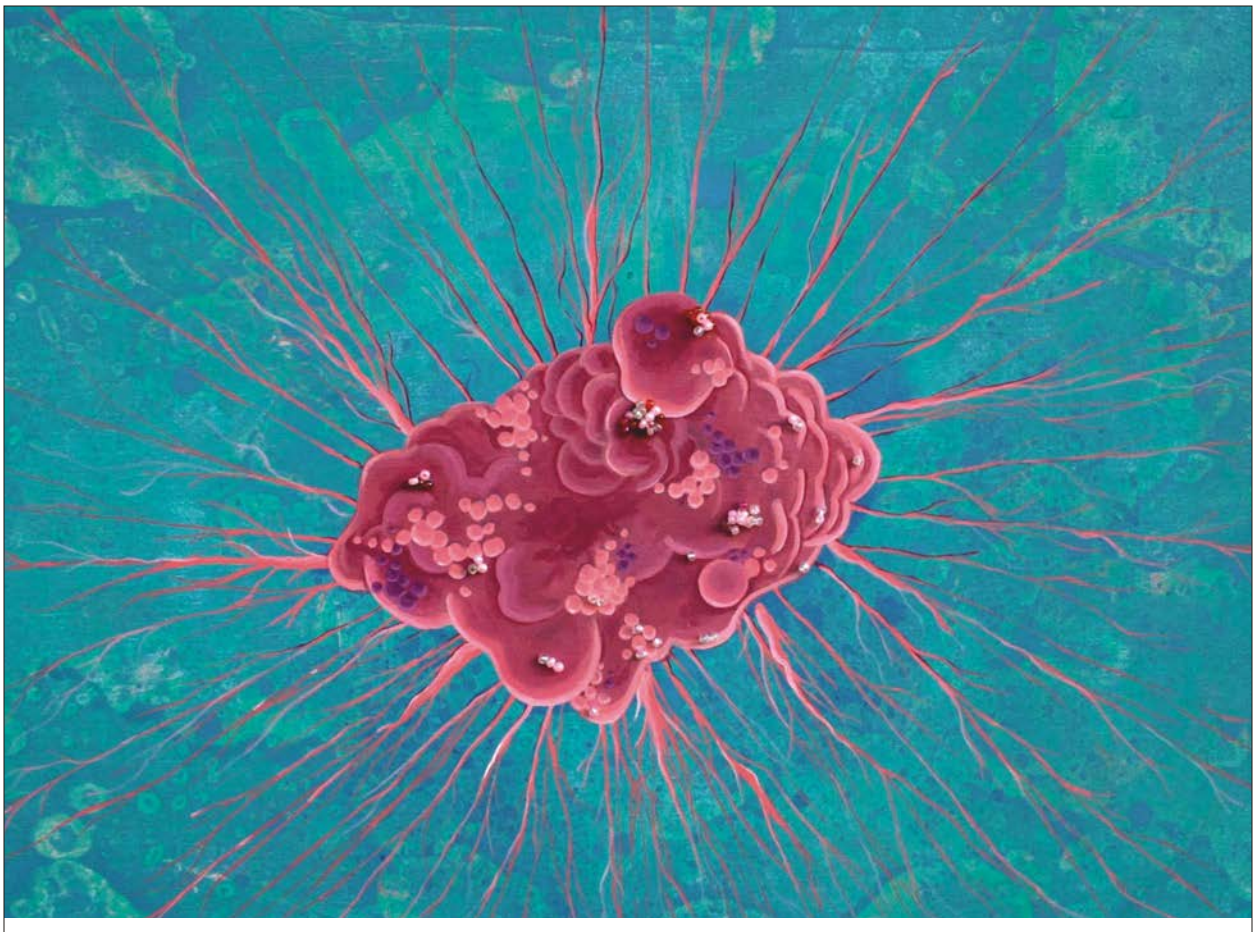

International Journal of Cancer Research

ISSN 1811-9727

Academic Journals Inc.

www.academicjournals.com

International Journal of Cancer Research 11 (1): 19-31, 2015
ISSN 1811-9727 / DOI: 10.3923/ijcr.2015.19.31

Gastric Cancer Protective Effect of Mangrove Oil, Derived from *Rhizophora apiculata* on Benzo(a)Pyrene Induced Cancer in Albino Mice

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ABSTRACT

Rhizophora apiculata, a mangrove specie is used in traditional medicine against several human diseases including tumor. To validate the ethnopharmacological claims against cancer, we examined the effects of *Rhizophora apiculata* leaf oil on the anti gastric cancer activities of *Benzo(a)pyrene* (BaP) induced gastric cancer in albino mice. The animals were divided into five groups. Group I were given BaP (40 mg kg^{-1} b.wt.) with 100 μL sesame oil by oral gavage twice a week for 4 consecutive weeks. Group II were given 100 μL sesame oil (SMO) treated (Vehicle treated control) a week for 4 consecutive weeks. Group III mice (BaP) (40 mg kg^{-1} b.wt.)+*R. apiculata* (50 μL) for 14 weeks. Group IV only *R. apiculata* (50 μL kg^{-1} b.wt.) dialy for two weeks. Goup V as the control non treated. At end of 14 weeks, all the animal were killed. Tumor incidence was observed to be 100% in mice that received only BaP. However, treatment with *R. apiculata* oil reduced the tumor incidence as observed in mice of BaP+*R. apiculata* oil group when compared to that of BaP group. Similarly, the tumor burden and body weight were seen to decrease by 137.21, respectively in mice of BaP+*R. apiculata* oil group when compared to those of BaP group. Diminished lipid peroxidation in the stomach tumor tissue was associated with enhanced antioxidant levels. In contrast to tumor tissue, enhanced lipid peroxidation with compromised antioxidant defences was found in the liver and erythrocytes of tumor bearing animals. Administration of *R. apiculata* oil significantly reduced the incidence of stomach tumors, modulated lipid peroxidation and enhanced antioxidant status in the stomach, liver and blood. From the results of our study, we suggest that *R. apiculata* oil may exert its chemopreventive effects by modulating lipid peroxidation and enhancing the antioxidant status in the stomach, liver and erythrocytes.

Key words: Gastric cancer, *R. apiculata*, mangrove oil, albino mice, Benzo(a)pyrene

INTRODUCTION

Gastric cancer is the second most common cause of cancer death worldwide. It is a major cause of mortality in Chennai, India. The development of stomach cancer is associated with sustained genetic mutations that leads to excessive cell proliferation, dysregulation of cellular differentiation,

evasion of apoptosis as well as sustained angiogenesis (Crew and Neugut, 2006). The gastric epithelium is exposed to toxic Reactive Oxygen Species (ROS) within the gastric lumen due to ingested food, cigarette smoke and inflammation due to the *Helicobacter pylori* infection. The dynamic balance between cell proliferation and apoptosis is essential for maintaining mucosal homeostasis. Decreased apoptosis with increased proliferation may favor the carcinogenic process. The prolonged survival of abnormal cells can support the accumulation of sequential genetic mutations, changes in gene expression profiles and protein structure and function which can result in tumor promotion (Smoot *et al*., 2000; Karam, 2008).

Cancer chemoprevention is a mean of cancer control by pharmacological intervention of the occurrence of the disease using chemical compounds. Recent events suggest that new emphasis in the development of medical treatment of human disease will be intimately connected to natural products. The use of medicinal plants in modern medicine for the prevention or treatment of cancer is an important aspect. For this reason, it is significant to identify anti-tumor-promoting agents present in medicinal plants commonly used by the human population which can inhibit the progression of tumor (Goyal *et al*., 2010).

Polycyclic Aromatic Hydrocarbons (PAHs) are environmental carcinogens present in the atmosphere from combustion sources such as diesel exhaust, cigarette smoke, residential heating processes, refuse burning, industrial coke production, volcanic eruption and oil contamination by effluents and oil spills (Mahadevan *et al*., 2005). They can also be generated by the pyrolysis of amino acids, fatty acids and carbohydrates during cooking process (Collins *et al*., 1998). After inhalation, ingestion is the second most important exposure route for PAHs to enter the human body (Van de Wiele *et al*., 2005). The BaP, a potent pro-carcinogen employed in initiating stomach cancer is the prototypical and best characterized member of PAHs family of chemical carcinogens (Singh *et al*., 1998). It has been shown to induce tumors of skin, lung, mammary glands and fore stomach tissues of various experimental animals (Athar *et al*., 1989). Since, BaP is an omnipresent environmental pollutant and is believed to be a risk factor for human chemical carcinogenesis, it is important to identify the naturally occurring/synthetic agents that could modulate BaP-induced tumorigenesis (Dasgupta *et al*., 2004).

Mangroves have been long used in folk medicine to treat diseases and very few mangrove plants are reported for possible source of anticancer drugs based on traditional knowledge and preliminary scientific study (Boopathy and Kathiresan, 2010). Mangrove, *Rhizophora apiculata* family of Rhizophoraceae is halophytic used as folk medicine based to on the fact that use of its root, leaf or stem extracts to a greater extent imparts an inhibitory effect on the growth of bacterial, viral and fungal pathogens (Premnathan *et al*., 1992; Antony *et al*., 2011). Polysaccharide extracted from the leaf of *R. apiculata* reported to inhibit HIV-1 or HIV-2 strains in various cell cultures (Premnathan *et al*., 1992). *Rhizophora apiculata* extract have a high content of flavanoids, tannins, catachin, anthroquinone, pyroligneous acid and syringol. Phytochemical analysis of methanolic extract of *R. apiculata* by GC/MS and LC/MS analysis shown the presence of pyrazole (alkaloid), ketone, thiazolidinediones and 4-pyrrolidinyl with a wide range of biological properties such as anti-tumor, anti-inflammatory, immunostimulatory and chemoprotectant activities (Prabhu and Guruvayoorappan, 2012a, b). Anti-metastasis activity of *R. apiculata* extract have not been reported elsewhere to provide validity to the claims that *R. apiculata* extract has numerous polyphenol compounds with potential health benefits. Anti-metastatic activity of methanolic extract of *R. apiculata* in B16F-10 melanoma cell induced lung metastasis

in C57BL/6 mice (Prabhu and Guruvayoorappan, 1833). Hence, in our present study, Benzo(a)pyrene induce anti gastric cancer effect of mangrove oil from *R. apiculata* leaf oil in albino mice.

MATERIALS AND METHODS

Animal care and handling: Random-bred Swiss albino mice (6-7 weeks old) were used for the present experiments. These animals were maintained in the animal house at room temperature of 24±3°C and 12 h light: 12 h dark cycle at Central Animal House, Department of Experimental Medicine, RMMC and H, Annamalai University. All mice were fed with standard pellet diet supplied by Hindustan Lever Ltd., Bangalore, India and water *ad libitum* for 1 week before the study. The study were carried out in accordance with Indian National law of animal care and use and committee for the purpose of control and supervision of animals at RMMCH (Reg.no.160/1999.CPCSEA) Annamalai University, Annamalai Nagar.

Chemical: Benzo(a)pyrene was procured from Sigma Chemical Company, St. Louis, MO, USA. The BaP-induced stomach tumorigenesis in mice was performed according to the method of (Sakagami *et al*., 1998) with minor modifications as suggested by Wattenberg (1981).

Collection of plant materials and oil extraction: The plant material of *Rhizophora apiculata* fresh leaves was collected from Ariyankuppam coastal region, Pondicherry, India and the collected plant material was botanically identified and confirmed by Herbaria of Centre of Advanced Study in Marine Biology, Annamalai University, Tamil Nadu, India.

Extraction of the essential oil: Essential oil was obtained from freshly harvested leaves 441.1 g by hydro distillation for 2 h in a Clevenger apparatus (Maison neuve, 1985). The oil was dried over anhydrous sodium sulfate and stored under nitrogen at 4°C.

Experimental protocol: The mice were divided into six groups of six mice each. The total duration of the experiment is 14 week, the details of groupings and feeding protocol are summarized as follows.

Preparation of drug and mode of administration:

- **Group I:** Carcinogen (BaP) treated (positive control) 1 mg of BaP in 100 μL sesame oil by oral gavage twice a week for 4 (Total eight administration)
- **Group II:** Sesame oil (SMO) treated (Vehicle treated control) twice a week for 4 consecutive weeks
- **Group III:** Carcinogen(BaP) (100 μL)+*R. apiculata* oil (50 μL kg^{-1} b.wt.) for 14 weeks
- **Group IV:** *Rhizophora apiculata* oil with the dose of 50 μL kg^{-1} day^{-1} b.wt. orally for 2 weeks)
- **Group V:** Sterile Tap Water (STW) treated (negative control) throughout the study period

The body weights were measured at the end of experiment. The experiment was terminated in the 14 week and all mice were killed after an overnight fast. Blood was collected and the plasma separated was used for analysis. The stomachs were excised to prepare a 10% homogenate for

biochemical measurements. Frozen gastric tissue was ground in liquid nitrogen and suspended in a homogenization buffer consisting of 50 mM Tris-HCl, pH 8.2, 1 mM EDTA, 0.1% Triton X-100 and proteinase inhibitor cocktail (Roche, Mannheim, Germany). After centrifugation in a micro centrifuge at 4°C, the supernatants were used to determine enzyme activity and protein concentration.

Preparation of tissue homogenate and erythrocyte lysate: The tissue samples after weighing were homogenized using appropriate buffer in a glass homogeniser with Teflon pestle. Blood samples were collected in heparinised tubes and the plasma was separated by centrifugation at 1000 g for 15 min. After centrifugation, the buffy coat was removed and the packed cells washed three times with physiological saline. The erythrocyte samples (0.5 mL) were lysed with 4.5 mL of hypotonic phosphate buffer with pH 7.4. The hemolysate was separated by centrifuging at 2500 g for 15 min at 2°C.

Biochemical methodology: Lipid peroxidation as evidenced by the formation of Thiobarbituric Acid Reactive Substances (TBARS) was assayed in tissues by the method described by Nagabhushan and Bhide (1987) in the plasma by the method of (Ohkawa *et al*., 1979) and in erythrocytes by the method of (Yagi, 1987). The pink coloured chromogen formed by the reaction of 2-thiobarbituric acid with the breakdown products of lipid peroxidation was read at 535 nm. Reduced glutathione (GSH) was determined by the method of (Buege and Aust, 1978) based on the development of a yellow colour when DTNB is added to compounds containing sulfhydryl groups. Glutathione Peroxidase (GPx) activity was assayed by the method of (Anderson, 1985) with modifications. A known amount of enzyme preparation was incubated with hydrogen peroxide in the presence of GSH for 10 min. The amount of hydrogen peroxide utilized was determined by estimating GSH content by the method of (Buege and Aust, 1978). The activity of Glutathione S-Transferase (GST) was determined as described by Rotruck *et al*. (1973) by following the increase in absorbance at 340 nm using CDNB as substrate. Glutathione Reductase (GR) activity was determined by the method of (Habig *et al*., 1974) using oxidized glutathione as substrate and FAD as cofactor.

The protein content was estimated by the method of (Lowry *et al*., 1951), plasma ascorbic acid was estimated by the method of (Omaye *et al*., 1979). This involves oxidation of ascorbic acid by copper followed by treatment with DNPH to form the derivative bis 2,4-dinitrophenylhydrazone that undergoes rearrangement to form a product with an absorption maximum at 520 nm. Plasma vitamin E was measured by the method of (Baker *et al*., 1980) on the basis of the reduction of ferric ions to ferrous ions by-tocopherol and the formation of a red coloured complex with 2,2'-dipyridyl at 520 nm. Haemoglobin in erythrocytes and hemolysate was measured according to the method of (Drabkin and Austin, 1932). Blood was diluted in an alkaline medium containing potassium cyanide and potassium ferricyanide. Haemoglobin oxidized to methemoglobin combines with cyanide to form cyanmethemoglobin which was measured at 540 nm.

Statistical analysis: Statistical analysis on the incidence of lesions was performed using Fisher's exact probability test. The body weight and biochemical parameters were analysed using ANOVA and the group means were compared by Duncan's Multiple Range Test (DMRT). The results were considered statistically significant if the $p<0.05$.

RESULTS

The incidence of gastric tumors in group 1 was 91.4% (6/7 animals) with a mean tumor burden of 137.21 mm^3. Although, no tumors were observed in groups 3, 4 and 5 animals, in group 2 had small multiple nodules. Figure 1 illustrate the effect of treatment with mangrove

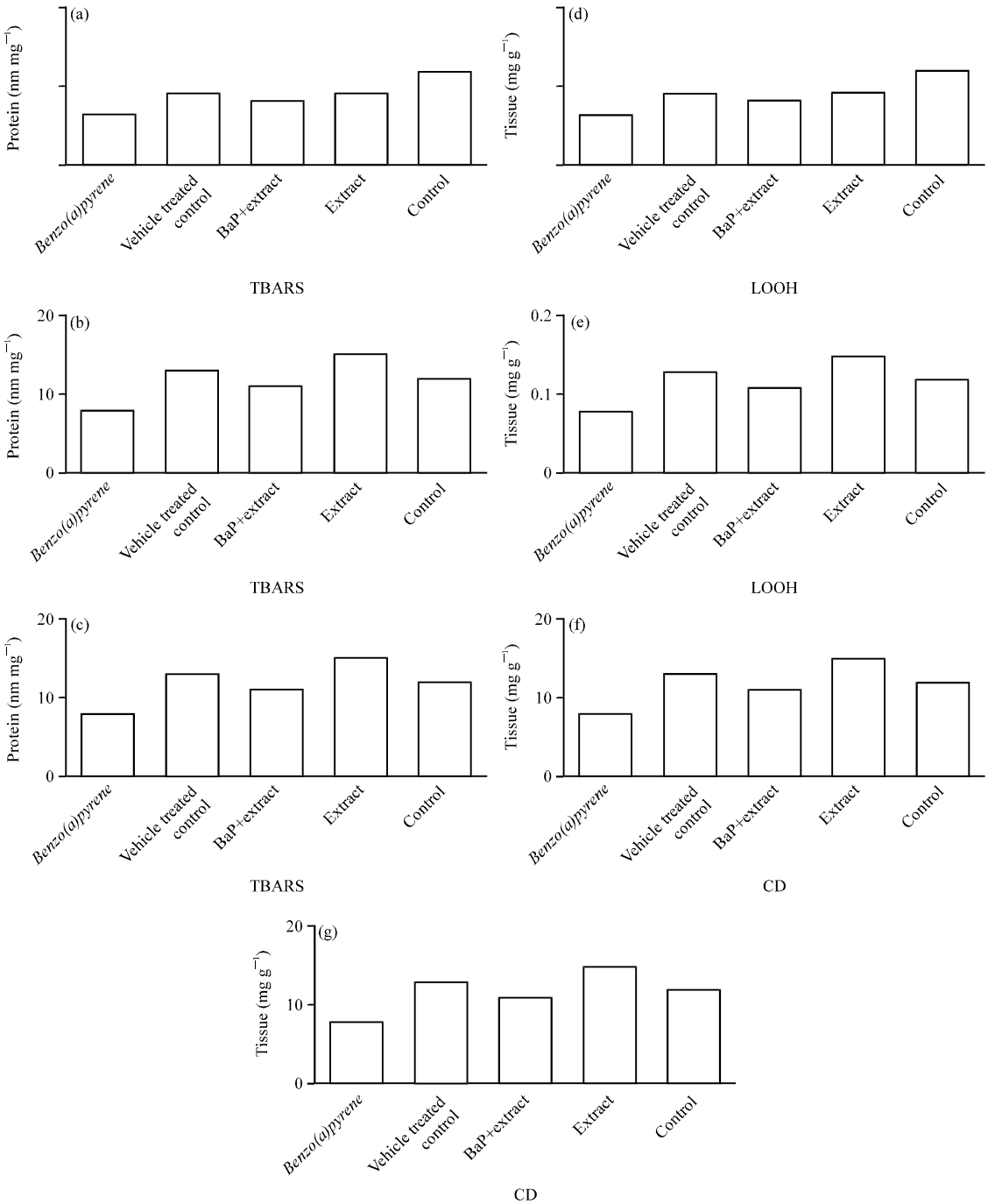

Fig. 1(a-g): Effects of *R. apiculata* oil on the levels of TBARS in (a) Stomach, (b) Liver and (c) Erythrocytes, levels of lipid hydroperoxides:(LOOH) in (d) Stomach and (e) Liver, and levels of conjugated dienes in (f) Stomach and (g) Liver, (Mean±SD, n = 7)

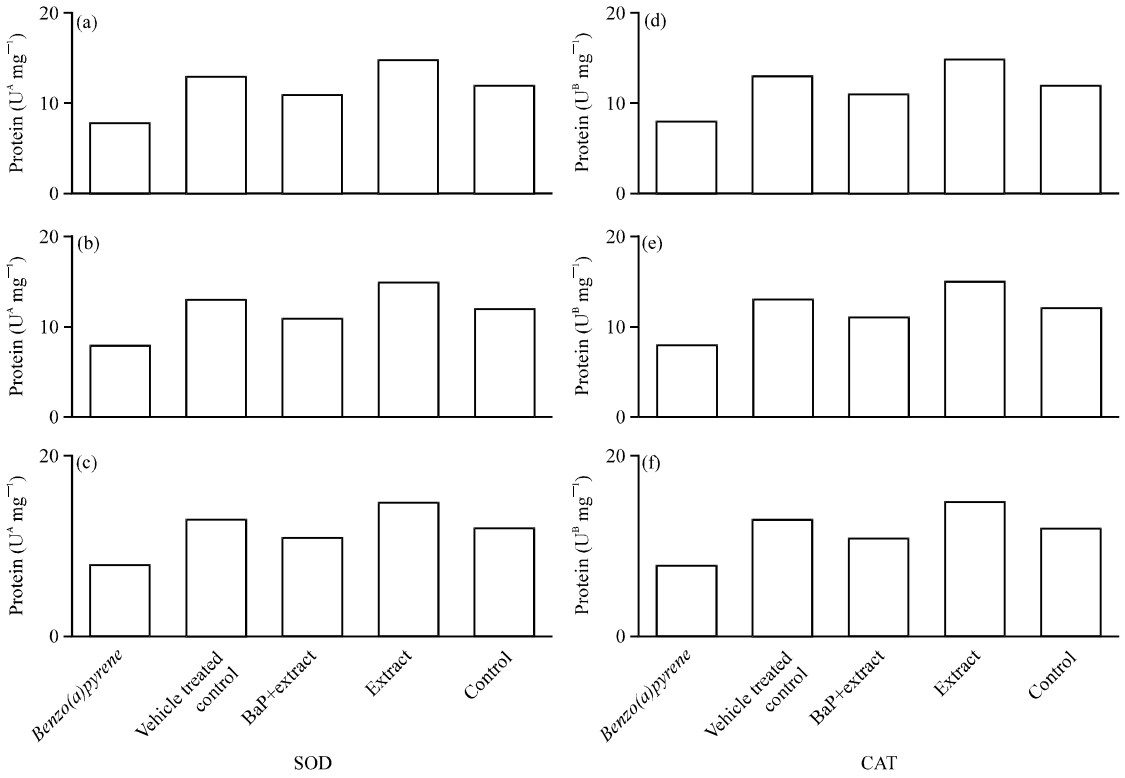

Fig. 2(a-f): Influence of treatment with *R. apiculata* oil on SOD and CAT activity in (a, d) Stomach, (b, e) Liver and (c, f) Erythrocytes, (Mean±SD, n = 7), A: Amount of enzyme required to give 50% inhibiton of NBT reduction and B: Moles of H_2O_2 utilised/sec

leaf oil on BaP-induced lipid peroxidation as evidenced by the formation of TBARS, lipid hydroperoxides and conjugated dienes in the stomach, liver and erythrocytes of experimental and control animals. Administration of BaP significantly lowered the extent of lipid peroxidation in the stomach of group 1 mice compared to control (group 5). Treatment with 50 μL mg^{-1} kg^{-1} b.wt. *Rhizophora apiculata* leaf oil significantly increased lipid peroxidation levels in group 3 animals as compared to group 1. In contrast to diminished lipid peroxidation in the stomach, the extent of lipid peroxidation in the liver and erythrocytes was significantly increased by BaP (group 1) compared to group 5. Treatment with *R. apiculata* leaf oil significantly reduced BaP-induced lipid peroxidation in group 3 animals compared to group 1. Administration of *R. apiculata* leaf oil alone (group 4) significantly reduced the extent of lipid peroxidation in the stomach, liver and erythrocytes compared to control. The influence of treatment with *R. apiculata* leaf oil on the antioxidant profile in the stomach, liver and erythrocytes are shown in Fig. 2-6. The concentrations of GSH and GSSG, GSH/GSSG ratio and the activities of SOD, CAT, GPx and GST were significantly increased in the stomach, whereas in the liver and erythrocytes all the antioxidants were significantly decreased in BaP treated animals compared to control (group 5). Treatment with *R. apiculata* leaf oil significantly increased all the antioxidants in group 3 animals compared to group 1. Administration of *R. apiculata* leaf oil alone (group 4) significantly enhanced the antioxidant status compared to group 1.

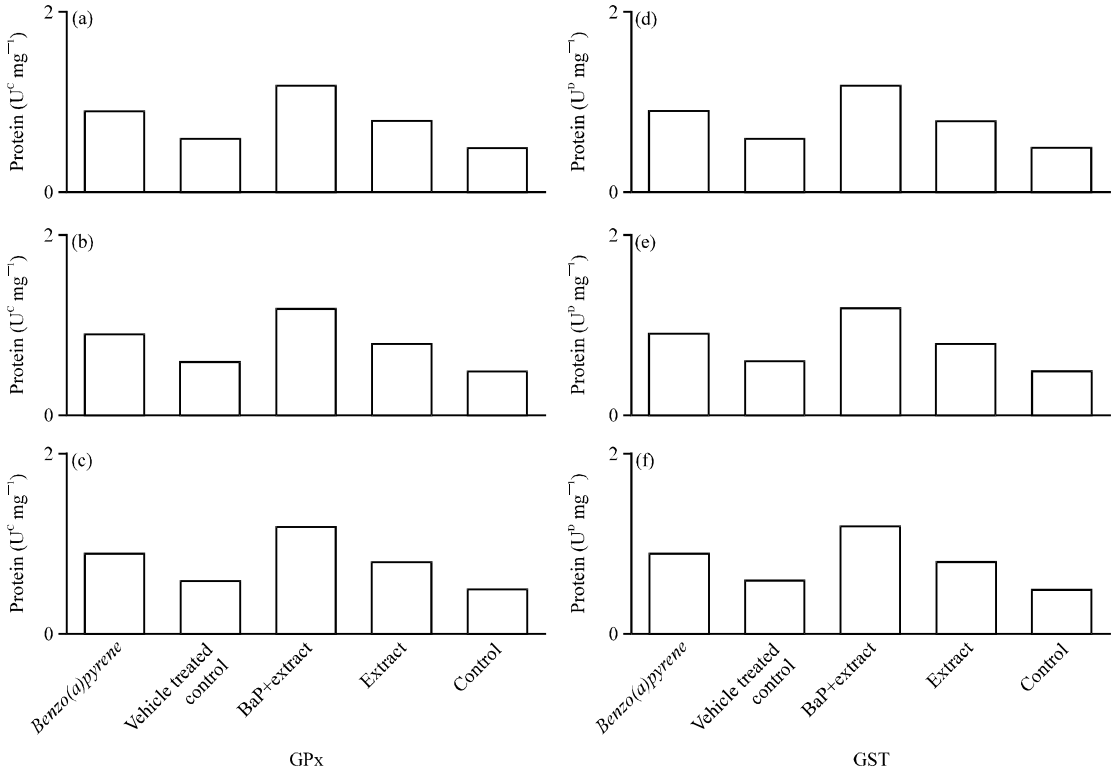

Fig. 3(a-f): Influence of treatment with *R. apiculata* oil on GPx and GST activities in the (a, d) Stomach, (b, e) Liver and (c, f) Erythrocytes. (Mean±SD, n = 7), C: Moles of GSH utilised/min and D: Moles of CDNB-GSH conjugate formed/min

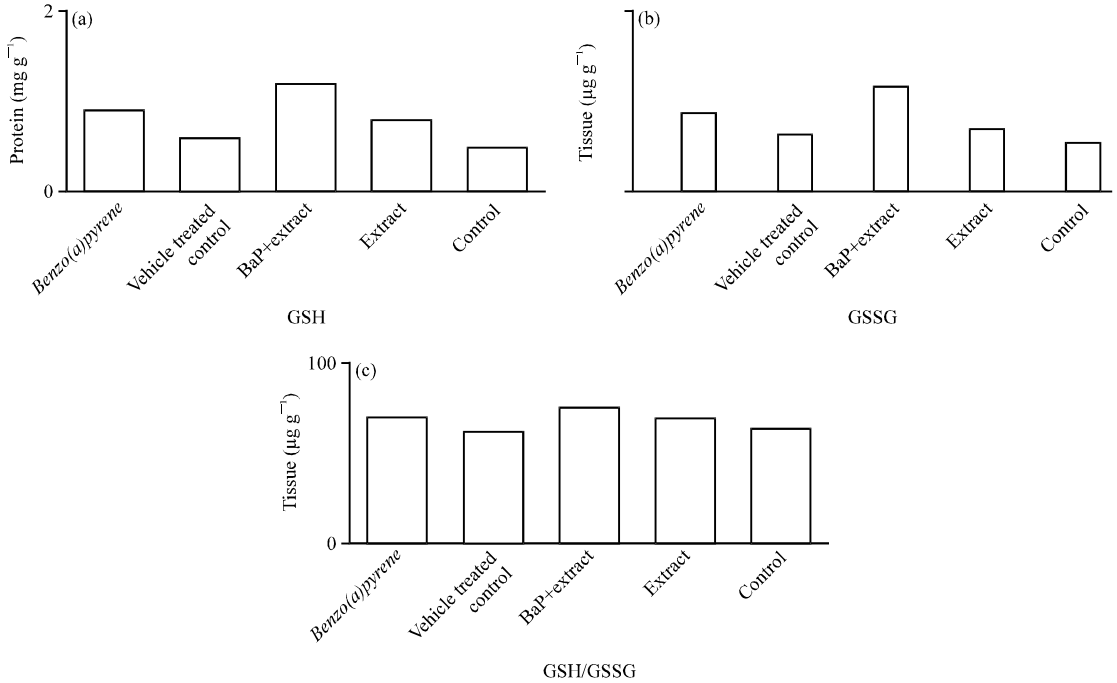

Fig. 4(a-c): Influence of treatment with *R. apiculata* oil on concentrations of (a) GSH, (b) GSSG and (c) GSH/GSSG ratio in the stomach (Mean±SD, n = 7)

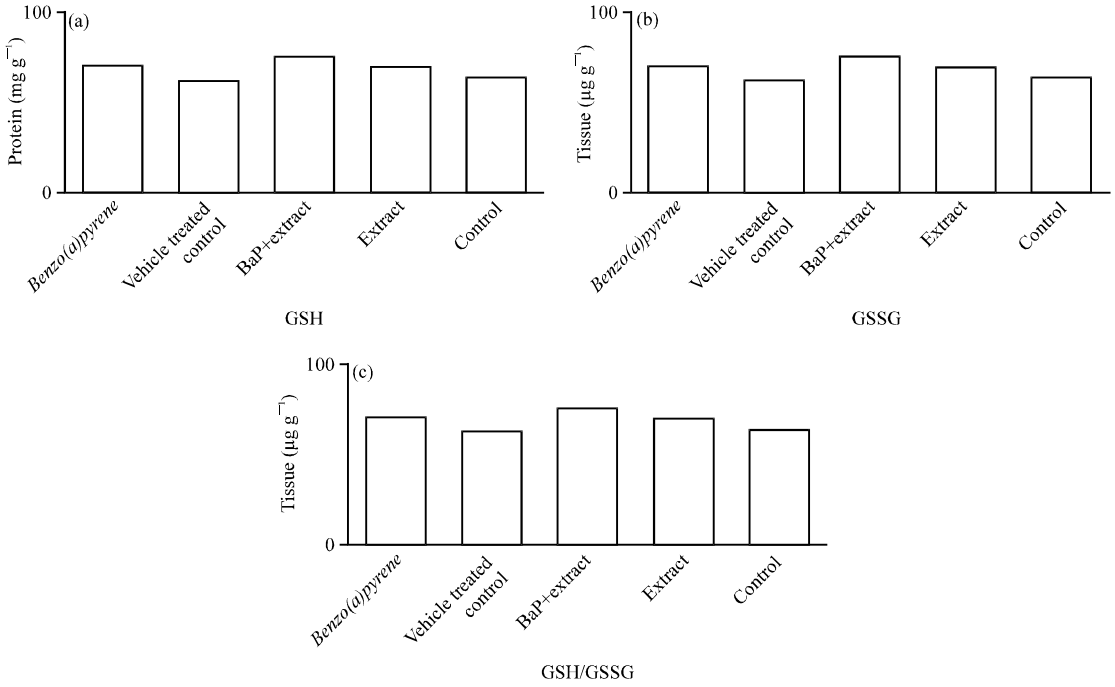

Fig. 5(a-c): Influence of treatment with *B. cylindrica* oil on concentrations of (a) GSH, (b) GSSG and (c) GSH/GSSG ratio in the liver (Mean±SD, n = 7)

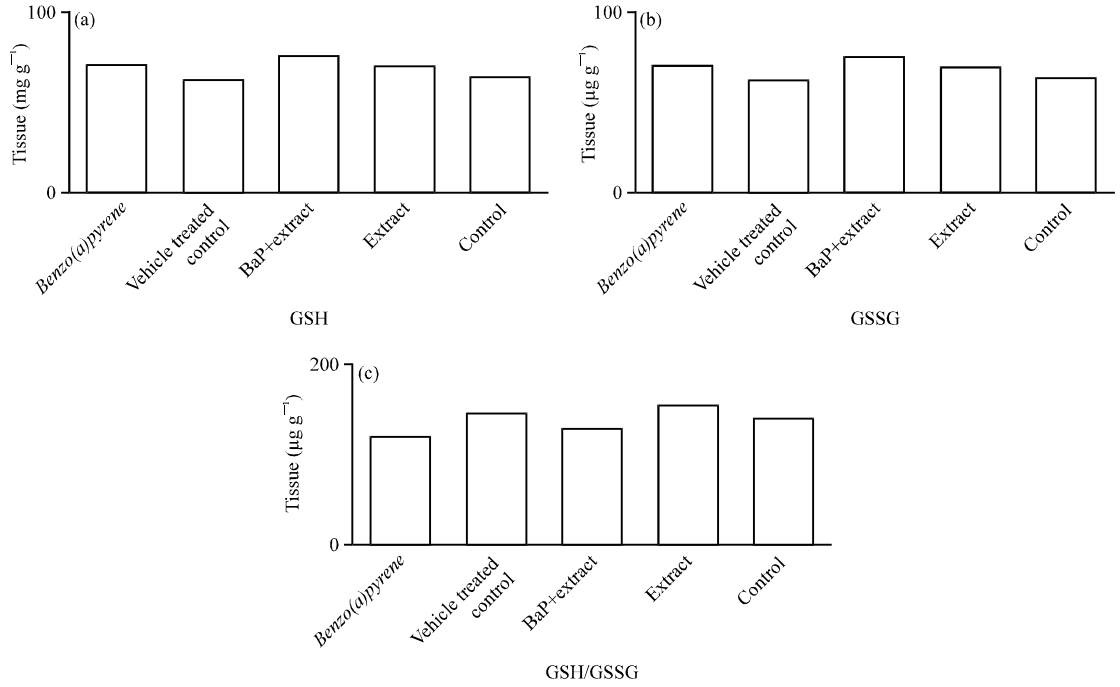

Fig. 6(a-c): Influence of treatment with *B. cylindrica* oil on concentrations of (a) GSH, (b) GSSG and (c) GSH/GSSG ratio in the erythrocytes (Mean±SD, n = 7)

DISCUSSION

The BaP an extremely potent pro-carcinogen is metabolized by biotransformation enzymes to a variety of metabolites that are responsible for initiating carcinogenesis (Choi *et al*., 1994). Biotransformation enzymes have broadly been divided into two categories namely phase-I and II. The former constitutes cytochrome P-450 based mono-oxygenase system which is responsible for initiating conversion of procarcinogens to several of their metabolites including ultimate carcinogens. Glutathione-S-Transferase (GST) is a major phase II detoxifying enzyme that primarily functions in catalyzing the active carcinogenic metabolites. To endogenous ligand-reduced glutathione (GSH) favoring their elimination from the body of the organisms (Hartman and Shankelm, 1990). The balance between the phase-I carcinogen activating enzymes and the phase-II detoxifying enzymesis critical to determining an individual's risk for cancer (Wilkinson and Clapper, 1997). There is substantial evidence that chemopreventive agents including medicinal plants exert their anti-carcinogenic effects by modulation of phase-I and II xenobiotic biotransformation enzymes (Wilkinson and Clapper, 1997).

In the present study, the exposure of mice to the carcinogen BaP caused high incidence of fore stomach tumors while the sesame oil treatment did not induce any tumor in the recipient animals. In BaP alone treated group tumor multiplicity, tumor incidence, tumor burden, tumor yield as well as cumulative number of papillomas was found to be quite high in comparison to *R. apiculata* leaf oil+BaP treated group (Experimental). The results of the present investigation are also supported by the others (Subapriya *et al*., 2005; Deshpande *et al*., 1997; Agha *et al*., 2001) who have used the different plant extracts to reduce chemical induced carcinogenesis in there finding.

The decreased susceptibility of stomach tumors to lipid peroxidation seen in the present study may be attributed to enhanced antioxidant capacities. Increased generation of ROS such as O_2 and H_2O_2 is recognized to induce SOD, CAT and GPx. Higher activities of antioxidant enzymes have been observed in malignant tumors compared to controls (Kumaraguruparan *et al*., 2002). In particular, synthesis of GSH which has a central role in the maintenance of the cellular redox status was found to be increased in rapidly proliferating tumors. GSH in conjunction with GPx and GST regulates cell proliferation (Obrador *et al*., 1997). Overexpression of GSH and GSH dependent enzymes has been documented in a wide range of tumors (Ghalia and Fouad, 2000). Thus, diminished lipid peroxidation combined with enhanced antioxidant capacity of BaP-induced gastric tumors may serve to maintain a reduced environment which facilitates cell proliferation offering a selective growth advantage to tumor cells.

The tumor and host tissue appear to comprise two separate metabolic compartments with respect to the cellularredox state. In contrast to BaP-induced stomach tumors, the liver and erythrocytes of tumor bearing animals showed enhanced lipid peroxidation associated with antioxidant depletion. A significant reduction in the activity of cytochrome P450 and cytochrome b5 (phase I enzymes) in hepatic tissue of mice upon AAILE treatment has been reported (Koul *et al*., 2006). *Rhizophora apiculata* leaf oil can exert down-regulatory effect on the activity of cytochrome P450 and cytochrome b5 in some organs like liver, kidney and forestomach of mice (Singh *et al*., 1998). The erythrocytes are major targets for lipid peroxidation because of their high content of polyunsaturated fatty acids and iron and their role as oxygen transporters (Hebbel, 1986). Compromised antioxidant defences in the host liver and erythrocytes may be due to increased utilization to scavenge lipid peroxides in these tissues as well as sequestration by the tumor cells.

Glutathione acts as a most important antioxidant in living systems because it is a remover of H_2O_2 lipid peroxides and their products like 4-hydroxinental (Bagchi *et al*., 2000). The GSH level was observed significantly higher in *R. apiculata* leaf oil treated mice than the carcinogen alone treated ones. But decrease in the level of GSH in stomach in the BaP treated mice has been observed and this may be because of the enhanced oxidative damage, enhanced use of GSH by the enzyme GPx and a reduction in the activities of the GSH-synthesizing enzymes such as glucose-6- phosphatedehydrogenase and GPx which neutralize the hydroxyl radicals and singlet oxygen. As it is present in high concentration in the cells, it protects cells from free radical damage (Gopalakrishnan *et al*., 1996). The chemo preventive agents including medicinal plants exert their anti-carcinogenic effects by modulation of phase I and II xenobiotic biotransformation enzymes. The results of the present study substantiate the anticarcinogenic and antioxidant activities of mangrove plants reported from our laboratory (Thirunavukkarasu *et al*., 2011; Shanmugapriya *et al*., 2012). Mangroves have long been used in fisher-folk medicine to treat diseases (Bandaranayake, 1998; Kathiresan, 2000). Marine floras are rich in biologically active and medicinally potent chemicals. Polyphenols and polysaccharides are the most predominant group of compounds which are applicable for antioxidant and anticancer activities. Polyphenols are widely distributed in plants and they are reportedly acting as free radical scavengers, antimicrobial and anticancer agents. The reducing properties are generally associated with the presence of reductones. Boopathy and Kathiresan (2010) reported that the antioxidant action of reductones is based on the breaking of the free radical chain by donating a hydrogen atom. The result presented here indicates that the marked antioxidant activity of bark extracts of *B. cylindrica* and *C. decandra* seems to be due to the presence of polyphenols which may act as reductones to convert free radicals into more stable products and terminate free radical chain reaction.

CONCLUSION

In this study, the effects of *R. apiculata* leaf oil on an gastric cancer animal model were assayed to explore the body weight by determination of SOD, CAT and GSH levels to explore its oxidative stress modifying effect and by liver, stomach and erythrocytes of control and *B. cylindrical* extract treated mice against BaP induced gastric cancer in albino mice. The results of this study have demonstrated that *R. apiculata* leaf oil induced potent anti-gastric cancer effects thought the induction of apoptosis and oxidative stress pathways. Based on this finding, *R. apiculata* oil may be a potential natural product for anti-gastric cancer treatment.

ACKNOWLEDGMENTS

We thanks the authorities of the Annamalai University for providing the all necessary facilities. The first author thanks the UGC-BSR fellowship for financial support during the period of study and we also thank to Dean and Director, Faculty of Marine Sciences, Annamalai University, Parangipettai.

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