Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 3, Suppl 5, 2011

Research Article

ANTICANCER EFFICACY OF *HOLOPTELEA INTEGRIFOLIA*, PLANCH. AGAINST 7, 12-DIMETHYL BENZ(A)ANTHRACENE INDUCED BREAST CARCINOMA IN EXPERIMENTAL RATS

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Received: 25 Jun 2011, Revised and Accepted: 5 Oct 2011

ABSTRACT

The present study was carried out to investigate Breast Cancer prevention property and Anti-oxidant status of ethanolic extract of *Holoptelea integrifolia*, Planch. against 7,12-dimethyl benz(a)anthracene induced breast carcinoma in experimental rats. *Holoptelea integrifolia*, Planch. is a widely used herb in traditional medical systems of India. The bark was defatted with petroleum ether and extracted with ethanol. Anticancer activity and Antioxidant Status of the ethanolic extract of bark of *Holoptelea integrifolia*, Planch against 7,12-dimethyl benz(a)anthracene induced breast carcinoma were assessed in Female Sprague-Dawley rats and the body weight of animals, tumour burden, tumour multiplicity, tumour volume of breast and antioxidant parameters like SOD,CAT,GSH and GPx levels were also measured. The extract administered orally at doses of 250 and 500 mg/kg p.o. showed significant ($P < 0.05, 0.01$) dose-dependent inhibition of breast cancer formation. A significant % inhibition of breast cancer formation by the ethanolic extract of leaves of *H. integrifolia*, Planch. and its almost nearby same % inhibition with Tamoxifen suggest its usefulness as an Anticancer agent.

Keywords*: Holoptelea integrifolia*, 7,12-dimethyl benz(a)anthracene, *Invivo* anti-tumor, Antioxidant parameters.

INTRODUCTION

Breast cancer is the leading cause of morbidity and mortality in women's lives. Worldwide, breast cancer is the second most common type of cancer after lung cancer and the fifth most common cause of cancer death. The numbers of breast cancer cases are greater in developed countries than developing countries. It has been reported that one in four new cancers diagnosed worldwide each year is a cancer of the female breast. Globally more than 700,000 women are diagnosed with breast cancer every year. In USA, approximately 182,460 new cases of invasive and 67,770 new cases of non-invasive (in situ) breast cancers were diagnosed in women. In India 70,000 new cases of breast cancer and 35,000 deaths due to this cancer are reported every year [1].

Polycyclic aromatic hydrocarbons (PAHs) are products formed by incomplete combustion of organic matter. Sources of PAHs include industrial and domestic oil furnaces, gasoline, and diesel engines. PAHs are widely distributed in our environment and are implicated in various types of cancer. Enzymatic activation of PAHs lead to the generation of active oxygen species such as peroxides and superoxide anion radicals, which induce oxidative stress in the form of lipid peroxidation. The PAH 7,12-dimethyl-benz[*a*]anthracene (DMBA) acts as a potent carcinogen by generating various reactive metabolic intermediates leading to oxidative stress.

Experimental investigations as well as clinical and epidemiological findings has provided evidence supporting the role of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anions ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) in the etiology of cancer. Reactive oxygen species (ROS) are seemingly involved in a variety of important pathophysiological conditions including mutagenesis and carcinogenesis [2]. Free radicals play an important role in tumor promotion by direct chemical reaction or alteration of cellular metabolic processes, and their scavengers (SOD, CAT, GPx) represent inhibitors at different stages of carcinogenesis [3]. Antioxidants may protect against the toxicity of reactive oxygen species (ROS) by the prevention of ROS formation. The analysis of antioxidant levels is considered essential as they neutralize oxygen-free radicals. Development of life threatening diseases like cancer is linked to the availability of these antioxidants. For all these reasons, it is important to develop a new strategy possessing anti-neoplastic and free radical scavenging properties. Therefore, it is appropriate to investigate various phytotherapeutic origins to detect anti-tumor and free radical scavenging activities. The available semisynthetic anticancer drugs have more side effects and are cytotoxic to human beings. Since modern medicine has no effective cure for the malignant cancers and tumors, scientists are interested in finding a potent phytotherapeutic agent with non-cytotoxic properties.

Various molecules can inhibit the formation of free radicals associated with carcinogenesis. Bioactive compounds from plant origin have the potential to subside the biochemical imbalances induced by various toxins associated with free radicals. They provide protection without causing any side effects and therefore, development of drugs from plant products is desired. Many plant extracts and plant products have been identified as good protectors against the free radicals by triggering antioxidant gene expression.

Holoptelea integrifolia, Planch. belongs to the family of Urticaceae. It is an important pollen allergen of India and sensitizes almost 10% of the atopic population in Delhi [4]. Some recent explorations have been reported on this plant in which antiviral activity [5], antioxidant, antimicrobial and wound healing activities [6] are important. Ethnomedically, the leaves and stem bark of this plant were used by local people for skin diseases, obesity, cancer [7] and for wound healing in the form of paste. The fresh material, stem bark of the plant, is applied as paste externally for cancer [8].

Considering the potential antitumour/antioxidant activity of phytochemicals, the present study was carried out to investigate the breast cancer prevention property of *H.integrifolia* against DMBA induced breast cancer in experimental rats.

MATERIALS AND METHODS

Chemicals

7,12-dimethyl benz(a)anthracene (DMBA) was purchased from Sigma chemical company, and all other chemicals including solvents were of high purity and of analytical grade marketed by SRL Ltd., India and tamoxifen from Ranbaxy, India.

Plant material and extraction

The bark of *Holoptelea integrifolia* Planch. were collected from Tirunelveli, Tamil Nadu, India and identified and authenticated by the Dr.V.Chelladurai, Research officer-Botany. The air-dried and coarsely powdered bark (400 g) were extracted successively with 1.5 L each of petroleum ether (60-80°C) and ethanol in a Soxhlet extractor for 72 hours. The extracts were concentrated to dryness under reduced pressure and controlled temperature (40-50°C).The petroleum ether extract yielded a yellowish brown sticky semisolid,

weighing 2 g (2%). The ethanol extracts yielded brown and semi-solid residues, weighing 10 g (10%).

Animals

Thirty Female Sprague-Dawley rats, 30 days of age, and 170-200 g body weight were procured from National Institute of Nutrition (NIN), Hyderabad, India and housed in standard isolation cages (45×35×25 cm) under environmentally controlled conditions with 12-hour light/12-hour dark cycle. They were allowed free access to water, standard laboratory chow (Hindustan Liver Pvt. Ltd, Mumbai) given food and water *ad libitum*. After a sufficient period of acclimatization (2 weeks), they were used to evaluate breast cancer activity. The experimental protocol was approved by CPCSEA and approval number being IAEC/118/2010.

Tumour induction

Tumour induction was carried out by following the method of Barros *et al*., 2004 [9]. DMBA was used as a carcinogen for the present investigation. Mammary tumour was induced to 45 days old rats by a single dose of 20 mg of DMBA dissolved in corn oil (1 ml) which was given orally using oral feeding needle. 120 days after DMBA administration, all the experimental animals were sacrificed.

Experimental design

A total of 30 Female Sprague-Dawley rats were divided into 5 groups of 6 rats each.

Group I: Animals were administered with normal saline.

Group II: Animals received a single dose of DMBA given orally.

Group III: Animals received a single dose of DMBA followed by *H.integrifolia* extract (250 mg/kg body weight) given for 120 days orally.

Group IV: Animals received a single dose of DMBA followed by *H.integrifolia* extract (500 mg/kg body weight) given for 120 days orally.

Group V : Animals received a single dose of DMBA followed by a standard drug tamoxifen (10 mg/kg body weight/day) which is dissolved in water given for 120 days orally.

After the experimental period of 120 days, oxidative stress parameters were assessed in serum of both control and experimental groups. The rats were anesthetized and sacrificed. Animals were starved overnight before sacrifice. Blood was collected and the serum was separated by centrifugation. Supernatant collected was used for the determination of non-enzymatic antioxidants Reduced glutathione (GSH) and enzymatic antioxidants such as Catalase (CAT), Superoxide dismutase (SOD), Glutathione peroxidase (GPx). In addition to this, body weight of animals, tumour burden, tumour multiplicity, tumour volume of breast were also assessed.

Tumour Burden

The tumour burden was calculated by multiplying the mean tumour volume ($4/3\ \pi r^3$) (r = 1/2 tumour diameter in mm) with the mean number of tumours.

Tumour Multiplicity

Tumor multiplicity was calculated as the average number of total tumors per rat in the group.

Tumour Volume

The volume of tumor was calculated by using the formula, $V = 4/3\pi r^3$, Where 'r' was the mean of r_1 and r_2 which are two independent radii of the tumor mass.

Biochemical analysis

Superoxide dismutase (SOD) activity was measured at absorbance 420 nm using a spectrophotometer as the degree of inhibition of autoxidation of pyrogallol in an alkaline pH according to the method of Marklund and Marklund, 1974 [10]. The catalase (CAT) activity was assayed by the method of Sinha, 1972 [11]. In this method, dichromate in acetic acid was reduced to chromic acetate when heated in the presence of hydrogen peroxide (H_2O_2) with the formation of perchloric acid as unstable intermediate and chromic acetate thus formed was measured spectrophotometrically at 570 nm. The results were expressed in terms of μmol H_2O_2 liberated/min/mg protein. The activity of glutathione peroxidase (GPx) was assayed by the method of Rotruck *et al*., 1973 [12]. The reaction mixture containing 0.2 ml of EDTA (0.8 mM, pH 7.0), 0.4 ml of phosphate buffer (10 mM), and 0.2 ml of enzyme source were incubated with 0.1M of H_2O_2 and 0.2 ml of glutathione for 10 min. Oxidation of glutathione by the enzyme was measured spectrophotometrically at 420 nm. The activity of GPx was expressed as μmol glutathione oxidized/min/mg protein. Reduced glutathione was determined by the method of Moron *et al*., 1979 [13]. The sample (1.0 ml) was precipitated with 1.0 ml of TCA and centrifuged at 1200×*g* for 20 min. To 0.5 ml of supernatant 2.0 ml of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was added and the colour developed was read immediately at 412 nm using a spectrophotometer.

Statistical analysis

The experimental results were expressed as the MEAN ± SEM. Data were assessed by the method of one-way ANOVA followed by Dunnet's test and were computed for statistical analysis by using Graph Pad Software (San Diego, CA).

RESULTS

Results of the preliminary phytochemical analysis, carried out on the crude ethanol extract, indicate the presence of alkaloids, glycosides, sterols, flavonoids, terpenoids, tannins and saponins. Table 1 presents the body weight and tumour parameters of control and experimental animals. The body weight was found to be significantly decreased in Group II tumour induced animals. Conversely, the administration of EEHI increased the body weight in Group III and Group IV ($P < 0.05$) animals when compared to Group II animals. The standard drug tamoxifen also increased the body weight significantly when compared to group II animals ($P < 0.05$). The tumour burden and tumour volume was found to be increased in Group II animals. The administration of EEHI decreased the tumour parameters in Group III and IV animals when compared to Group II animals ($P < 0.05$). The standard drug tamoxifen also decreased the tumour parameters significantly when compared to group II animals ($P < 0.05$). Administration of EEHI decreased the tumour parameters significantly ($P < 0.05$). The tumour multiplicity was found to be 1 per group.

Table 1: Table shows the effect of EEHI on body weight and tumour parameters of control and experimental animals

Groups	Body weight (g)	Tumour Burden (mm^3)	Tumour volume (mm^3)
I	182.3 ± 2.65	0	0
II	130.5 ± 2.29	8797.45 ± 162.10	8797.45 ± 162.10
III	142.0 ± 1.57 *	2635.59 ± 53.60 ***	2635.59 ± 53.60 ***
IV	151.2 ± 2.41***	1742.19 ± 56.82***	1742.19 ± 56.82***
V	170.7 ± 3.15***	721.61 ± 7.55***	721.61 ± 7.55***

Values are expressed in terms of mean ± SEM, n=6 in each group. Value comparisons were made between Negative control Vs Group III, IV, V ($p < 0.05$). *** - value is highly significant, * - value is less significant.

Serum of Group II cancer-bearing animals shows a significant decrease in SOD and GPx levels. However, the enzymatic levels were increased significantly in Group III, IV and Group V animals when compared with Group II animals (Table 2). Treatment of rats with EEHI in Group III and IV ($P < 0.01$) animals significantly increased the antioxidant levels when compared to Group II animals. Administration of tamoxifen in Group V animals significantly ($P < 0.01$) increased the antioxidant levels to normal level when compared to Group II animals.

Table 2: Table shows the effect of EEHI on SOD and GPx levels

Groups	SOD(Units/min/mg protein)	GPx (µmol glutathione oxidized/min/mg protein)
I	12.03 ± 0.14	9.68 ± 0.11
II	7.46 ± 0.18	7.03 ± 0.19
III	8.12 ± 0.10 **	7.65 ± 0.13*
IV	9.20 ± 0.18***	8.15 ± 0.09***
V	10.88 ±0.14***	8.93 ± 0.09***

Values are expressed in terms of mean ± SEM, n=6 in each group. Value comparisons were made between Negative control Vs Group III, IV, V ($p < 0.01$). *** - value is highly significant, * - value is less significant.

Table 3 presents the activity of CAT and GSH levels. Serum of Group II cancer-bearing animals shows a significant decrease in CAT and GSH levels. However, the levels were increased significantly in Group III, IV and Group V animals when compared with Group II animals. Administration of EEHI in Group III and IV ($P < 0.05$) animals significantly increased the antioxidant levels when compared to Group II animals. Administration of tamoxifen in Group V animals significantly ($P < 0.05$) increased the enzymatic levels to normal level when compared to Group II animals.

Table 3: Table shows the effect of EEHI on CAT and GSH levels

Groups	CAT(µmol H_2O_2 consumed/min/mg protein)	GSH(µg/mg protein)
I	63.57 ± 1.61	11.15 ± 0.30
II	35.47 ± 0.91	6.85 ± 0.15
III	40.12 ± 0.82 *	7.65 ± 0.08*
IV	48.85 ±0.52***	8.23 ± 0.14***
V	58.73 ±1.09***	9.80 ± 0.20***

Values are expressed in terms of mean ± SEM, n=6 in each group. Value comparisons were made between Negative control Vs Group III, IV, V ($p < 0.05$). *** - value is highly significant, * - value is less significant.

DISCUSSION

Animal experimental systems are particularly useful for the study of human mammary carcinogenesis. Since the rat mammary gland shows a high susceptibility to develop neoplasms which closely mimic human breast cancer, they have been selected in comparison to other animal models. DMBA-induced mammary gland tumor in rodent has been widely used as an animal model for development of chemopreventive drugs for breast cancer in humans. The mammary gland of the rat undergoes extensive development after 32–35 days of age, first appearing as terminal end buds that subsequently evolve into alveolar buds and eventually into terminal ducts; this occurs at 40–60 days of age. Under normal conditions, DMBA is most effective in producing tumors during the most active transition period of terminal end bud evolution into alveolar buds. The greater incidence and tumor yield in the DMBA-treated animals occurred because transition in the mammary tissue was occurring in the normal window of DMBA sensitivity. Such a mechanistic proposition has been put forth by Anderson et al., 2000 [14], who studied the effect of constant light on DMBA-induced mammary tumorigenesis in rats.

One mechanism of DMBA carcinogenesis is that DMBA is metabolized through an oxidative process that damages DNA. DMBA is metabolized in the microsomes of liver in addition to the rat mammary gland. Toxic manifestation of DMBA is associated with its oxidative metabolism leading to the formation of reactive metabolites (epoxides and quinines) capable of generating free radicals. Metabolism of PAHs like 7,12-dimethyl benz(*a*)anthracene by the mixed function oxidases system (MFO) often results in the formation of oxyradicals "O_2 •–, $^1O_2$, H_2O_2, •OH," which bind covalently to nucleophillic sites on cellular macromolecules thereby eliciting cancerous responses. The generation of ROS and the peroxidation of membrane lipids are well associated with the initiation of carcinogenesis affecting the normal biochemical process, which further leads to the reduction of body weight [15]. Oxidative stress induced due to the generation of free radicals and/or decreased antioxidant level in the target cells and tissues has been suggested to play an important role in carcinogenesis. Increased incidence of oxidative stress and lipid peroxidation are implicated in carcinogenic processes [16]. MDA is a low-molecular weight aldehyde that can be produced from free radical attack on polyunsaturated fatty acids. Hence, it is of interest to assess MDA as a marker of oxidative stress and the role played by lipid peroxidation and the modulation of antioxidants during the progression of breast cancer. It is evident from the results that decreased levels of antioxidants was found in cancer-bearing animals when compared to control group. On the contrary, increased levels of enzymes were observed in EEHI (Ethanolic Extract of bark of *Holoptelea integrifolia*) treated animals indicating that it is a good free radical scavenger.

Antioxidants act as the primary line of defence against ROS and suggest their usefulness in estimating the risk of oxidative damage induced during carcinogenesis. During cell membrane damage, various enzymes leak down to the circulatory fluid and their assessment in serum serves as markers in clinical studies. Even in healthy animals enzyme activities are present in serum because of physiological wear and replacement of body cells. The enzyme activity rises in the serum more than the normal level, which indicates serious tissue damage. SOD is the first antioxidant enzyme to deal with oxyradicals by accelerating the dismutation of superoxide (O_2-) to hydrogen peroxide (H_2O_2) CAT is a peroxisomal haem protein that catalyses the removal of H_2O_2 formed during the reaction catalysed by SOD. Thus, SOD and CAT acts mutually supportive antioxidative enzymes, which provide protective defence against reactive oxygen species [17]. Increased level of superoxide radicals in tumour cells decreases the antioxidant activity when compare to normal cells. The present study reveals that SOD levels are decreased in the cancer-bearing animal, which may be due to altered antioxidant status caused by carcinogenesis. Decreased level of CAT activity was measured in patients with breast cancer and benign breast disease conditions [18]. Our study also shows that decreased level of CAT observed in Group II cancer-bearing animals may be due to the utilization of antioxidant enzymes in the removal of H_2O_2 by DMBA. GPx is an important defence enzyme against oxidative damage and this in turn requires glutathione as a cofactor. GPx catalyses the oxidation of GSH to GSSG at the expense of H_2O_2. Decreased GPx activity was also observed in cancerous conditions [19]. Our findings agree well with this observation and also the activity of GPx significantly decreased in cancer-bearing animals.

The non-enzymic antioxidant systems are the second line of defence against free radical damage. GSH is an important non-protein cellular thiol that in conjunction with GPx plays a regulatory role in cell proliferation. GSH and GSH dependent enzymes are involved in scavenging the electrophilic moieties involved in the cancer initiation. Also observed decreased activity of GSH in cancer-bearing animals. GSH serves as a marker for evaluation of oxidative stress and it acts as an antioxidant at both extra cellular and intracellular levels. The EEHI increased the GSH levels, which clearly suggest their antioxidant property. Ascorbic acid is a good scavenger of free radicals and it protects cellular membranes thereby preventing degenerative disease like cancer [20]. Decreased levels of water soluble antioxidants found in cancer-bearing animal may be due to the utilization of antioxidants to scavenge the free radicals.

Tamoxifen is the effective standard drug in preventing the occurrence of rat breast cancer chemically induced by DMBA**.** The phenomenon that tamoxifen can reduce the occurrence of breast cancer of rats coincides with the rule that tamoxifen prevents human breast atypical hyperplasia from developing breast cancer [21].

Flavonoids and catechins were first shown to be apoptotic in human carcinoma cells [22]. Similar observation has since been extended to lung tumor cell lines, colon cancer cells, breast cancer cells, prostate cancer cells stomach cancer cells, brain tumor cells, head and neck squamous carcinoma and cervical cancer cells. Genistein, quercetin, rutin, and other food flavonoids have been shown to inhibit carcinogenesis in animal models [23]. They all induce apoptosis in tumor cells. It appears that these flavonoids can also differentially induce apoptosis in cancer cells, but not in their normal counterparts. It has been proposed that flavonoids terminate chain

radical reactions by donating hydrogen atoms to the peroxy radical forming a flavonoid radical which in turn reacts with free radicals thus terminating the propagating chain.

Thus, it is suggested that the EEHI significantly attenuated the SOD activity and resulted in increased antioxidant levels to normal levels. All these data point to the possibility of developing an ethanolic extract of bark of *Holoptelea integrifolia* as a novel, potential agent in the area of cancer chemotherapy. The phytochemical study indicated the presence of flavonoids, alkaloids and terpenoids in EEHI. Further, flavonoids have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis [24]. Thus, antitumour effects produced by the EEHI may be due to flavonoids as well as its antioxidant potential. The ethanolic extract of *Holoptelea integrifolia* decreases tumour burden, tumour multiplicity, tumour volume of breast. Our study suggests that EEHI possess potent breast cancer prevention activity.

In conclusion, the present study demonstrates that EEHI exert inhibitory effects on DMBA-induced Breast Carcinoma that may attributed to phytochemicals with anticarcinogenic properties. The biochemical alterations observed in cancer bearing animals in the present study may be due to the reduction of antioxidant level following carcinogen administration. However, administration of EEHI significantly reversed the alteration to near normal level in cancer-bearing animals. From the results it can be inferred that *Holoptelea integrifolia* positively modulated the antioxidant activity by quenching and detoxifying the free radicals induced by DMBA. The attenuation of DMBA induced oxidative stress by the plant extract could be attributed to the antioxidants activity of triterpenes, alkaloids and flavonoids of *H. integrifolia* plant, which is known to quench the free radicals by maintaining antioxidants levels. Considering the antioxidant property of *H. integrifolia* the bioactive compounds derived from this plant can be supplemented with anticancer medicines and thus additional studies are underway to isolate and characterize the plant active ingredients that contributes to its preventive effects.

ACKNOWLEDGEMENTS

The author thanks to the Department of Pharmacology, SRM University, Tamil Nadu for providing facilities and grants necessary to carry out the work.

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