Evaluation of Antioxidant and Antitumor Activities of *Wrightia arborea*

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Abstract: *Wrightia arborea* (Br.) (Family-Apocynaceae), locally known as Sathkurchi, is a small deciduous tree with small branches and densely velvety leaves. This study was designed to investigate the antioxidant and anti tumor activities of leaves of *W. arborea*.

Antioxidant potential was evaluated *in vitro* by DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging- and reducing power-assay method which was concentration dependent that was 25-200 µg/ml. The extract showed scavenging activity with IC_{50} value of 15.23 ± 4.10 µg/ml for DPPH and also showed reducing activity in dose dependent manner. In addition, total phenol and flavonoid content were determined by Folin-Ciocalteu reagent and were found to be 112.54 ± 9.74 mg/g plant extract (in GAE) and 247.14 ± 15.45 mg/g plant extract (in quercetin equivalent), respectively. The antioxidant capacity was evaluated by phosphomolybdenum method and was found to be 117.27 ± 12.36 mg/g plant extract (in ascorbic acid equivalents). The anti tumor effect of the methanol extract was determined with doses of 5, 10 and 20 mg/kg b.wt against Ehrlich ascites carcinoma (EAC) in mice with respect to the determination of tumor volume, tumor weight, % of cell growth inhibition, % increase in life span (%ILS), and hematological parameter (WBC, RBC and hemoglobin).

The methanol extract of *W. arborea* decreased the tumor weight significantly compared to control group at all the mentioned doses and the highest was observed at the dose of 20 mg/kg b. wt. (1.28 ± 0.15 g). The % of cell growth inhibition increased in dose dependent manner like 36.43 ± 7.45, 58.76 ± 9.43 and 98.43 ± 12.45 at the doses of 5, 10, 20 mg/kg b.wt. respectively. The %ILS was also enhanced in all the doses where 20 mg/kg b.wt showed maximum effect (105.5 ± 3.57). Heamoglobin (Hb) content was significantly increased 8.12 ± 2.6, 10.23 ± 1.62 and 12.12 ± 2.09 g/dL at the doses of 5, 10 and 20 mg/kg b.wt. respectively, compared to EAC control mice (4.95 ± 1.80 g). There was a significant ($p < 0.05$, $p < 0.01$, $p < 0.001$) decrease in RBC count and increase in WBC counts in extract treated animals when compared to EAC control animals.

This is the first report of biological activities of leaves of *W. arborea* and it showed significant antioxidant as well as antitumor activity.

Keywords: Antitumor, Ehrlich ascites carcinoma, free radical, phenol, flavonoid, *Wrightia arborea.*

INTRODUCTION

Cancer is one of the most dreaded diseases of centuries, characterized by uncontrolled cellular proliferation, tissue invasion and metastases [1]. Overproduction of peroxides and free radicals (reactive oxygen species, ROS) due to aberrant metabolism involving redox enzymes and/or exposure to a plethora of exogeneous chemicals/ factors can cause damage to cellular macromolecules such as nucleic acids, proteins or lipids leading to many degenerative diseases including cancer, Alzheimer's disease, arthritis and ischemic reperfusion [2-5]. More and more recent evidences suggest that this potentially cancer-inducing oxidative damage or 'oxidative stress' can be prevented or limited in part by natural antioxidants which may mediate their effects by directly quenching the ROS or chelating the catalytic metal ions of redox enzymes [6]. Natural products including fruits and vegetables have drawn considerable interest as chemotherapeutics because of their widespread antioxidant and anticancer principles. Among the natural antioxidants polyphenolic compounds such as flavonoids, flavonols and terpenoids etc. from plant origin have appeared as favored choice. By virtue of being electron rich these molecules can donate electrons to ROS and neutralize these chemical species [7-9].

The genus *Wrightia* comprises of 23 flowering plants (Apocynaceae family), which are native to tropical Africa, Asia and Australia. Plants belonging to *Wrightia* are known in the traditional medicine for anti-cancer activity along with other broad indications including in snake and scorpion bites, renal

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complications, menstrual disorders etc. Based on our comprehensive search using Scifinder, most of the studies on *Wrightia* species were concentrated on *W. tinctoria* and *W. tomentosa*. Triterpenes such as α/β-amyrins and their acetates, ursolic acid, oleanolic acid, wrightial and other cycloartanes were abundant in some *Wrightia* species suggesting conserved biosynthetic pathways across the genus. Besides triterpenes, *Wrightia* species also contain chemically interesting steroid/ indole-type alkaloids, and flavonoids. Extracts of various parts of *Wrightia* plants possess a wide range of bioactivity including anticancer, antimicrobial, amoebicidal, analgesic and anti-inflammatory. Isolated compounds such as β-amyrin derivatives were antidyslipidemic, oleanolic and urosolic acids showed anti-breast cancer potentials; Wrightiadione (isoflavone) was cytotoxic against murine P388 lymphocytic leukemia cell line whilst Wrightiamines A and B (pregnane alkaloids), were cytotoxic against vincristine-resistant murine leukemia P388 cells [10, 11]. Some synergistic actions of isolates such as antifungal indirubin from *W. tinctoria* and *W. tomentosa* have also been documented to potentiate antibiotics like ciprofloxacin. Notably, the indirubin derivatives of synthetic origin were more recently found to induce apoptosis *via* caspase-9 dependent programmed cell death or *via* G2/M cell cycle arrest by up-regulating p21 [12].

W. arborea (locally known as Sathkurchi) is a small deciduous tree, distributed in the southern part of Indian subcontinent including Chittagong and Sylhet districts of Bangladesh. The plant is virtually unexplored; some preliminary studies have suggested antibacterial and cytotoxic potentials of the leaves. However, the lack of more detailed investigations including phytochemical works limits a scientific basis of its ethnopharmacological values.

We have tested the leaf extract of *W. arborea* for the presence of polyphenolic compounds preparation of the bark made from *W. arborea* is found useful in menstrual, renal complaints [13, 14] and antibacterial properties [15]. From the genus specially the species *Wrigthia tinctoria* contains wrightial, a triterpenoid chemical [16], along with cycloartenone, cycloeu-calenol, β-amyrin, and β-sitosterol isolated from the methanol extract of the immature seed pods. As not enough phytochemical and biological studies have been carried out with the *W. arborea,* the present study was conducted to evaluate the antioxidant and antitumor activities of methanol extract of the leaves of *W. arborea* against Ehrlich ascites carcinoma (EAC) in mice.

MATERIALS AND METHODS

Plant Material

The leaves of the *W. arborea* were collected from Banderban Hill Tracts, Chittagong, Bangladesh during February 2010 and were identified by Taxonomist Md. Boctiar Uddin, Associate Professor, Department of Botany and University of Chittagong, Bangladesh where a voucher specimen number (Voucher No # 147) has been deposited.

Chemicals

Ammonium molybdate, folin-chiocaltu phenol reagent, sodium chloride, propylene glycol, trypan blue, methyl violet, sodium sulphate, methylene blue, bleomycin were purchased from Merck Limited, Mumbai, India. 1,1-diphenyl- 2-picryl-hydrazyl (DPPH), ascorbic acid, quercetin, gallic acid and potassium ferric cyanide, was purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals and reagents used were of highest analytical grade.

Preparation of Extract

The air dried and powdered plant material (700 g) was extracted in a Soxhlet apparatus with methanol (60-80°C). The extract was filtered through a fresh cotton plug followed by Whatman no.1 filter paper. The filtrate was then concentrated with a Buchii rotavapor at low temperature and pressure to afford methanol extract (120 g approx.).

Preliminary Phytochemical Investigation

The extract was subjected to qualitative chemical investigation for the identification of different phytoconstituents like steroids, saponins, alkaloids, phenol, flavonoids, tannins and terpenoids [17].

Animal

Swiss albino mice (25-30 g) of both sexes were used for assessing the biological activity. The animals were maintained under standard laboratory conditions and had free access to food and water *ad libitum.* The animals were allowed to acclimatize to the environment for 7 days prior to experimental session. The animals were divided into different groups, each consisting of twelve animals which were fasted overnight prior to the experiments. Experiments with the animals were performed in accordance with guidelines of the

Institutional Animal Ethics Committee, Department of Applied Chemistry & Chemical Engineering, University of Rajshahi, Rajshahi, Bangladesh.

Acute Toxicity

The acute oral toxicity of the plant extract in Swiss albino mice was studied as per established protocol [18].

In Vitro Antioxidant Activity

The Amount of Phenolic Compounds and Flavonoids

The total phenolic and flavonoid content of methanol extract of *W. arborea* were determined using Folin–Ciocalteu reagent [19] and aluminium chloride colorimetric method [20], respectively. The content of total phenolic in the extract was calculated from regression equation of the calibration curve ($y = 0.013x + 0.127$, $r^2 = 0.988$) and is expressed as galic acid equivalents (GAE) and the flavonoid contents of the extract in terms of quercetin equivalent (the standard curve eqation: $y = 0.009x – 0.036$).

Determination of Total Antioxidant Capacity

The antioxidant activity of the methanol extract was evaluated by the phosphomolybdenum method [21]. The assay is based on the reduction of Mo (VI)–Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Free Radical Scavenging Activity Measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH)

The free radical scavenging activity of methanol extract, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined by the method described by Braca [22]. The percentage inhibition activity was calculated from $[(A_0–A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/ standard. IC_{50} value was calculated from the equation of line obtained by plotting a graph of concentration (μg/ml) versus % inhibition.

Reducing Power Activity

The reducing power was determined according to the Oyaizu method [23]. Increased absorbance of the reaction mixture indicated increased reducing power.

In Vivo Antitumor Activity

Transplantation of Tumor

Ehrlich ascites carcinoma (EAC) cells were obtained from Indian Institute of Chemical Biology (IICB), Kolkata, India. The EAC cells were maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation of 2×10^6 cells per mouse after every 10 days. Ascitic fluid was drawn out from EAC tumor bearing mouse at the log phase (days 7–8 of cell implantation) of the tumor cells. Each animal received 0.1 ml of tumor cell suspension containing 2×10^6 tumor cells intraperitoneally.

Treatment Schedule

72 Swiss albino mice were divided into six groups (n = 12) and given food and water *ad libitum*. All the animals in each group except Group-I received EAC cells (2×10^6 cells/mouse i.p.). This was taken at day ‘0’. Group-I served as normal saline control (5 ml/kg i.p.) and Group-II served as EAC control. After 24-h of EAC transplantation, Group-III, Group-IV and Group-V received methanol extract of *W. arborea* leaves at 5 mg/kg, 10 mg/kg and 20 mg/kg i.p. for nine consecutive days, respectively. Group-VI received reference drug Bleomycin (0.3 mg/kg i.p) for nine consecutive days [24]. After 24 hours of last dose and 18 h of fasting, 6 animals from each group were sacrificed by cervical dislocation to measure antitumor and hematological parameters and the rest were kept with food and water ad libitum to check percentage increase in life span of the tumor bearing host. The antitumor activity of the methanol extract of *W. arborea* was measured in EAC animals with respect to the following parameters.

Determination of Tumor Volume and Weight

The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube and weighed immediately.

Tumor Cell Count

The ascitic fluid was taken in a WBC pipette and diluted 100 times with normal saline and then a drop of the diluted cell suspension was placed on the Neubauer’s counting chamber and the number of cells in the 64 small squares was counted.

Viable/Nonviable Tumor Cell Count

The viability and non viability of the cells were checked by trypan blue assay with the help of microscope. The cells were stained with trypan blue

(0.4% in normal saline) dye. The cells that did not take up the dye were viable and those that took the dye were nonviable. These viable and nonviable cells were counted using the following equation:

Cell count = (Number of cells × dilution factor)/ (Area × thickness of liquid film)

Determination of Median Survival Time and Percentage Increase in Life Span

The mortality was monitored by recording percentage increase in life span (%ILS) and median survival time (MST) [25].

Hematological Parameters

Collected blood was used for the estimation of hemoglobin (Hb) content, red blood cell (RBC) and white blood cell count [26].

Statistical Analysis

Antitumor data are expressed as mean ± SEM. (n = 6 mice per groups). Statistical significance (P) calculated by ANOVA done in SPSS version 15.0 followed by Dunnet's T test. ***P<0.001, **P<0.01 and *P<0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

The results of the phytochemical screening of the leaves of *W. arborea* are shown in (Table **1**). It indicates the presence of alkaloids, phenolics, flavonoids, saponins and tannins.

The acute toxicity study was conducted to establish the therapeutic index, i.e., the ratio between the pharmacologically effective dose and the lethal dose on the same strain and species. The methanol extract of *W. arborea* was safe up to a dose of 500 mg/kg (p.o.) body weight. The extract did not cause mortality in mice during 48h of observation but little behavioral changes, locomotors ataxia, diarrhea and weight loss were observed. Food and water intake had no significant difference among the groups studied.

A large number of *in vitro* methods have been developed for evaluating antioxidant activity.

Total phenols and flavonoids content were found to be 112.54 ± 9.74 mg/g leaves extract (in GAE) and 247.14 ± 15.45 mg/g leaves extract (in quercetin equivalent), respectively, in methanol extract of *W arborea*; presented in Table **2**. In the study this possibility is supported by the estimation of total phenols and flavonoids [27] which was found to be present in high concentration in the *Phaseoloides* sp. extracts. Our study also supported to the previous study [28].

The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the compounds having antioxidant property and is successfully used to quantify vitamin E in seeds [29]. Total antioxidant capacity (given in Table **2**) of the methanol extract of *W. arborea* leaves is expressed as the number of equivalents of ascorbic acid and was found to be 117.27 ± 12.36 mg/g equivalent of ascorbic acid.

DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule [30] and is usually used as a substrate to evaluate the antioxidant activity of a compound [20]. It was revealed that the methanol extract did show the proton donating ability and could serve as free radical inhibitor or scavenger. The percentage (%) scavenging of DPPH radical was found to be concentration dependent i.e. concentration of the extract between 25-200 μg/ml greatly increasing the

Table 1: Result of Chemical Group Tests of the Methanol Extract of *Wrightia arborea* (MEWA)

Extract	Tannin	Saponin	Flavonoid	Phenol	Steroid	Alkaloid	Terpenoids
MEWA	+++	++	+++	++	+	+	+

(+): Present; (+ + +): Reaction intensity is high; (+ +): Reaction intensity is medium; (+): Reaction intensity is normal.

Table 2: Total Amount of Plant Phenolic Compounds, Flavonoids and Total Antioxidant Capacity of Methanol Extract of *Wrightia arborea* (MEWA)

Group	Total antioxidant content (mg/g, Ascorbic acid equivalents)	Total Phenol content (mg/g, Gallic acid equivalents)	Total flavonoid content (mg/g, quercetin equivalents)
MEWA	117.27 ± 12.36	112.54 ± 9.74	247.14 ± 15.45

inhibition activity. The IC_{50} value of the methanol extract of *W. arborea* was 15.23 ± 4.10 µg/ml, while ascorbic acid showed the value of 12.45 ± 3.36 µg/ml (Figure **1**).

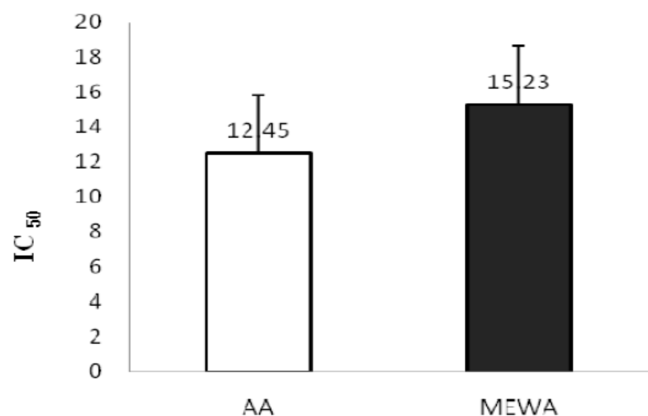

Figure 1: IC_{50} value of the methanol extract of *Wrightia arborea* (MEWA) and ascorbic acid (AA) for DPPH radicals.

For the measurement of the reductive ability, we investigated the Fe^{3+} to Fe^{2+} transformation in the presence of methanol extract of *W. arborea*. Like the antioxidant activity, the reducing power of the extract increased with increasing concentration. Figure **2** shows the reductive capabilities of the methanol extract of *W. arborea* compared with ascorbic acid.

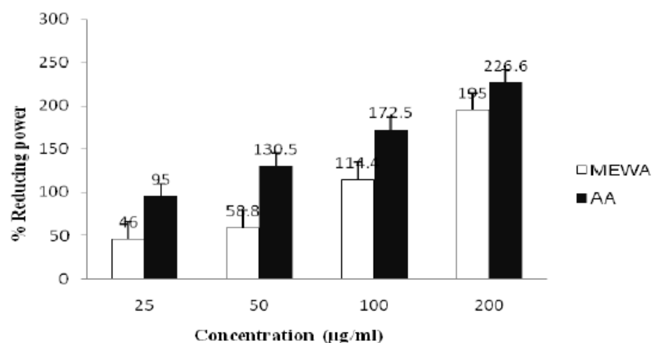

Figure 2: Reducing power of the methanol extract of *Wrightia arborea* (MEWA) and ascorbic acid (AA) by spectrophotometric detection of Fe^{3+} to Fe^{2+} transformation.

The reducing properties are generally associated with the presence of reductones [31]. Moreover, it has been reported that the phenol and polyphenolic compound (flavonoids) constituents of the plant possess antioxidant properties mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. In addition, they have a metal chelating potential [32, 33]. The methanol extracts showed antioxidant activity and the activity of the extract might be attributed to the phenolic and flavonoids, which detected by phytochemical analysis in our study and also previously reported [34-36].

Antitumor activity of the methanol extract against EAC tumor bearing mice was assessed by the parameters such as tumor volume, tumor weight, cell count (viable and non viable), mean survival time, % increase in life span and % of cell growth inhibition. The results are shown in Table **3**. The tumor volume, tumor weight and viable cell count were found to be increased and non-viable cell count was low in EAC control animals when compared with normal control animals. Administration of the methanol extract at doses of 5 mg/kg, 10 mg/kg and 20 mg/kg significantly decreased the tumor volume, tumor weight and viable cell count. Furthermore, the median survival time (MST) was increased to 27 ± 1.7, 35 ± 1.9 and 42 ± 2.5 (%ILS = 36.5 ± 2.1, 76.25 ± 3.4 and 105.5 ± 3.57) on administration of the methanol extract at 5 mg/kg, 10 mg/kg and 20 mg/kg, respectively. All these results clearly indicate that the methanol extract had a capacity to inhibit the growth of solid tumor induced by EAC cell line in experimental animals.

Hematological parameters (Table **4**) of tumor bearing mice on 14 day were found to be significantly altered compared to the normal group. The total WBC count was found to be increased with a reduction of Hb content of RBC. The total number of RBC showed a modest change. At the same time the methanol extract at 5 mg/kg, 10 mg/kg and 20 mg/kg b.wt. restored all the altered hematological parameters to almost close to normal.

In EAC tumor bearing mice, a regular rapid increase in ascetic tumor volume was observed. Ascetic fluid is the direct nutritional source for tumor cells and a rapid increase in ascetic fluid with tumor growth would be a way to meet the nutritional requirement of tumor cells [37]. Treatment with methanol extract of *W. arborea* reduced the intraperitonial tumor burden, thereby reducing the tumor volume, tumor weight, viable tumor cell count and increased the life span of the tumor bearing mice. The steadfast criteria for judging the potency of any anticancer drug are prolongation of life span of animals [38]. It can therefore be inferred that the methanol extract increased the life span of EAC bearing mice may be due to decrease the nutritional fluid volume and delay the cell division [39].

Reduction in viable cell count and increased non viable cell count towards normal in tumor host suggest antitumor effect against EAC cell in mice. These

Table 3: Effect of the Methanol Extract of *Wrightia arborea* (MEWA) on Tumor Volume, Tumor Weight, Mean Survival Time (MST), Percentage Increase in Life Span (% ILS), % of Cell Growth Inhibition in EAC Bearing Mice

Parameter	EAC control	MEWA (5 mg/Kg b.wt.)	MEWA (10 mg/Kg b.wt.)	MEWA (20 mg/Kg b.wt.)	Bleomycin (0.3 mg/Kg b.wt.)
Tumor volume (ml)	3.8 ± 0.15	2.35 ± 0.05*	1.15 ± 0.04***	0.55 ± 0.06***	0.49 ± 0.05***
Tumor weight (g)	3.99 ± 0.13	3.03 ± 0.10*	2.01 ± 0.11***	1.28 ± 0.15***	0.47 ± 0.11***
MST (days)	23 ± 2.1	27.0 ± 1.7**	35.0 ± 1.9***	42.0 ± 2.5***	48.6 ± 2.5***
% ILS	0.00	36.5 ± 2.1	76.25 ± 3.4	105.5 ± 3.57	144.0 ± 4.5
% of cell growth inhibition	0.00	36.43 ± 7.45	58.76 ± 9.43	98.43 ± 12.45	95.32 ± 8.53

Each point represent the mean ± SEM. (n=6 mice per group), *$p<0.05$, **$p<0.01$, ***$p<0.001$ statistically significant when compared with EAC control group.

Table 4: Effect of the Methanol Extract of *Wrightia arborea* (MEWA) on Hematological Parameters in EAC Bearing Mice

Treatment	RBC (cell × 10^3/mm^3)	WBC (cell × 10^3/mm^3)	Hemoglobin (g %)
Normal control	5.78 ± 0.22	4.12 ± 0.32	13.90 ± 3.1
EAC control	3.11 ± 0.80*	5.84 ± 0.52*	4.95 ± 1.80*
MEWA (5 mg/kg)	3.94 ± 0.12*	4.95 ± 0.15*	8.12 ± 2.6**
MEWA (10 mg/kg)	4.33 ± 0.76**	4.29 ± 0.32**	10.23 ± 1.62**
MEWA (20 mg/kg)	5.07 ± 0.15**	3.89 ± 0.19**	12.12 ± 2.09**
Bleomycin (0.3 mg/kg)	5.58 ± 0.11**	3.15 ± 0.83**	12.89 ± 2.93**

Each point represent the mean ± SEM. (n=6 mice per group), *$p<0.05$, **$p<0.01$, ***$p<0.001$ statistically significant when compared with control group.

demonstrated that the methanol extract have direct relationship with tumor cells as these tumor cells are absorbed the anticancer drug by direct absorption in peritoneal cavity and this anticancer agent lysis the cells by direct cytotoxic mechanism [40]. Anemia is encountered in ascites carcinoma mainly due to iron deficiency, either by haemolytic or myelopathic conditions which finally lead to reduced RBC number [41]. Treatment with methanol extract brought back the hemoglobin level, RBC and WBC count more or less to normal levels, thus supporting its haematopoietic protective activity without inducing myelotoxicity, the most common side effects of cancer chemotherapy.

Preliminary phytochemical studies indicated the presence of alkaloid, phenolic and flavonoid compounds in methanol extract of *W. arborea.* Literature survey shows that most of the polar compound i.e., alkaloids, flavonoids etc. are biologically active. A number of scientific reports indicated that certain phenolic compounds and flavonoids have chemo preventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis [42]. Furthermore, flavonoids such as quercetin, kaemferol and their glycosides have been shown to possess antimutagenic and antimalignant effect [43]. The antitumor activities of methanol extract of *W. arborea* are probably due to one or more of alkaloids, phenolic compounds, flavonoids present in the extract.

Based on the result, it is possible to conclude that the methanol extract of *W. arborea* not only exhibited antioxidant activity but also significantly reduced tumor growth and viability of tumor cells, normalized the hematological profiles and increased life span as compared with those of EAC treated control mice. As it is a less known medicinal plant in the literature, our next aim will be an attempt to isolate the bioactive compounds responsible for these bioactivities.

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