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Evaluation of Antitumor Activity of *Leea indica* (Burm.f.) Merr. extract against Ehrlich Ascites Carcinoma (EAC) Bearing Mice

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Abstract

Most of the conventional anti tumor drugs are designed to have selective toxicity to rapidly dividing cells. Among these agents the focus of many studies are compounds obtained from natural products that significantly reduce tumor size and enhance the survival time. In this study the crude methanolic extract of the leaves of *Leea indica* (*L. indica*) was examined for their anti tumor, anti-oxidant and cytotoxic activity. In vivo anti tumor activity was studied against Ehrlich Ascites Carcinoma (EAC) cells in Swiss albino mice by monitoring parameters like tumor weight measurement, survival time and tumor cell growth inhibition. It has been found that the compound at the dose of 40 mg/kg/day (i.p) significantly decreases tumor weight, increases life span and reduces tumor cell growth rate in comparison to those of EAC bearing mice receiving no extract. Bleomycin was used as a positive control (0.3 mg /kg). In vitro anti-oxidant potentiality was tested using DPPH radical scavenging test, total phenol and flavonoid content and reducing power determination assays. The extracts showed moderate antioxidant activity in a dose dependent manner. The cytotoxic activity of the extract was assessed by brine shrimp lethality bioassay technique which showed significant result (LC_{50} less than 25µg/ml). Anti tumor properties of *L.indica* could be linked with the presence of these antioxidant and cytotoxic activity. These outcomes indicate the possible potential use of *L.indica* as anti tumor agent.

Keywords: Antitumor activity, EAC cell, antioxidant, cytotoxicity, *Leea indica*.

1. Introduction

After cardiovascular diseases, cancer is the second major cause of death in the western world accounting for 24% of all deaths. In European countries each year over three quarters of a million people die from cancer [1]. Bangladesh has an immense wealth of medicinal plant species, both endemic and non-endemic. The most extensive screening for anticancer compounds obtained from plant species was done by National Cancer Institute (NCI) in the USA. More than 35,000 species (or about 14% of the estimated number of higher plant species on earth) collected from different rain forests in Asia, Africa and the Amazon were screened by the NCI alone [2]. Many natural plant extracts and phytochemicals have been reported to induce apoptosis in cancer cell lines [3-5]. From the screening process, taxol from *Taxas brevifolia* was discovered and developed into one of the most successful plant based anti cancer drug. Other anticancer drugs originated from plants include vinblastine and vincristine derived from *Catharatus roseus* and etoposide from *Podophyllum peltatum*, to name a few. Although chemotherapy is effective in detecting cancer at a very early stage, the side effects and resistance towards drug are a major problem. Hence new drugs or treatments are needed. Identification of new cytotoxic compounds led the development of anticancer therapeutics for several decades. The induction of apoptosis with cytotoxic compound is known to be an efficient and promising strategy to kill cancer cells [6]. Compounds of natural origin have often provided new leads in the novelty of structures with anticancer activity. In the present study, *Leea indica* was selected for the evaluation of its anti tumor potential against EAC cell line.

Leea indica (Burm.f.) Merr. (Family Leeaceae) is an evergreen perennial shrub with stout, soft wooded, glabrous stems indigenous to tropical Asia, Australasia, Pacific and grown mostly in Bangladesh, India, China, Bhutan and Malaysia. The leaves are claimed to have some medicinal values such as anticancer, antidiabetic, antidiarrhoeal, antidysenteric and antispasmodic based on local uses [7-9]. The ointment prepared from roasted leaves relieves vertigo [10]. The whole plant is used traditionally for headache, body pains and skin complaints [7, 8]. Marma tribes of Chittagong Hill Tracts, Bangladesh, prescribe combined root paste of this plant along with the root of *Oreocnide integrifolia* and *Cissus repens* in bubo and boils [11]. Previous biological studies have shown that it possesses strong antioxidant [12], CNS depression [13], phosphodiesterase and nitric oxide synthase inhibitory activities [14]. Apart from the initial screening against breast cancer cell line [15] and Ca Ski Human Cervical Epidermoid Carcinoma Cell line [16] still there is no report showing the anti tumor activity of *L.indica* against EAC cell lines. Moreover, no detailed mechanism of action underlying the anti tumor activity of *L.indica* had been delineated.

2. Materials and Methods

2.1 Drugs and chemicals

All the chemicals and reagents used throughout the investigation were of reagent grade. DPPH (1, 1-diphenyl, 2-picrylhydrazyl), Methanol and DMSO (dimethyl sulfooxide) were purchased from Sigma Chemical Co. USA, Ascorbic acid and Gallic acid were from SD Fine Chem. Ltd. India.

2.2 Plant material

The plant leaves were collected from the hill forest of Chittagong district in November 2010 when leaves were in their maximum densities. The plant parts were thoroughly washed with water and were dried in hot air oven at room temperature for 7 days and at 40°C for the next 2 days.

2.3 Preparation of plant Extract

The dried leaves were coarsely powdered and about 1000 g of powdered material was macerated with 99% methanol at room temperature for a period of 7 days accompanying occasional shaking and stirring. The whole mixture was then filtered and the filtrate thus obtained was concentrated by using a rotary evaporator (Bibby RE200, Sterlin Ltd, UK) to get a viscous mass. The viscous mass was then kept at room temperature under a ceiling fan to get a dried extract (about 10% of the whole mass). Sample for antitumor assay was prepared

by dissolving the dried methanolic extract in 0.2% DMSO. From this solution, different working dilutions were prepared to get concentration range for 20, 30, 40 mg/kg doses. The standard drug bleomycin was also dissolved in same solvent to make dose of 0.3mg/kg body weight.

2.4 Animals

White albino male mice (Swiss-webstar strain, 20-25 g body weight) were collected from the animal research branch of the International Center for Diarrheal Disease and Research, Bangladesh (ICDDRB). The animals were provided with standard laboratory food and tap water ad libitum and maintained at natural day night cycle. The animals were acclimatized to laboratory condition for one week prior to experimentation.

2.4 Tumor cells

Ehrlich Ascites Carcinoma (EAC) cells were obtained by the Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi-6205, Bangladesh and were maintained by weekly intraperitoneal (i.p.) inoculation of 10^5cells/mouse in the laboratory.

2.5 Ethical clearance

Protocol used in this study for the use of mice as animal model for cancer research was approved by the Rajshahi University Animal Ethical committee (27/08/RUBCMB).

This research work was approved by Ethical Review Committee of Research cell of Rajshahi Medical College, Bangladesh (ref. RMC/ER/2010-2013/01).

2.6 Determination of median lethal dose (LD_{50})

The LD_{50} value was determined following conventional methods [17]. The test compound was dissolved in distilled water and injected intraperitoneally to six groups of mice (each containing 5 mice) at different doses (20, 50, 100, 200, 250 and 400 mg/kg). LD_{50} was evaluated by recording mortality after 24 hours.

2.7 Evaluation of Anticancer potentiality

Anticancer potentiality of methanol extract of the aerial parts of *L.indica* was evaluated by measuring tumor cell growth inhibition, regression of tumor size and increase of survival time.

2.8 Cell growth inhibition

In vivo tumor cell growth inhibition was carried out by the method as described by Sur et al. [18]. For this study, 5 groups of mice (5 in each group) were used. For therapeutic evaluation 14 $\times 10^5$ cells/mouse were inoculated into each group of mice on the first day. Treatment was started after 24 hours of tumor inoculation and continued for 5 days. Group 1 to 3 received the test compound at the doses of 20 mg/kg (i.p.), 30 mg/kg (i.p.) and 40 mg/kg (i.p.) respectively per day per mouse. In each case the volume of the test solution injected (i.p.) were 0.1ml/day per mouse. Group 4 received bleomycin (0.3 mg/kg, i.p.) and finally group 5 was treated with the vehicle (normal saline) and was considered as untreated control. The mice were sacrificed on the 6th day after transplantation and tumor cells were collected by repeated intraperitoneal wash with 0.9% saline. Viable tumor cells per mouse of the treated group were compared with those of control.

The cell growth inhibition was calculated by using the formula,

$$\% \text{ Cell growth inhibition} = \left(1 - \frac{Tw}{Cw}\right) \times 100$$

Where, Tw = Mean of number of tumor cells of the treated group of mice and

Cw = Mean of number of tumor cells of the control group of mice.

2.9 Average tumor weight and survival time

These parameters were measured under similar experimental conditions as stated in the previous experiment. Tumor growth was monitored daily by measuring weight change. The host survival time was recorded and expressed as mean survival time in days and percent increase of life span was calculated [19] as follows:

Mean survival time (MST) =

$$\frac{\sum \text{Survival time in days of each mouse group}}{\text{Total number}}$$

Percent increase of life span (ILS) % =

$$\left(\frac{\text{MST of treated group}}{\text{MST of control group}} - 1\right) \times 100$$

2.10 Antioxidant capacity determination

Antioxidant potentiality of methanol extract of the leaves of *L.indica* was evaluated by determining DPPH radical scavenging activity, total phenol and flavonoid content determination and reducing capacity assays.

DPPH radical scavenging activity: The free radical scavenging capacity of the extracts was determined using DPPH [20, 21]. A methanolic solution of DPPH (0.004% w/v) was mixed with solutions of different concentrations (0 to 500µg) of *L.indica* extracts and after 10 minutes the absorbance was read at 515nm using a spectrophotometer (Shimadzu UVmini-1240, Japan). Ascorbic acid was used as a standard. The inhibition curve was plotted and IC_{50} values were calculated.

Determination of total phenolic content: The total phenolic content of extracts was determined using Folin-Ciocalteu method [22]. The extracts were oxidized with Folin-Ciocalteu reagent and were neutralized with sodium carbonate. The absorbance of the resulting blue color solution was measured at 760nm after 60min using Gallic acid (GA) as standard. Total phenolic content was expressed as mg GA equivalent/gm of extract.

Determination of total flavonoid content: The flavonoid content was determined using a method described by Kumaran and Karunakaran [23] using quercetin as a reference compound. 1 mg of plant extract in methanol was mixed with 1ml aluminium trichloride in Ethanol (20mg/ml) and a drop of acetic acid, and then diluted with Ethanol. The absorption at 415nm was read after 40 minute. The absorptions of blank samples and standard quercetin solution (0.5mg/ml) in methanol were measured under the same conditions.

Reducing power: The reducing power activity of *L.indica* extract was determined according to the method described by Oyaizu [24]. Different concentrations of methanol extract of the leaves of *L.indica* in 1ml of distilled water was mixed with phosphate buffer (2.5ml, 0.2M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5ml, 1%). The mixture was incubated at 50°C for 20min. A portion (2.5ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3,000rpm for 10min. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5ml) and $FeCl_3$ (0.5ml, 0.1%) and the absorbance was measured at 700nm. Ascorbic acid was used as a reference standard. Phosphate buffer was used as blank solution.

2.11 Brine shrimp lethality bioassay (cytotoxicity)

Brine shrimp lethality bioassay was carried out to investigate the cytoxicity of plant extracts [25]. 50mg of *Artemia salina* (Leach) eggs were added to a hatching chamber containing sea water (75ml). The hatching chamber was kept under an inflorescent bulb for 48 hours for the eggs to hatch into shrimp larvae. The matured nauplii were then used in the experiment. For the experiment 3mg of the compound was dissolved in 0.6 mL (600 µL) of distilled water to get a concentration of 5 µg/µL and by serial dilution technique, solutions of varying concentrations such as 5, 10, 20, 40, 80 and 100.0 µg/ml were obtained. After 24 hours of incubation, the vials were observed using a magnifying glass and the number of surviving nauplii in each vial were counted and noted. From this data, the percentage of mortality of the nauplii was calculated for each concentration and the LC_{50} value was determined using Probit analysis as described in the literature [26].

2.12 Statistical analysis

In all the experiments, data were expressed as means ± standard error of mean. A significant difference from the respective control for each experiment was assessed by using one way ANOVA followed by Dunnett 't' test with *P* values <0.05 being regarded as statistically significant.

3. Result

3.1 Median Lethal Dose (LD_{50})

No mortality was noticed up to 250 mg/kg body weight (i.p.), whereas, 100% mortality was noticed at the dose of 400 mg/kg (i.p.). The LD_{50}

of the extracts was found to be 300 mg/kg body weight (i.p.). One-tenth of this dose was selected as the therapeutic dose [27] for the evaluation of anti tumor activity.

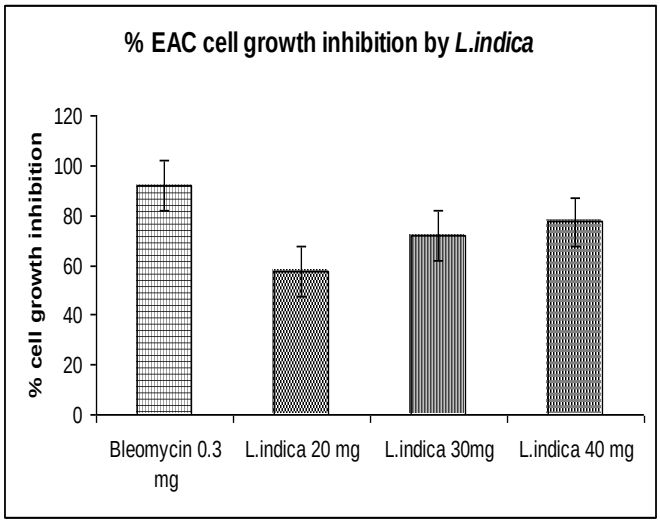

Figure 1. Effect of methanol extract of the leaves of *L.indica* on %EAC cell growth inhibition (In vivo). Values are mean ± SEM, (n = 5); where significant value is **p<0.001, Dunnet test as compared to control.

3.2 Cell growth inhibition

The effects of the different doses of methanol extract of the leaves of *L.indica* at the dose of 20 mg/kg (i.p), 30 mg/kg (i.p), and 40 mg/kg (i.p) and bleomycin at 0.3mg/kg (i.p) per mouse per day on EAC cell growth inhibition (*In vivo*) was observed. Among the three doses tested, the methanol extract at the dose of 40mg/kg body weight showed maximum antitumor activity with 77.29% inhibition of cell growth. This activity was comparable to that of standard drug bleomycin, which showed 92.02% cell growth inhibition when administered similarly at a dose of 0.3 mg/kg (i.p). Whereas the same extract at the dose of 20mg/kg and 30 mg/kg body weight also showed significant activity in a dose dependent manner with 57.48% and 71.98% cell growth inhibition respectively (Figure 1).

3.3 Average tumor weight and survival time

In vivo tumor weight of EAC cell bearing mice after treatment with methanol extract of *L.indica* at the dose of 20mg/kg, 30mg/kg and 40 mg/kg for 20 days was calculated. It was found that tumor weight decreases approximately in a similar manner with bleomycin (0.3 mg/kg). Highest tumor weight reduction (7.90g) was observed at the dose of 40 mg/kg (i.p) compared to the standard drug bleomycin (7.05g). Same extract at the dose of 20 and 30mg/kg (i.p.) showed moderate reduction of tumor cell weight (Figure 2).

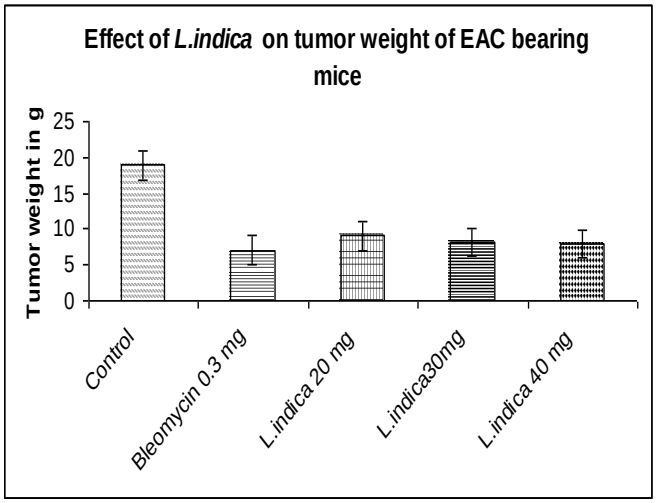

Figure 2. Effect of methanol extract of the leaves of *L.indica* on tumor weight of EAC cell bearing mice. Values are mean ± SEM, (n = 5); where significant value is **p<0.001, Dunnet test as compared to control.

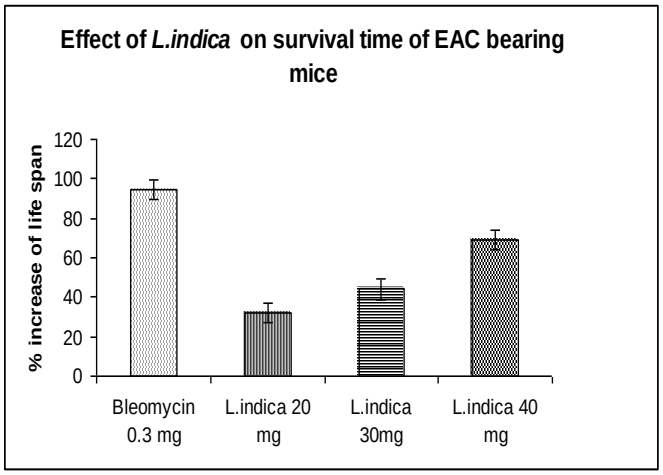

Figure 3. Effect of methanol extract of the leaves of *L.indica* on survival time of EAC cell bearing mice. Values are mean ± SEM, (n = 5); where significant values are,*p<0.01 and **p<0.001, Dunnet test as compared to control.

Mean survival time (MST) of the untreated tumor bearing mice was 15 days. With the treatment of the three different doses of methanol extract of *L.indica*, this value increased remarkably. Maximum 69.33% enhancement of life span was found at the dose of 40 mg/kg (i.p.) whereas at the dose of 20 mg/kg (i.p) and 30 mg/kg (i.p.) the plant extract showed moderate effect– 32% and 44% respectively (Figure 3). Under the same experimental condition, bleomycin at the dose of 0.3 mg/kg (i.p.) increased the survival time to 94.66 % ($p<0.01$).

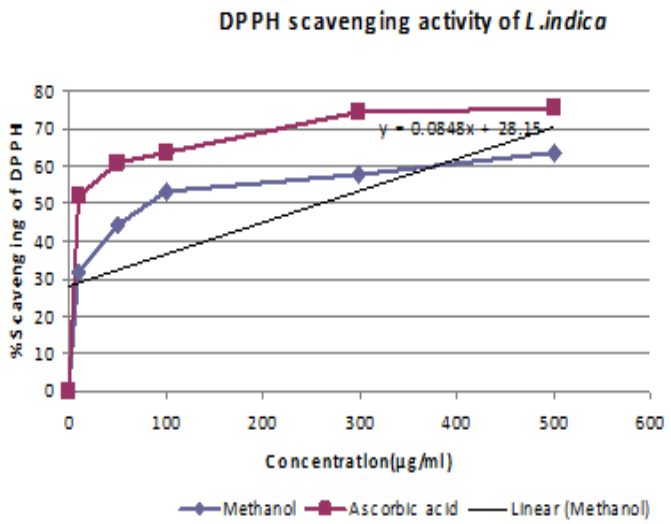

Figure 4. DPPH radical scavenging activity of methanol extract of the leaves of L.indica.

3.4 DPPH radical scavenging activity

The DPPH radical scavenging activity of *L.indica* is shown in Figure 4. This activity was found to increase with increasing concentration of the extracts. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515-517nm and also for a visible deep purple color. The IC_{50} value of the methanol extract was 257.66µg/ml while the IC_{50} value of ascorbic acid was 11.24µg/ml.

3.5 Total phenol and flavonoid content

Table 1 shows the results of total phenol and flavonoid content of methanol extracts of the aerial parts of L.indica. The total phenol and total flavonoid content of the extracts were expressed in gallic acid and quercetin equivalents respectively. The content of phenolics in the extracts under this investigation correlates with the antioxidant activity; it showed moderate results (120.88 mg/g GAE). Flavonoid content of the extract was also found significant (150.14 mg/g quercetin equivalent).

Table 1. Total amount of phenol and flavonoid content of the leave extract of L.indica

Extracts	Total phenol (In mg/g, Gallic acid Equivalents)	Total flavonoid (In mg/g, Quercetin Equivalents)
Methanol extract of the leaves of *L.indica*	120.88	150.14

3.6 Reducing power

Figure 5 represent the reductive capabilities of the plant extracts compared to Ascorbic acid which was determined using the potassium ferricyanide reduction method. The reducing power of the extracts was moderately strong while increasing dose it showed remarkable increment.

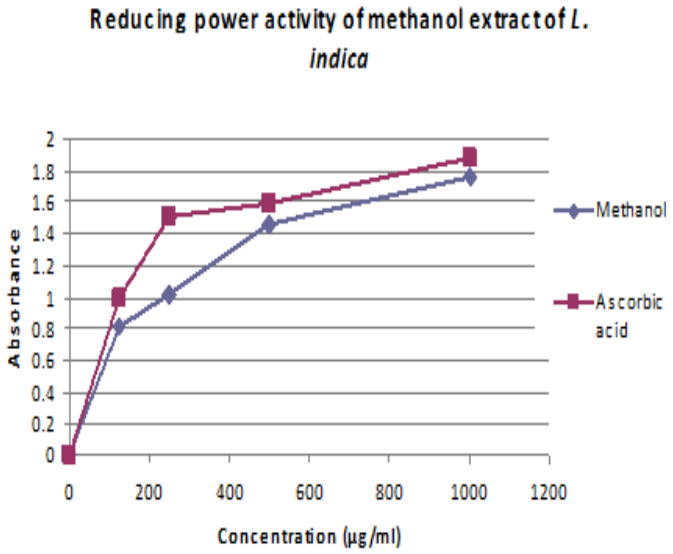

Figure 5. Reducing power activity of the methanol extracts of the leaves of *L.indica* and ascorbic acid.

3.7 Brine shrimp lethality bioassay (LC_{50})

The brine shrimp lethality bioassay was done to assess the *in vitro* cytotoxic effect of the plant extract. Median lethal concentration (LC_{50}) of brine shrimp lethality was found to be 11.56 µg/ml (Figure 6).

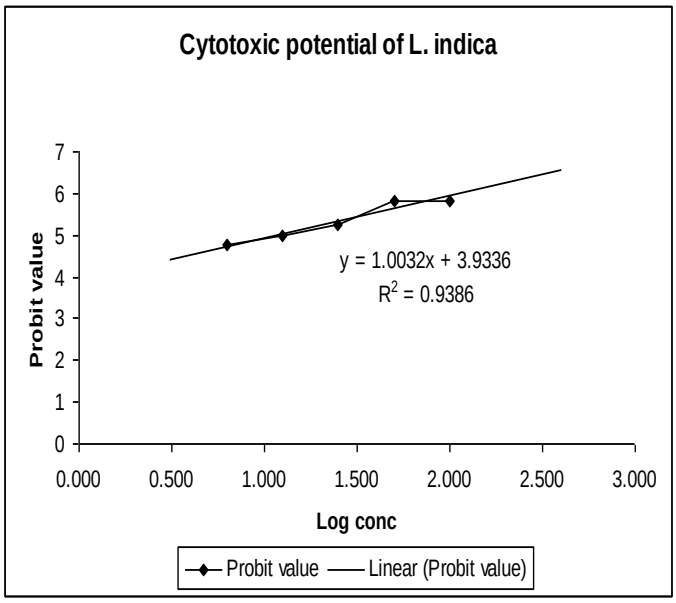

Figure 6: Effects of various log concentrations of **m**ethanolic extract of *L.indica* against brine shrimp nauplii after 24 hrs of incubation.

4. Discussion

Experimental results presented above proved that the methanol extract of *L.indica* at its different doses (maximum effect observed at the dose of 40 mg/kg) can slow down the growth of tumor satisfactorily, reduce tumor weight markedly and increase life span considerably. Our present study results also reflected that the same extract of *L.indica* showed moderate antioxidant and significant cytotoxic activity. All these are measured as very important aspects in justifying the effectiveness of a compound in cancer chemotherapy [28]. Cancer is recognized primarily as a disease of uncontrolled cell division. Hence, all efforts are directed toward the identification of antiproliferative compounds. Accordingly, regression of tumor size and increase of survival time has been recognized as the primary objective end point of effectiveness in preclinical and clinical testing. Conventional screening models for anticancer agents are geared toward the selection of antioxidant and cytotoxic drugs. Antioxidants have been extensively studied for their ability to prevent cancer in human [29]. Several plant species rich in antioxidant flavonoids are reported to reduce disease risk and have therapeutic properties. Their consumption can reduce the risk of cancer [30, 31]. Previous data also prove that flavonoids are biologically active against different strains of bacteria and many human cancer cell lines [32, 33, 34]. Moreover plant phenolics have drawn increasing attention due to their potent antioxidant properties and their marked effects in the prevention of various oxidative stress associated diseases such as cancer [35]. Remarkable cytotoxic activity shown by *L.indica* extracts can be attributed mainly to phenol, flavonoids and Gallic acid. Gallic acid is isolated from the leaves of *L.indica* [36] and active against several cancer cell lines [37]. Cytotoxic compounds triggers apoptosis through two signaling mechanisms – the activation and release of mitochondrial pro-apoptotic proteins known as caspases under the control of Bcl-2 family of proteins or up regulated expression of pro-apoptotic receptors on cancer cells, whose subsequent interaction with their ligands activates apoptotic signaling pathways. These receptors include the Fas (also called APO-1 or CD95) and the tumor necrosis factor (TNF)-related apoptosis-inducing ligands (TRAIL) receptors. Numerous animal studies have been published demonstrating decreased tumor size and/or increased longevity with the combination of chemotherapy and antioxidants [38]. Antitumor activity recorded in the present study is in accordance with this finding, since the antioxidant and cytotoxic study indicated the presence of phenol, flavonoid and Gallic acid in the crude methanol extract of *L.indica*. Although most chemotherapeutic agents cause cellular damage by making lethal injury to DNA through initiating free radical oxidants; however, recent evidence indicates that a sizeable amount of chemotherapy damage is by other mechanism, for example radiation induced failure of mitosis and the inhibition of cellular proliferation, which kill cancer cells [39].

Moreover, apoptosis can be generated by the effect of radiation on cell membrane apparently through lipid peroxidation [40]. Resistance to many chemotherapeutic agents is thought to be due to reduced accumulation in tumor cell [41]. Recent research has focused on the ability of flavonoid type antioxidant compound to increase the concentration of chemotherapeutics in tumor cells. From literature review it was found that the methanol extract of the leaves of *L.indica* possess eleven hydrocarbons, phthalic acid, palmitic acid, 1-eicosanol, solanesol, farnesol, three phthalic acid esters, lupeol, _-sitosterol and ursolic acid in addition to gallic acid [42]. Though the results obtained from the antioxidant, cytotoxicity and antitumor activity study of this plant revealed that methanol extract of *L.indica* could be a rich source of anticancer drugs but still there is no consensus about which substances are exactly responsible for these effects. So, much more investigation including hematological studies have to be carried out with this extract using higher animal models, in order to confirm it as a potent anticancer drug resource.

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