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International Journal of Pharmacy and Pharmaceutical Science Research

ISSN: 2249-0337

Original Article

Chemoprevention of Gastric Cancer by mangrove plant species *Bruguiera cylindrica* against Benzo (a) pyrene induced gastric cancer in albino mice

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Received 12 December 2013; accepted 02 January 2014

Abstract

*Bruguiera cylindrica*isa mangrove species. It is used in traditional medicine against several human diseases, including tumour. To validate the ethno pharmacological claims against cancer.We examined the effects of *B. cylindrica*leaves extract 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 bw)with 100µl sesame oil by oral gavage twice a week for 4 consecutive weeks. Group II were given 100µl Sesame oil(SMO) treated (Vehicle treatedControl) a week for 4 consecutive weeks. Group III mice (BaP) (40 mg/kg bw)+ *B.cylindrica* (200 mg/kg/bw) for 14 weeks. Group IV only *B.cylindrica*(200 mg/kg/bw) daily for two weeks. Group V as the control. All the animals were sacrificed at the end of the 14th weeks.

Tumor incidence was observed to be 100% in mice that received only B(a)P. However, treatmentwith*B. cylindrica*extract reduced the tumor incidence as observed in mice of B(a)P + *B. cylindrica* group compared to that of B(a)P group. Similarly, the tumor burden and body weight were seen to decrease by 126.44 respectively in mice of B(a)P + *B. cylindrica* group when compared to those of B(a)P group. Diminished lipid peroxidation in the stomach tumour tissue was associated with enhanced antioxidant levels. In contrast to tumour tissue, enhanced lipid peroxidation with compromised antioxidant defenses was found in the liver and erythrocytes of tumour bearing animals. Administration of *B. cylindrica*significantly reduced the incidence of stomach tumours, modulated lipid peroxidation and enhanced antioxidant status in the stomach, liver and blood. From the results of our study, we suggest that *B. cylindrica*extract may exert its chemopreventive effects by modulating lipid peroxidation and enhancing the antioxidant status in the stomach, liver and erythrocytes.

1. Introduction

Gastric cancer is the second most common cause of cancer death worldwide, is a major cause of mortality in Chennai, India. The development of stomach cancer is associated with a sustained genetic mutation 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 (Wu, 2006).

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).B(a)P, a potent pro-carcinogen employed in initiating stomach cancer, is the *prototypical* and best characterized member of PAHs family of chemical carcinogens(Srivastava*et al.,* 2000). It has been shown to induce tumors of skin, lung, mammary glands and fore stomach tissues of various experimentalanimals(Athar*et al.,* 1989). Since B(a)P is an omnipresent environmental pollutant and is believed to be a risk factor for humanchemical carcinogenesis, it is important to identify the naturally occurring/synthetic agents that could modulate B(a)P-induced tumourigenesis(Dasgupta*et al*., 2004).

Bruguieracylindrica (L.)Blume, (family: Rhizophoraceae) is a rare tree mangrove found along the western coast of India. The bark of *B.cylindrica* is used to stop hemorrhage and applied to malignant ulcers (Kirtikar and Basu,1999).Pentacyclictriterpenoid esters from the fruits (Mahadevan*et al.,*2005) and brugine alkaloid from stem and bark (Takahashi, 1975)have been isolated. *Ceriopsisdecandra* (Griff.) Ding Hou (Family: Rhizophoraceae) is reported to cure hepatitis. (Magwa*et al.,*2005) Earlier, antiviral activity of *B. cylindrica* and *C. decandra* leaves was studied (Premnathan*et al.,* 1992). A study was carried out to evaluate phenolic content, DPPH (1,1 - diphenyl - 2-picryl hydroxyl) reduction activity and reducing power of leaves on *B. cylindrica* and *C. decandra* leaves (Agoramoorthy*et al.,* 2008). Leaf extract of *C. decandra* exhibited antidiabetic activity inalloxan induced diabetic rats (Nabeel*et al.,* 2010). Leaf of *C. decandra* was found to have radical scavenging activity against superoxide anion (Sakagami*et al.,* 1998). However, there has been no detailed *in vitro* study on antioxidant properties of stem bark of these mangrove medicinal plants from Pichavaram forest, Tamil Nadu, India. Hence, in the present study, we aimed to evaluate the total phenolic content and total flavonoid content and to examine the antioxidant activities. In our present study Benzo (a) pyrene induce Anti gastric cancer effect of extract from *B.cylindrica*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° and 12 hrs light: 12 hrs dark cycles in Central Animal House, Department of Experimental Medicine, RMMC&H, Annamalai University.All mice werefed with standard pellet diet supplied by (Hindustan Lever Ltd., Bangalore, India) and water *ad libitum* for 1 week before the study. The study will be 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 Wattenberg et al (1981) with minor modifications as suggested by Nagabhushan and Bhide (1987).

Collection of Plant Materials:

The plant material of *Bruguiera cylindrinca* leaves was collected from Ariyankukkuappam 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. The plant material were dried under shade with occasional shifting and then powdered with a mechanical grinder and stored in an airtight container. The powder obtained was subjected to successive Soxhlet extraction with the organic solvents with increasing order of polarity.

Extraction

The 100g of plant powder was extracted with 99.5 % methanol in 24 hours at 35^0C. The extraction was repeated many times to obtain a sizable quantity of extract. The extracts were filtered and concentrated by using rotary evaporator (BuchiRotavapor R-124). The resultant residues were kept of 4^0C until further use.

2.4. Experimental Protocol

The mice were divided into five 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) 1mg 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*) +B.cylindrica (*200 mg/kg/bw) for 14 weeks

Group IV -*B.cylindrica*extract with the dose of 200 mg/kg/b.w/day orally for 2 weeks).

Group V- Sterile tap water (STW) treated (Negative Control) throughout the study period.

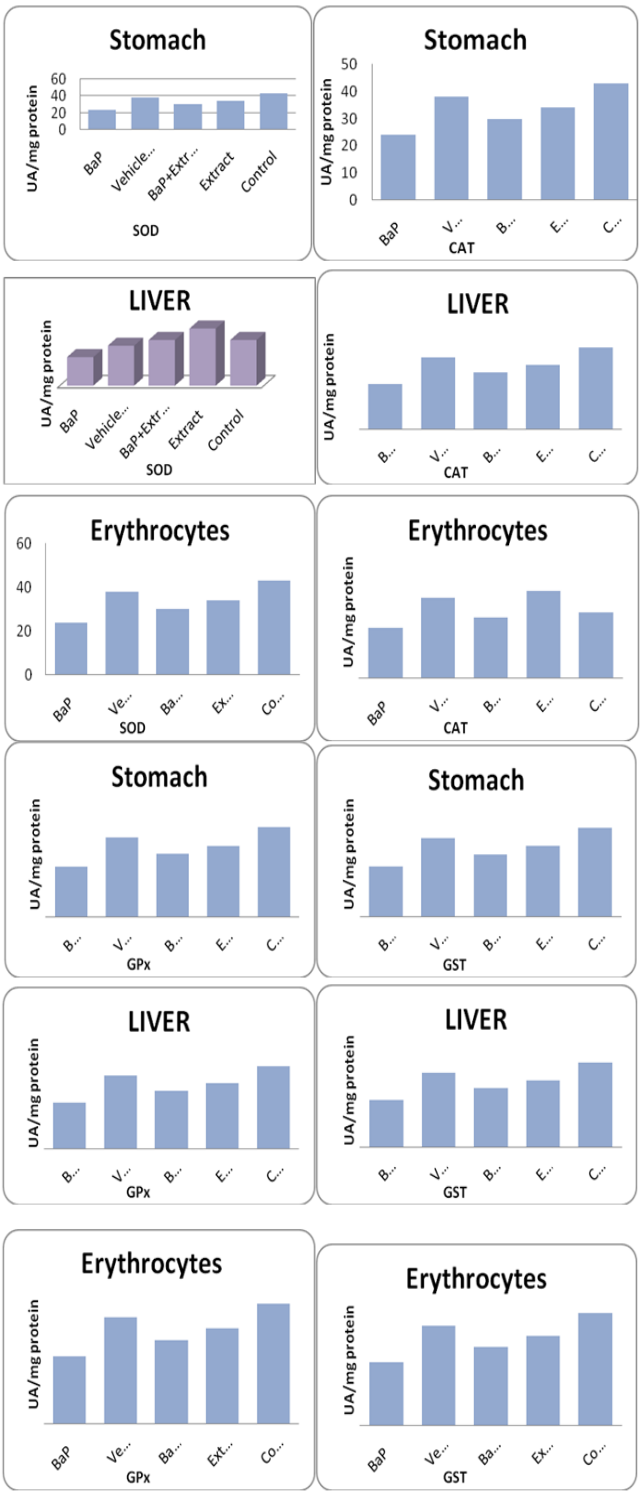
The body weights were measured at the end of experiment. The experiment was terminated in the 14th week, and all mice were killed after an overnight fast. Blood was collected, and the plasma separated was used for analysis. The stomachs were excised to prepare a 10% homogenate for biochemical measurements. Frozen gastric tissue was ground in liquid nitrogen and suspended in a homogenization buffer consisting of 50 mMTris–HCl, pH 8.2, 1 mM EDTA, 0.1% Triton X-100, and proteinase inhibitor cocktail (Roche, Mannheim, Germany). After centrifugation in amicrocentrifuge at 4^0C, the supernatants were used to determine enzyme activity and protein concentration.

2.4. Preparation of tissue homogenate and erythrocyte lysate

The tissue samples after weighing were homogenized using appropriate buffer in a glass homogenizer with Teflon pestle. Blood samples were collected in heparinised tubes and the plasma was separated by centrifugation at 1000 g for 15 minutes. After

Table 1: Shows the mean body weight, incidence of gastric tumours and the mean tumour burden in control andexperimental animals. The mean body weights of BaP treated animals (group 1) were significantly lower than that of control (group 5). No significant differences in body weights were observed in groups 3 through 5.

Groups	Initial weight (g)	Final weight (g)	Tumour incidence (%)	Tumour burden (mm3)
I –BaP	31.9 ± 11.6	41.4 ± 22.6	126.44	5/7 (855)
II –Vehicle	32.7 ± 10.8	44.3 ± 20.1		
III– BaP+*B.cylindrica*	34.3 ± 12.7	46.5 ± 23.5		
IV – *B.cylindrica*	33.4 ± 13.4	48.2 ± 24.6		
V –Control	33.5± 09.5	40.1 ± 19.4		



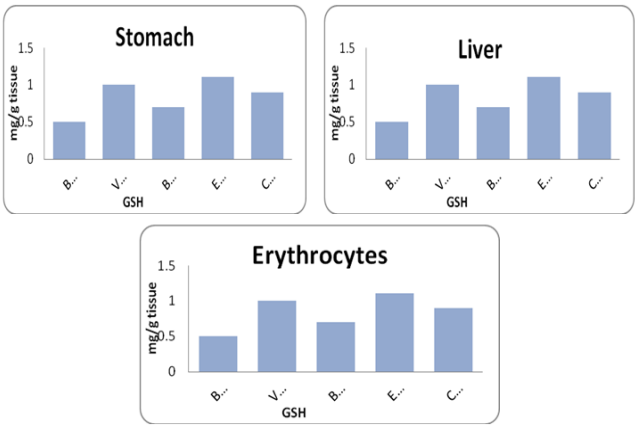


Figure 1. The Influence of Treatment with B. *cylindrical* Leaf Extract on SOD and CAT Activities in the Stomach,Liver and Erythrocytes. (Mean ± SD; n=7).

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, pH 7.4. The hemolysate was separated by centrifuging at 2500 g for 15 min at 2^{o}C.

2.5. Biochemical methodology

Lipid peroxidation as evidenced by the formation of thiobarbituric acid reactive substances (TBARS) was assayed in tissues by the method described by Ohkawa *et al.*, (1979) in the plasma by the method of Yagi(1978) and inerythrocytes by the method of Buege and Aust (1987). The pink coloredchromogen 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 Anderson (1985) 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 Rotruck*et al*., (1973) with modifications. A known amount of enzyme preparation was incubated with hydrogen peroxide in the presence of GSH for 10 minutes. The amount of hydrogen peroxide utilized was determined by estimating GSH content by the method of Anderson(1985). The activity of glutathione S-transferase (GST) was determined as described by Habig*et al.*,(1974) by following the increase in absorbance at 340 nm using CDNB as substrate. Glutathione reductase (GR) activity was determined by the method of Carlberg and Mannervik(1985), 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 colored complex with 2,2'-dipyridyl at 520 nm. Hemoglobin 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.

4. Statistical analysis

Statistical analysis on the incidence of lesions was performed using Fisher's exact probability test. The body weight and biochemical parameters were analyzed 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 tumours in group 1 was 85.5 per cent (6/7animals) with a mean tumour burden of 126.44 mm3. Although no tumours were observed in groups 3 and 4 and 5 two of six animals in group 2 had small multiple nodules.**Figures 1** illustrate the effect of treatment with methanol Mangrove leaf extract 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 B(a)P significantly lowered the extent of lipid peroxidation in the stomach of group 1 mice compared to control (group 5). Treatment with 200 mg/kg/bw methanolic mangrove leaf extract 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 B(a)P (group 1) compared to group 5.Treatment with methanolic mangrove leaf extract significantly reduced BaP-induced lipid peroxidation in group 3 animals compared to group 1.Administration of methanolicmangrove leaf extract alone (group 4) significantly reduced the extent of lipid peroxidation in the stomach, liver and erythrocytes compared to control. The influence of treatment with mangrove leaf extract on the antioxidant profile in the stomach, liver anderythrocytes are shown in Figures 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 methanolic mangrove leaf extract significantly increased all the antioxidants in group 3 animals compared to group 1Administration of methanolicmangrove leaf extract alone (group4) significantly enhanced the antioxidant status compared togroup 1.

Discussion

B(a)P, 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 phase-II. The former constitutes cytochrome P-450 based mono-oxygenase system which is responsible for initiating conversion of pro-carcinogens 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*et al.,* 1990). The balance between the phase-I carcinogenactivating enzymes and the phase-II detoxifying enzymesis critical to determining an individual's risk for cancer (Wilkinson *et al.,* 1997). There is substantial evidence that chemopreventive agents including medicinal plants exerttheir anti-carcinogenic effects by modulation of phase-Iand phase-II xenobiotic biotransformation enzymes (Subapriya*et al.,* 2005).

In the present study, the exposure of mice to the carcinogen B(a)P caused high incidence of forestomachtumors, 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 papillomaswas found to be quite high in comparison to mangrove lead extract B(a)Ptreated group (Experimental). The results of the present investigation are also supported by the others (Wattenberg *et al.,* 1980; Azuine and Bhide, 1992; Deshpande*et al.*,1997; Agha *et al.,* 2001) who have used the different plant extracts to reduce chemical induced carcinogenesis in there finding (Dasgupta*et al.,* 2004; Gangar*et al.,* 2006).

The decreased susceptibility of stomach tumours to lipid peroxidation seen in the present study may be attributed toenhanced 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 tumours compared to controls (Kumaraguruparan*et al.,* 2002; Izutani*et al.,* 1996). 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 tumours. GSH in conjunction with GPx and GST, regulates cell proliferation (Obrador*et al.,* 1997). Overexpression of GSH and GSHdependent enzymes has been documented in a wide range of tumours (Kumaraguruparan*et al.,* 2002; Ghalia and Fouad, 2000). Thus, diminished lipid peroxidation combined with enhanced antioxidant capacity of BaP-induced gastric tumours may serve to maintain a reduced environment

which facilitates cell proliferation offering a selective growth advantage to tumour cells.

The tumour and host tissue appear to comprise two separate metabolic compartments with respect to the cellularredox state. In contrast to B(a)P-induced stomach tumours, the liver and erythrocytes of tumour 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. B. cylindrical leaf extract can exert down-regulatory effect on the activity of cytochrome P450 and cytochrome b5 in some organs like liver, kidney and forestomach of miceThe 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 tumour 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).GSH level was observed significantly higher in mangrove extract 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).Thechemo preventive agents including medicinal plants exert their anti-carcinogenic effects by modulation of phase I and phase II xenobiotic biotransformation enzymes(Subapriya*et al.,* 2005).

The results of the present study substantiate the anticarcinogenic and antioxidant activities of mangrove plants reported from our laboratory and other (Thirunavukkarasu*et al.,*2011; Shanmugapriya*et al.,* 2012; Sitharagaboopathy*et al.,* 2010). 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 polysaccharidesare 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 (Shahidi and Wanasundara,1992; Sanchez Moreno *et al.,* 1999).

The reducing properties are generally associated with the presence of reductones. (Gordan,1990) 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 *B. cylindrical* extract onan 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 *B. cylindrical* extractinduced potent anti-gastric cancer effects thought the induction of apoptosis, and oxidative stress pathways. Based on this finding, *B. cylindrical* extractmay be a potentialnatural product for anti-gastric cancer treatment.

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Source of support: Nil; Conflict of interest: None declared