, such as the oxidative degradation of fatty acids and the synthesis of unsaturated fatty acids, plasminogens and prostaglandins. However, studies in this area are limited. Oxidation of LDL-cholesterol is catalyzed by iron present in atherosclerotic gruel[12]. Serum deficient in iron has minimal oxidative capacity that increases with iron repletion. Several studies have been conducted in developed countries to assess the association of iron with coronary heart disease or AMI. Iron besides promoting lipid peroxidation could increase the risk of AMI through the elevation of blood haematocrit and blood hemoglobin levels. This in turn increases the viscosity of blood and has a direct thrombogenic effect[22]. Ferritin is the storage form of iron and high levels of ferritin are always associated with CAD[23]. The concentration of ferritin in the serum is directly proportional to the levels of body iron stores. The body iron status in turn related to the concentration of the ferritin, its storage form and transferring, the transport form. The total iron binding capacity (TIBC) is also considered to be an indicator to know the iron status of the body.

The results of the present study have shown increased values of iron, %transferrin saturation and ferritin in AMI patients when compared to that of the normal healthy individuals. The TIBC value was found to be decreased. The value of ferritin was found to be more in patients with time of onset of chest pain from 12 noon to 6 pm and the value of % transferrin saturation was found to be more in AMI patients with a family history of AMI. However all the other parameters

did not show much alteration between the groups. So the present study indicate the increased levels of iron, %transferrin saturation and TIBC and decreased values of TIBC in AMI patients.

The results of the present study indicate the deficiency of the micronutrients such as vitamin E, vitamin C, Zn and Mg in AMI patients and increased values of iron, %transferrin saturation and ferritin and decreased values of TIBC in AMI patients compared to that of the normal controls. These together constitute the further complications and the severity of AMI in these patients.

Table: 1

Values of micronutrients and trace elements in normal and in AMI patients.

Parameters	Groups	
	Normal (n=100)	AMI (n=300)
Vitamin E (mg/dl)	5.94 ± 0.82	2.89 ± 0.75**
Vitamin C (mg/dl)	1.19 ± 0.22	0.24 ± 0.13**
Zn (mg /dl)	88.82 ± 12.04	48.71 ± 8.71**
Mg(mg/dl)	2.31 ± 0.30	0.73 ± 0.36**

Values are mean ±SD

**p<0.01

Table : 2

Values of micronutrients and trace elements in normal and in AMI patients according to the age and sex.

Parameters	Age (years)						Sex			
	< 40		40 - 60		> 60		Male		Female	
	Normal (n=22)	AMI (n=27)	Normal (n=54)	AMI (n=160)	Normal (n=24)	AMI (n=113)	Normal (n=85)	AMI (n=261)	Normal (n=15)	AMI (n=39)
VitaminE (mg/dl)	5.80 ± 0.86	2.56 ± 0.11**	6.03 ± 0.80	2.98 ± 0.78**	5.86 ± 0.79	2.84 ± 0.55**	5.95 ± 0.82	2.89 ± 0.78**	5.88 ± 0.80	2.84 ± 0.56**
VitaminC (mg/dl)	1.15 ± 0.24	0.19 ± 0.13bc**	1.21 ± 0.22	0.23 ± 0.14b**	1.19 ± 0.21	0.27 ± 0.12a**	1.19 ± 0.23	0.23 ± 0.13b**	1.20 ± 0.22	0.29 ± 0.12a**
Zn(mg / dl)	89.47± 12.08	51.00 ± 8.42**	88.62± 11.74	48.08± 9.01**	88.69± 12.64	49.04± 8.31**	89.15± 11.95	48.57± 8.84**	87.00± 12.36	49.64± 8.01**
Mg (mg/dl)	2.19 ± 0.27	0.72 ± 0.29b**	2.36 ± 0.29	0.63 ± 0.31bc**	2.29 ± 0.30	0.87 ± 0.39ba**	2.30 ± 0.30	0.71± 0.35b**	2.36 ± 0.29	0.90± 0.37a**

Values are mean ±SD

**p<0.01

Inter group comparisons were done by one way ANOVA. Same alphabets denote homogenous values and different alphabets denote heterogenous values

Table : 3

Values of micronutrients and trace elements in normal and in AMI patients according to the time of onset of chest pain.

Parameters		Time of onset of chest pain			
	Normal (n=100)	AMI			
		12midnight to 6am (n=50)	6am to 12noon (n=94)	12noon to 6pm (n=90)	6pm to 12 midnight (n=66)
Vitamin E (mg/dl)	5.94 ± 0.82	2.51 ± 0.88bcd**	2.79 ± 0.67bcd**	3.25 ± 0.75a**	2.82 ± 0.55cdb**
Vitamin C (mg/dl)	1.19 ± 0.22	0.21 ± 0.14cbd**	0.24 ± 0.16b**	0.22 ± 0.11bc**	0.29 ± 0.11a**
Zn(mg/dl)	88.82 ± 12.04	49.13 ± 7.93**	47.66 ± 8.01**	48.60 ± 10.39**	50.03 ± 7.54**
Mg(mg/dl)	2.31 ± 0.30	0.67 ± 0.26c**	0.65 ± 0.35cd**	0.71 ± 0.35bcd**	0.92 ± 0.38a**

Values are mean ±SD

**p<0.01

Inter group comparisons were done by one way ANOVA. Same alphabets denote homogenous values and different alphabets denote heterogenous values.

Table : 4
Values of micronutrients and trace elements in normal and in AMI patients with the history of diabetes and hypertension.

Parameters	Normal (n=100)	AMI			
		Diabetes		Hypertension	
		Diabetic (n=101)	Nondiabetic (n=199)	Hypertensive (n=62)	Non Hypertensive (n=238)
Vitamin E (mg/dl)	5.94 ± 0.82	2.51 ± 0.74b**	3.08 ± 0.69a**	2.62 ± 0.56**	2.95 ± 0.78**
Vitamin C (mg/dl)	1.19 ± 0.22	0.21 ± 0.15b**	0.25 ± 0.12a**	0.22 ± 0.16**	0.24 ± 0.13**
Zn(mg / dl)	88.82 ± 12.04	47.65 ± 8.24**	49.24 ± 8.88**	46.55 ± 8.48b**	49.27 ± 8.71a**
Mg(mg/dl)	2.31 ± 0.30	0.65 ± 0.28b**	0.77 ± 0.38a**	0.65 ± 0.32b**	0.75 ± 0.37a**

Values are mean ±SD
**p<0.01
Inter group comparisons were done by one way ANOVA. Same alphabets denote homogenous values and different alphabets denote heterogenous values.

Table: 5
Values of micronutrients and trace elements in normal and in AMI patients according to the values of cholesterol.

Parameters	Cholesterol (mg/dl)		
	Normal (n=100)	AMI	
		>200 (n=105)	<200 (n=195)
Vitamin E (mg/dl)	5.94 ± 0.82	2.51 ± 0.72b**	3.09 ± 0.69a**
Vitamin C (mg/dl)	1.19 ± 0.22	0.21 ± 0.16**	0.25 ± 0.12**
Zn (mg / dl)	88.82 ± 12.04	47.22 ± 8.36b**	49.51 ± 8.77a**
Mg(mg/dl)	2.31 ± 0.30	0.64 ± 0.28b**	0.78 ± 0.38a**

Values are mean ±SD
**p<0.01
Inter group comparisons were done by one way ANOVA. Same alphabet denote homogenous values and different alphabets denote heterogenous values.

Table : 6
Values of micronutrients and trace elements in normal and in AMI patients according to the habits of smoking and alcohol intake.

Parameters	Smoking				Alcohol intake			
	Yes		No		Yes		No	
	Normal (n=62)	AMI (n=222)	Normal (n=38)	AMI (n=78)	Normal (n=20)	AMI (n=160)	Normal (n=80)	AMI (n=140)
Vitamin E (mg/dl)	5.90 ± 0.81	2.91 ± 0.82**	6.00 ± 0.81	2.82 ± 0.54**	5.85 ± 0.89	2.99 ± 0.80**	5.96 ± 0.79	2.80 ± 0.69**
Vitamin C (mg/dl)	1.18 ± 0.23	0.22 ± 0.14b**	1.20 ± 0.21	0.28 ± 0.12a**	1.15 ± 0.25	0.22 ± 0.15**	1.20 ± 0.22	0.25 ± 0.11**
Zn (mg/dl)	89.17 ± 11.40	48.42 ± 9.03**	88.26 ± 13.03	49.52 ± 7.81**	89.24 ± 12.64	48.70 ± 8.66**	88.71 ± 11.91	48.72 ± 8.81**
Mg (mg/dl)	2.28 ± 0.29	0.68 ± 0.33b**	2.36 ± 0.30	0.88 ± 0.39a**	2.17 ± 0.27	0.66 ± 0.32b**	2.35 ± 0.29	0.81 ± 0.39a**

Values are mean ±SD
**p<0.01
Inter group comparisons were done by one way ANOVA. Same alphabets denote homogenous values and different alphabets denote heterogenous values.

Table: 7
Values of micronutrients and trace elements in normal and in AMI patients according to the food habits and family history.

Parameters	Food habits				Family history		
	Vegetarians		Non Vegetarians			AMI	
	Normal (n=16)	AMI (n=27)	Normal (n=84)	AMI (n=273)	Normal (n=100)	+ ve (n=82)	- ve (n=218)
Vitamin E (mg/dl)	5.84 ± 0.78	3.88 ± 0.60a**	5.96 ± 0.82	2.79 ± 0.69b**	5.94 ± 0.82	3.36 ± 0.73a**	2.71 ± 0.69b**
Vitamin C (mg/dl)	1.22 ± 0.22	0.21 ± 0.11**	1.19 ± 0.23	0.24 ± 0.14**	1.19 ± 0.22	0.25 ± 0.15**	0.23 ± 0.13**
Zn (mg/dl)	87.34 ± 12.04	46.32 ± 9.43**	89.11 ± 12.06	48.94 ± 8.60**	88.82 ± 12.04	47.85 ± 8.66**	49.03 ± 8.71**
Mg (mg/dl)	2.39 ± 0.31	1.02 ± 0.32a**	2.29 ± 0.29	0.70 ± 0.35b**	2.31 ± 0.30	0.74 ± 0.35**	0.73 ± 0.36**

Values are mean±SD
**p<0.01
Inter group comparisons were done by one way ANOVA. Same alphabets denote homogenous values and different alphabets denote heterogenous values.

Table: 8

Values of Iron, TIBC, % transferrin saturation and Ferritin in normal and in AMI patients.

Parameters	Groups	
	Normal (n=100)	AMI(n=300)
Iron (mg/dl)	99.67 ± 13.51	154.96 ± 23.08**
TIBC (mg/dl)	324.74 ± 15.84	256.21 ± 24.16**
%Transferrin saturation	30.68 ± 4.35	59.87 ± 4.00**
Ferritin (ng/ml)	65.78 ± 12.78	131.33 ± 24.76**

Values are mean ±SD

**p<0.01

Table : 9
Values of micronutrients and trace elements in normal and in AMI patients according to the age and sex.

Parameters	Age (years)						Sex			
	< 40		40 - 60		> 60		Male		Female	
	Normal (n=22)	AMI (n=27)	Normal (n=54)	AMI (n=160)	Normal (n=24)	AMI (n=113)	Normal (n=85)	AMI (n=261)	Normal (n=15)	AMI (n=39)
Iron (mg/dl)	99.16± 11.34	151.57± 24.57**	100.71± 13.62	155.83 ± 23.17**	97.80± 14.79	154.52 ± 22.45**	100.07± 12.93	155.67 ± 23.23**	97.39± 16.19	150.20± 21.34**
TIBC (mg/dl)	315.72± 15.91	251.56 ± 24.57**	329.52± 14.96	257.32 ± 24.10**	322.25± 13.30	255.74 ± 24.01**	325.19± 16.03	256.74 ± 24.22**	322.20± 14.42	252.64± 23.45**
%Transferrin saturation	31.12± 3.46	59.85 ± 3.92**	30.58 ± 4.47	60.37 ± 4.16**	30.51± 4.76	60.22 ± 3.78**	30.62 ± 4.35	60.36 ± 4.09**	31.05 ± 4.33	59.62± 3.30**
Ferritin (ng/ml)	67.55 ± 8.85	128.98 ± 40.17**	69.81± 10.44	131.89 ± 22.98**	55.08± 14.48	131.09 ± 22.19**	67.88± 11.74	131.76 ± 25.37**	53.86± 11.87	128.42± 20.01**

Values are mean±SD
**p<0.01

Table : 10
Values of Iron, TIBC, % transferrin saturation and Ferritin in normal and in AMI patients according to the time of onset of chest pain.

Parameters	Time of onset chest of pain				
	Normal (n=100)	AMI			
		12midnight to 6am (n=50)	6am to 12noon (n=94)	12noon to 6pm (n=90)	6pm to 12 midnight (n=66)
Iron (mg/dl)	99.67 ± 13.51	154.84 ± 26.36**	156.11 ± 22.52**	154.28 ± 22.11**	154.31 ± 22.40**
TIBC (mg/dl)	324.74 ± 15.84	254.54 ± 26.66**	258.28 ± 24.04**	256.24 ± 23.01**	254.47 ± 23.68**
%Transferrin saturation	30.68 ± 4.35	60.27 ± 4.28**	60.16 ± 4.33**	60.48 ± 3.83**	60.11 ± 3.48**
Ferritin (mg/ml)	65.78 ± 12.78	128.33 ± 35.52d**	129.82 ± 21.94bd**	136.29 ± 22.00ad**	129.73 ± 20.88abcd**

Values are mean ±SD
**p<0.01
Inter group comparisons were done by one way ANOVA. Same alphabets denote homogenous values and different alphabets denote heterogenous values.

Table : 11
Value of Iron, TIBC, % transferrin saturation and Ferritin in normal and in AMI patients with the history of diabetics and hypertension.

Parameters	Normal (n=100)	AMI			
		Diabetes		Hypertension	
		Diabetic (n=101)	Nondiabetic (n=199)	Hypertensive (n=62)	Non Hypertensive (n=238)
Iron (mg/dl)	99.67 ± 13.51	154.80 ± 24.47**	155.03 ± 22.33**	155.19 ± 25.07**	154.89 ± 22.51**
TIBC (mg/dl)	324.74 ± 15.84	255.92 ± 23.06**	256.78 ± 26.17**	255.89 ± 24.43**	256.29 ± 24.10**
%Transferrin saturation	30.68 ± 4.35	59.88 ± 4.57**	60.46 ± 3.67**	60.06 ± 3.87**	60.32 ± 4.04**
Ferritin (ng/ml)	65.78 ± 12.78	127.25 ± 29.42**	133.39 ± 21.73**	128.76 ± 23.15**	132.00 ± 25.11**

Values are mean ±SD
**p<0.01

Table: 12
Values of Iron, TIBC, % transferrin saturation and Ferritin in normal and in AMI patients according to the values of cholesterol.

Parameters	Cholesterol (mg/dl)		
		AMI	
	Normal (n=100)	>200(n=105)	<200(n=195)
Iron (mg/dl)	99.67 ± 13.51	155.02 ± 24.89**	154.92 ± 22.02**
TIBC (mg/dl)	324.74 ± 15.84	257.38 ± 25.98**	255.57 ± 23.11**
%Transferrin saturation	30.68 ± 4.35	60.01 ± 4.54**	60.41 ± 3.68**
Ferritin (mg/ml)	65.78 ± 12.78	127.58 ± 29.10**	133.34 ± 21.79**

Values are mean ±SD
**p<0.01

Table : 13
Value of Iron, TIBC, % transferrin saturation and Ferritin in normal and in AMI patients according to the habits of smoking and alcohol intake.

Parameters	Smoking				Alcohol intake			
	Yes		No		Yes		No	
	Normal (n=62)	AMI (n=222)	Normal (n=38)	AMI (n=78)	Normal (n=20)	AMI (n=160)	Normal (n=80)	AMI (n=140)
Iron (mg/dl)	100.99± 11.78	155.58 ± 23.21**	97.51 ± 15.62	153.15 ± 22.53**	98.53 ± 11.53	156.18 ± 23.83**	99.95 ± 13.92	153.56 ± 22.09**
TIBC (mg/dl)	324.95± 16.76	256.77 ± 24.26**	324.41 ± 14.17	254.61 ± 23.81**	315.83 ± 16.64	257.22 ± 24.47**	326.97 ± 14.79	255.06 ± 23.76**
%Transferrin saturation	31.01± 3.84	60.39 ± 4.10**	30.14 ± 5.03	59.91 ± 3.69**	30.98 ± 3.51	60.30 ± 4.20**	30.61 ± 4.53	60.22 ± 3.76**
Ferritin (ng/ml)	68.40± 10.69	132.13 ± 25.90**	61.50 ± 14.59	129.06 ± 21.00**	66.80 ± 8.81	130.17 ± 26.62**	65.53 ± 13.56	132.65 ± 22.36**

Values are mean±SD
**p<0.01

Table : 14

Values of Iron, TIBC, % transferrin saturation and Ferritin in normal and in AMI patients according to the food habits and family history.

Parameters	Food habits				Family history		
	Vegetarians		Non Vegetarians			AMI	
	Normal (n=16)	AMI (n=27)	Normal (n=84)	AMI (n=273)	Normal (n=100)	+ ve (n=82)	- ve (n=218)
Iron (mg/dl)	97.57 ± 15.68	152.68 ± 22.49**	100.07 ± 13.01	155.18 ± 23.11**	99.67 ± 13.51	156.88 ± 20.97**	154.23 ± 20.97**
TIBC (mg/dl)	322.94 ± 14.24	254.36 ± 24.81**	325.09 ± 16.09	256.39 ± 24.08**	324.74 ± 15.84	257.04 ± 23.97**	255.89 ± 24.22**
%Transferrin saturation	30.38 ± 4.93	59.73 ± 3.32**	30.74 ± 4.22	60.32 ± 4.06**	30.68 ± 4.35	61.12 ± 3.69a**	59.94 ± 4.07b**
Ferritin (ng/ml)	54.18 ± 11.53	131.46 ± 18.56**	67.99 ± 11.77	131.32 ± 25.27**	65.78 ± 12.78	134.58 ± 21.42**	130.11 ± 25.79**

Values are mean±SD

**p<0.01

Inter group comparisons were done by one way ANOVA. Same alphabets denote homogenous values and different alphabets denote heterogenous values.

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IMPAIRED CELL MEDIATED AND HUMORAL IMMUNITY IN UNTREATED HODGKIN LYMPHOMA PATIENTS

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ABSTRACT

A study was undertaken to assess the role of cellular and humoral immunity in the etiopathogenesis of Hodgkin's Lymphoma (HL) patients by enumeration of total and high affinity rosette forming cells (TRFC and HARFC) and estimation of circulating Immune complexes. The mean values of total and high affinity rosette forming cells in HL patients were significantly decreased than in controls ($p>0.001$), indicating impaired cellular immunity. On the other hand, the mean value of circulating immune complex (CIC) was significantly increased in HL patients when compared to controls, thereby indicating an impairment of humoral immunity also. Thus, untreated HL patients in all stages exhibited an immune defect characterized by markedly impaired cellular and humoral immunity. It is presumed that a defective immune system responsible for may be result of the neoplasm. Conversely, an inherent genetically determined defect resulting in defective immunity may be permissive to the development of HL.

INTRODUCTION

Hodgkin's Lymphoma (HL) is a malignant lymphoma affecting primarily the lymphoid tissues. At the regional Cancer Centre, Trivandrum, HL represents 1.2% of approximately 10,000 new cancer cases registered every year. The cell of origin for this malignancy still eludes us, although multiple groups are investigating its etiology. Probably not much work has been done on the etiology of HL in India. It has been suggested that HL may be a heterogeneous condition with different etiological factors in the different age groups. Patients with primary immunodeficiency syndromes have a markedly increased risk of various lymphoreticular neoplasms (Kersey *et al.*, 1973; Loiue and Schwartz, 1978). Immunodeficiency status has been implicated to be a risk factor for HL. It is not clear whether cellular and humoral immunity play any role in the etiology of HL. Hence a study was undertaken to elucidate the role of cellular and humoral immunity in the development of HL. The specific objectives were to enumerate the total and high affinity rosette forming cells (TRFC and HARFC) and estimation of circulating immune complexes(CIC) in HL patients.

MATERIALS AND METHODS

A total of 82 HL patients consisting of 70 adult cases (15-74 years age range) and 12 paediatric cases (5-14 years age range) who attended the outpatient clinics of Regional Cancer Centre, Trivandrum were included in this study. For comparison, 85 age and sex matched healthy controls (selected from patient's relatives, visitors, RCC staff etc.) were also included. The sex and age distribution of the study subjects are given in Table-1.

SAMPLE COLLECTION AND PROCESSING

Fifteen milliliters of blood was collected from each patient and control by venipuncture under sterile conditions, for immunological studies. Of this, 10 ml blood was collected in sterile heparinized tubes for the enumeration of total and high affinity rosette forming cells(TRFC and HARFC). The rest 5 ml was collected in siliconized tubes without any anticoagulant. Serum was separated and stored at -80°C for serological quantitation of circulating immune complexes (CIC).

The method of Weese *et al* (1980) was followed for enumeration of TRFC and HARFC. In short, 10 ml heparinized blood was diluted 1:3 with phosphate buffered saline(pH 7.4) and layered into Lymphoprep (Nyegaard and Co., Norway). After centrifugation at 1500 rpm (400Xg) for 30 mts, the mononuclear cell interface was carefully collected and washed twice in 50 ml of saline and centrifuged for 10 mts at 1500 rpm. The lymphocytes were suspended at a concentration of 4X 10^6 cells/ml in RPMI 1640 medium containing 10 ml of Hepes buffer(Sigma, USA) and 1% streptomycin. Sheep erythrocytes, drawn once weekly and preserved in Alseiver's solution at 4°C, were washed 3 times with PBS and resuspended at a concentration of 1X 10^8 cells/ml in RPMI 1640. 0.1 ml of the lymphocyte solution, 0.2 ml of heat inactivated (56°C, 30 minutes) fetal bovine serum and 0.2 ml of sheep erythrocytes solution were added in sequence to 12 X 75 mm plastic tubes. These were gently vortexed and incubated for 5 minutes at 37°C in centrifuge holders and were then centrifuged at 100 X g for 5 mts. The tubes were then incubated overnight in a 29°C water bath. Approximately, 18 hrs later, the cell pellets were resuspended by gentle multi-axle rotation. Lymphocytes binding at least 3 erythrocytes were visually quantitated which gave the number of high affinity rosette forming cells.

Quantitation of "total cell levels" was performed in a similar way. The technique used differed from that in two points. In this assay, sheep erythrocytes were used at a concentration of 3X 10^8 cells/ml. Additionally, the overnight incubation was performed at 4- 6°C instead of at 29°C. Quantitation was similar to the above described method.

ESTIMATION OF CIRCULATING IMMUNE COMPLEXES (CIC)

A modified method of Creighton *et al* (1973) employing precipitation with Poly Ethylene Glycol (PEG) was followed for estimation of CIC level. Here 5 ml of venous blood collected from the subjects were allowed to clot at 37°C for 3 hrs to avoid precipitation of cryoglobulins, serum was separated and stored at -70°C till use. The Circulating Immune Complexes (CIC) was then precipitated by Poly Ethylene Glycol (PEG) 6000. Two ml of 3.3% PEG 6000 was added to 0.2 ml of serum and the mixture was incubated at room temperature for 2 hours and then centrifuged at 2500X g for 30 minutes at 4°C. The precipitate was washed thrice with 3% ice cold PEG 6000 and the pellet was dissolved in 0.2 ml of distilled water and diluted to 2 ml with NaOH (0.1N). The protein content of the solution was estimated by Lowry's method (1951).

RESULTS

To assess the cellular immunity, the capability of the T-lymphocyte population to form rosettes (Total and High affinity rosette forming cells (TRFC & HARFC) with sheep red blood cells (SRBC) were assayed in this study on 82 HL

patients. For comparison, 85 age and sex matched controls were included. The mean TRFC was 56.829 ± 4.976 in HI patients and 70.741 ± 4.457 in controls. With respect to control value, HL patients showed significant decrease in TRFC (P< 0.001). Regarding HARFC, HL patients had a mean value of 52.271 ± 4.075. Thus the HARFC values were significantly reduced (P<0.001) in HI patients (Table-2) HL patients in all the 4 subtypes, different stages of the disease and different age groups exhibited significant decrease in both TRFC and HARFC values (P< 0.001). The values decreased progressively with the progression of the disease.

CIRCULATING IMMUNE COMPLEXES

The concentration of Circulating Immune Complexes is expressed as mg of proteins/dl of serum. The level of CIC in the sera from HL patients ranged from 75.38 – 315.23 with a mean value of 210.905 ± 61.526 whereas in controls, the CIC levels ranged from 65.11 – 101.60 with a mean value of 79.868 ± 0.091. The level of CIC was significantly (P<0.001) increased in the sera of HL patients as shown in Table-3 irrespective of histological subtype, clinical stages of the disease or different age groups of patients.

DISCUSSION

The comprehensive knowledge relating to the components of both humoral and cell mediated immune responses may be of immense help in the study of malignancies. In the present study, the role of humoral and cellular immunity in the etiopathogenesis of Hodgkin's Lymphoma was assessed.

Patients with untreated HL in all stages were reported to exhibit reduced cellular immunity with relatively intact humoral immune responses (Romagnani *et al.,* 1985; Griesinger *et al.*, 1990). Decreased E-rosette formation is considered as a manifestation of cellular immune defect (Mukhopadhyay *et al.*, 1987). Levy *et al* (1984) reported depressed natural killer (NK) cell cytotoxicity in patients with untreated HL. Patients with advanced disease (stages III or IV) have been reported to have an inherent T-lymphocyte defect (van Rijswijk *et al.*, 1986). Much effort has been given to evaluate the cellular immune responses of cancer patients by enumerating the rosette forming cells (West *et al.*, 1976). The rosette formation assay is considered to be a simple but potentially useful method for diagnosis and monitoring cancer patients, especially those undergoing immunosuppressive and immuno-stimulatory therapy (Djeu *et al.*, 1977).

E-rosette forming cells in human peripheral blood can be subdivided in to two fractions on the basis of their relative affinity for sheep red blood cells. Low affinity E-RFC and high affinity E-RFC. Later, Weese *et al* (1980) employed a modified method by which two types of RFCs could be identified and differentiated. The total rosette forming cells (TRFCs) included all T cells which form rosettes with SRBC at low temperature on prolonged incubation whereas the high affinity rosette forming cells (HARFCs) form rosettes at 29°C with fewer number of SRBC. West *et al* (1976) reported a decrease in T cells in cancer patients but the study on HL patients, the TRFC value decreased progressively with the progression of the disease (P<0.001).

In the current study, majority of the normal control subjects had HARFC values higher than 47% which is in agreement with previous reports (Weese *et al.*, 1980; Bashford and Gough, 1983; Vijayakumar *et al.*, 1985). A progressive decrease in the percentage of HARFC with the progression

of the clinical stages was also observed in the present study. The changes in TRFC and HARFC values were found to correlate well with the clinical stages and histological subtypes of HL patients. No significant difference was observed between patients and controls with regard to different age group. A depression in cell mediated immunity characterized by decrease in TRFC and HARFC levels was observed in these HL patients. Fisher and Young (1978) and Susan *et al* (1980) reported that there is a decrease in general immunity in cancer patients, especially in lymphomas.

Tumour associated antigens may be released from a developing tumour into the extra cellular environment and subsequently be found in free form and/ or as circulating immune complexes (CIC) in serum and other body fluids (Price and Baldwin, 1977). Soluble tumour antigens are believed to create a protection against the attack of specific antibodies and lymphocytes by blocking antigen receptors (Bellido *et al.*, 1981). A variety of methods has been developed for the detection and quantitation of circulating immune comp0lexes in the sera of patients with malignant and other diseases (Celeda *et al.*, 1982; Basalar *et al.*, 1984). The method employing precipitation with Poly Ethylene Glycol 6000(PEG) has been found to be easy and reliable and hence this method was employed in the present study.

In the present investigation, elevated levels of circulating immune complexes were detected in the sera of HL patients. Moreover, the levels of CIC were found to be increasing with progression of clinical stages. These findings are in agreement with previous reports in which a correlation between levels of immune complexes, tumour burden and prognosis had been demonstrated (Hoffken *et al.*, 1978; Amblot *et al.*, 1978; Gupta *et al.*, 1979). The observation of increased levels of CIC in advanced stages of HL patients in the present study is contradictory to the report of Nerurkar et al (1993), who reported elevated levels of CIC only in stage I and stage II of HL patients. Heier *et al* (1979) reported that the frequency of circulating immune complexes in the sera of HL patients was somewhat higher among patient with advanced disease than among those with localized disease. Amlot *et al* (1976) reported increased quantities of immune complexes in the plasma of untreated HL patients. They reported a correlation between CIC levels and general symptoms such as fever, night sweats and weight loss in Hl and suggested that CIC induced some of these symptoms. A fall in CIC levels following treatment and remission had been reported in HL by Brown *et al* (1978). According to Scully (1982), the alterations in humoral immunity, in cancer patients may be a reflection of altered cell mediated immunity.

Even though the nature of immune complexes is quite unknown, Heimer *et al* (1976) showed that besides immunoglobulins, nonimmunoglobulins components were present in the complexes (1977). The observation of a depression in humoral immunity is characterized by an increase in CIC levels, in the present study is in favour of Hellstrom *et al* (1977) who suggested that CIC block cell mediated immune responses. Overproduction of CIC might be due to the constant production of antibody with the continuous supply of antigens by the disease. There is no clear understanding of the host's response leading to elevated levels of CIC in the sera of HL patients. The elevated levels of CIC may be attributable to the changes in the complement fixing and non complement fixing of tumour specific antibodies. Thus the results from

this study clearly demonstrated a defective immune system in HL patients. However, these results did not give any conclusive evidence on the role of immune system in the etiopathology of HL. The persistent cellular immune alterations characteristic of HL may be the result of the neoplasm. Conversely, an inherent genetically determined defect resulting in defective cellular immunity may be permissive to the development of HL.

Table-1
Sex and age distribution of subjects

Subjects	No	Sex ratio Male: Female	Age in years	
			Range	Mean ± SD
Controls	85	64: 21	3-75	34.1 ± 19.4
HL patients	82	62: 20	5- 74	34.8 ± 18.8

Table-2
Enumeration of Total and High Affinity rosette forming cells (TRFC & HARFC) in HL patients and Controls

Subjects	Percentage of TRFC Mean ± SD	Percentage of HARFC Mean ± SD
Controls	70.74 ± 4.457	52.271 ± 4.075
HL patients(n= 82)	56.829 ± 4.976*	31.781 ± 5.202*

P< 0.001 compared to controls

Table-3

Circulating Immune Complexes in the sera of Hodgkin's disease patients and controls

Subjects	Circulating Immune Complexes (CIC) in mg/dl	
	Range	Mean ± SD
Controls (n=85)	65.11- 101.60	79.868 ± 9.091
HL patients (n= 82)	75.38 ± 315.23	210.905 ± 61.526*

* Compared to controls, $P < 0.001$

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XI. RESEARCH PUBLICATIONS (LAST FIVE YEARS - (1997 - 2003)

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109. K.S.Sreekanth, M.C.Sabu, Leyon Varghese, C.Manesh, G.Kuttan and R.Kuttan. Antioxidant activity of Smoke Shield and in vitro and in vivo, Journal of Pharmacy and Pharmacology, 55, 847-853 (2003).

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121. Sabu M.C. and R.Kuttan. Antidiabetic activity of Aegle marmelos and its relationship with antioxidant properties, Indian Journal of Physiology and Pharmacology (In press).

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124. K.T.Santhosh, S.Joseph and R.Kuttan. Potent suppressive effect of green tea polyphenols and tobacco induced mutagenicity. Phytomedicine (Accepted).

125. K..Harikumar and R.Kuttan. Protective effect of an extract of Phyllanthus amarus against radiation induced damage in mice. Journal of Radiation Research (Accepted).126. Sabu M.C. and R.Kuttan. Oxidants, antioxidants and Cancer. Ed.by Vandana Rai, Jaunpur, U.P.

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129. Sheena N, Ajith T.A. and Janardhanan K.K. Prevention of nephrotoxicity induced by the anticancer drug Cisplatin, using Ganoderma lucidum a medicinal mushroom occurring in South India. Current Science, 85 (4), 478-482 (2003).

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131. Sreekanth K.S, Geever Zachariah and Annamalai P.T. C-reactive protein and other inflammatory markers in Acute Coronary Syndromes. The Kuwait Medical Journal (in press)

132. Effect of S-allyl Cysteine Sulphoxide on lipid metabolism & free radical scavengers in alcohol fed rats. Publised - "Current Science", Vol. 82, No. 6, 25 March 2002.

133. Combined effect of alcohol & smoke on lipid peroxidation & antioxidafiont status in albimo rats. Indian Journal of Biochemistry & Biophysics, Bindu M.P., Annamali P.T. (accepted)

134. Effect of swimming stress on lipid metabolism and antioxidant status in experimental animals. Indian Journal of Experimenta Biology. (accepted) Bindu M.P., Annamali P.T.

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XII. PAPERS PRESENTED AT THE MEETING (1997-2003)

1. Kuttan R, Joseph C.D, Praveenkumar V, Lata G Menon, Jeena Jose and Kuttan G. Role of Rasayanas in cancer therapy. 16th Annual Convention of IACR and National Symposium on New sights into the molecular mechanism of oncogenesis, January (1997).
2. Girija Kuttan, Lata G Menon, Antony S and Kuttan R. Role of naturally occuring compounds in the inhibition of cancer cell metastasis. 16th Annual Convention of IACR and National symposium on new sights into the molecular mechanism of oncogenesis, January (1997).
3. Sini Antony, Lata G Menon, Kuttan R and Kuttan G. Antimetastatic and anticarcinogenic activity of Viscum album. 16th Annual Convention of IACR and National symposium on new sights into the molecular mechanism of oncogenesis. January (1997).
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Ph.D.THESIS SUBMITTED DURING THE LAST FIVE YEARS

1. Dr.K.T.Mary (Guide - Dr.R.Kuttan), M.G.University. Title of the thesis: Anticancer activity of medicinal plants.
2. Dr.Ruby John Anto (Guide - Dr.R.Kuttan), M.G.University, Title of the thesis: Pharmacological properties of curcuminoids and related compounds.
3. Dr.V.Praveenkumar (Guide - Dr.R.Kuttan/Dr.Girija Kuttan), Calicut University, Title of the thesis : Anticancer activity of selected indigenous drugs.
4. Dr.Josely George (Guide - Dr.R.Kuttan) Calicut University. Title of the Thesis: Assessment of cancer causation by industrial chemicals.
5. Dr.K.C.Thressiamma (Guide - Dr.R.Kuttan), M.G.University. Title of the thesis : Studies on the protective effect of antioxidants on the chemical and radiation-induced damage.
6. Dr.T.D.Babu (Guide - Dr.Padikkala), M.G.University, Title of the thesis: A study of anticancer activity and mechanism of action of selected medicinal plants.
7. C.R.Achuthan (Guide - Dr.Padikkala), M.G.University. Title of the thesis: A study on the antiatherogenic effect of proteoglycans and active principles from natural sources.
8. Lata G Menon (Guide - Dr.G.Kuttan), M.G.University. Title of the thesis : Anticancer properties of polyphenolic compounds.
9. Jeena Jose (Guide - Dr.R.Kuttan), M.G.University. Title of the thesis : A study of antioxidants present in plants.
10. K.L.Joy (Guide - Dr.R.Kuttan), Calicut University. Title of the thesis: Antioxidant activity of selected medicinal plants and their application in Medicine.
11. Anis K.V. (Guide - Dr.R.Kuttan), M.G.University. Title of the thesis: Investigation on the use of herbal drugs in cancer therapy.
12. Rekha, P.S. (Guide - Dr.R.Kuttan), M.G.University. Title of the thesis: Role of Rasayanas in ameliorating radiation and chemotherapy induced toxicity.
13. Mary N.K. (Guide - Dr.Jose Padikkala). M.G.University. Title of the thesis: A study of antiatherogenic effect of selected medicinal plants.
14. Harish Babu.B. (Guide - Dr.Jose Padikkala) M.G.University. Title of the thesis: Study on the production of antineoplastic secondary metabolites from selected medicinal plants using cell and tissue culture technique (with special reference to Camptothecin and Gossypin).
15. Sini Antony (Guide - Dr.Girija Kuttan) M.G.University. Title of the thesis : Study on the effect of immunomodulators in the inhibition of metastasis.
16. Shylesh, B.S. (Guide - Dr.Jose Padikkala), M.G.University. Title of the thesis: Study on the anticancer activity of secondary metabolites from selected medicinal plants employing cell and tissue culture techniques.
17. Mr.Rajeshkumar (Guide - Dr.R.Kuttan), M.G.University. Title of the thesis : The effect of natural products in chemical carcinogenesis.
18. Mrs.Leemol Davis (Guide - Dr.Girija Kuttan), M.G.University). Title of the thesis: Immunomodulatory activity of Withania somnifera - its implication in the treatment of cancer.

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