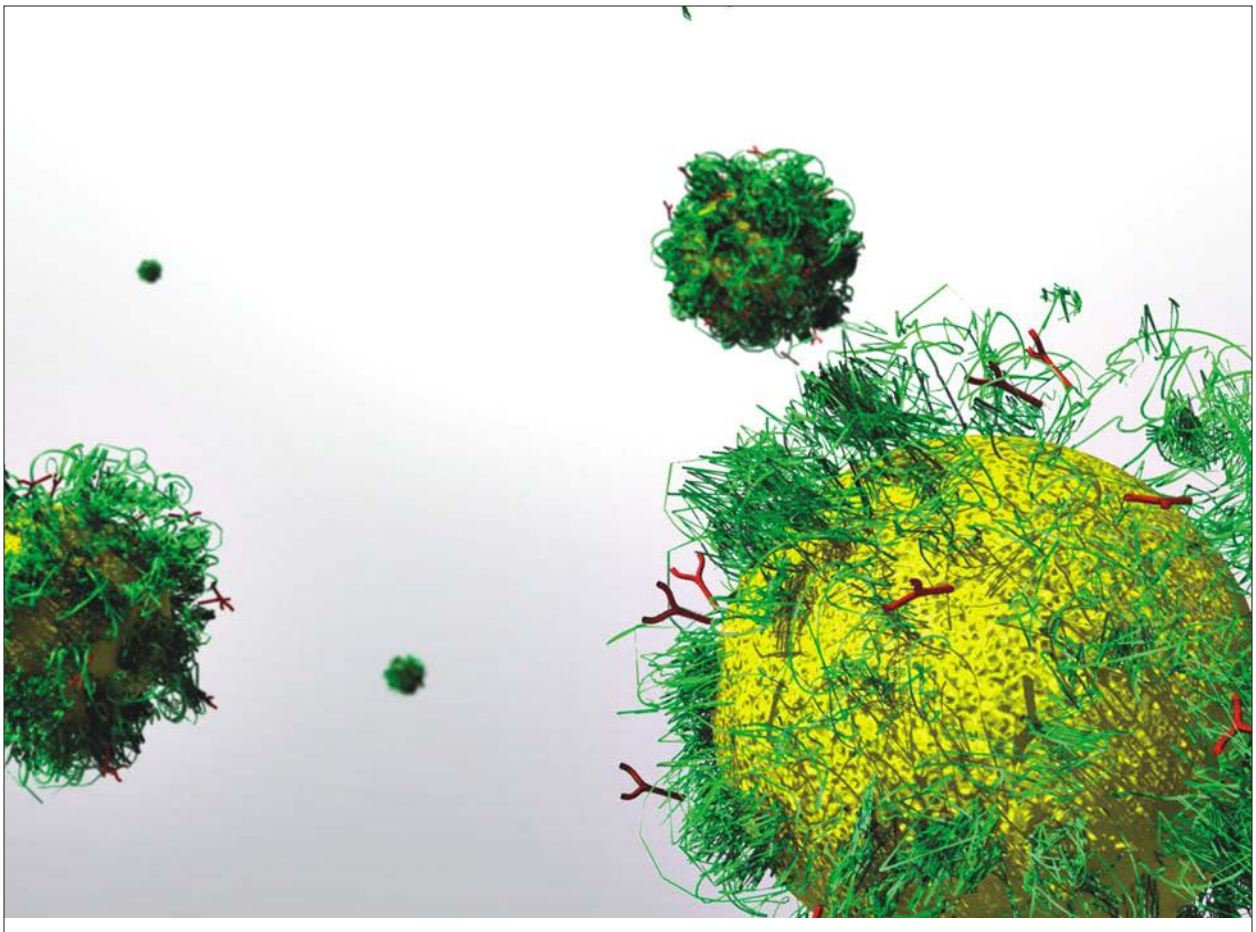

# American Journal of Drug Discovery and Development

ISSN 2150-427X

Academic Journals Inc.

www.academicjournals.com

American Journal of Drug Discovery and Development 2 (3): 124-134, 2012
ISSN 2150-427x / DOI: 10.3923/ajdd.2012.124.134


# *Plumbago zeylanica* L. Root Induced Apoptosis of Ehrlich Ascites Carcinoma Cell

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## ABSTRACT

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 for the discovery of a new anticancer drug. Dried roots from *P. zeylanica* were powdered and extracted with methanol. The root extract then at the dose of 20, 30 and 40 mg $kg^{-1}$ day (i.p.) was evaluated for antiproliferative activity against Ehrlich Ascites Carcinoma (EAC) cells in swiss albino mice. The experimental parameters like tumor cell count, mean survival time and increase in life span were evaluated to assess antiproliferative activity. The extract was administered intrapretoneally for 14 consecutive days to EAC cell bearing group of mice. Bleomycin at the dose of 0.3 mg $kg^{-1}$ (i.p.) was used as a positive control. It has been found that the root extract at the dose of 40 mg $kg^{-1}$ day (i.p.) significantly ($p<0.05$) decreases tumor weight, increases life span and reduces tumor cell growth rate in comparison to those of EAC bearing mice receiving no extract (negative control) in a dose-dependant manner. *In vitro* antioxidant and cytotoxic activity of the same extract were also assessed to link the finding with the strong antiproliferative activity.

**Key words:** Antiproliferative activity, EAC cell, antioxidant, cytotoxicity, *P. zeylenica*

## INTRODUCTION

Cancer is a major public health problem in the United States and many other parts of the world. Currently, one in 4 deaths in the United States is due to cancer (Rebecca *et al*., 2011). In European countries each year over three quarters of a million people die from cancer (Cancer Research Campaign, 1992). A number of natural products have been screened for anticancer activity on various experimental models (Rana *et al*., 2002; Chandrashekhar *et al*., 2011; Saha *et al*., 2011). 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.

*Plumbago zeylanica* L. (Family: Plumbaginaceae) is a pretty perennial shrub with semi woody stems and numerous branches (Chopra *et al.*, 1956). Plant roots are cylindrical irregularly bent having transverse shallow fissures at bents. The plant is popularly known as "Bookchita"or "Chitrak" in Bangladesh. The crude extracts of *P. zeylanica* have been used in China and other Asian countries as folk medicine for the treatment of cancer, rheumatoid arthritis and dysmenorrhea (Itoigawa *et al.*, 1991). Some recent explorations to appraise its use in traditional medicine have been reported in which wound healing (Devender *et al.*, 2011), antibacterial (Jeyachandran *et al.*, 2009), central nervous system stimulatory (Bopaiah and Pradhan, 2001) antiplasmodial (Simonsen *et al.*, 2001), antimicrobial (Ahmad *et al.*, 2000), antifungal (Mehmood *et al.*, 1999), antihyperglycemic (Olagunju *et al.*, 1999), anti-inflammatory (Oyedapo, 1996), hypolipidaemic and antiatherosclerotic activities (Sharma *et al.*, 1991) were comprehensively emphasized. Alkaloid "plumbagin" and five coumarins-seselin (Kostova *et al.*, 2001), 5-methoxyseselin (Kofinas *et al.*, 1998), suberosin (Uchiyama *et al.*, 2002), xanthyletin and xanthoxyletin have been isolated so far from the roots of *Plumbago zeylanica* L. (Lin *et al.*, 2003). Plumbagin (5-hydroxy-2-methylnaphthalene-1, 4-dione) is a naturally occurring yellow pigment accumulated mostly in root (Van Der Vijver, 1972) and some plumbagin derivatives are effective against EAC (Hazra *et al.*, 2008). Literature data indicated that *P. zeylanica* leave extract are effective against EAC cell line (Sachin *et al.*, 2010) but still there is no report showing the effectiveness of root extract of *P. zeylanica* against EAC. In this study we have attempted to investigate the antiproliferative activity of the root extract that will certainly help to proceed with all individual isolated compounds from the root to assess which other compounds in addition to "plumbagin" are exactly responsible for this apoptotic activity of EAC cell. Overall, this research work may lead to explore new cancer chemotherapeutic agents with novel structures and/or mechanisms of action against EAC.

## MATERIALS AND METHODS

**Drugs and chemicals:** All the chemicals and reagents used through out 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.

**Plant material:** The plant roots were collected from the forest of Meherpur district in May 2011 when roots were in their maximum densities. The plant roots were thoroughly washed with water, dried in hot air oven at room temperature for 7 days and at 40°C for the next 2 days.

**Preparation of plant extract:** The dried roots 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%). The extract thus prepared was used for study.

**Animals:** White albino male mice (Swiss-webstar strain, 20-25 g body weight) were collected from the animal research branch of Pharmacy Department, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh. 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.

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

**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).

**Acute toxicity study ($LD_{50}$):** The $LD_{50}$ value was determined following conventional methods (Litchfield and Wilcoxon, 1949). The test compound was dissolved in distilled water and injected intraperitoneally to six groups of mice (each group containing 5 mice) at different doses (20, 50, 100, 200, 300 and 500 mg $kg^{-1}$). $LD_{50}$ was evaluated by recording mortality after 24 h.

**Evaluation of anticancer potentiality:** Anticancer potentiality of methanol extract of the aerial parts of *P. zeylanica* was evaluated by measuring tumor cell growth inhibition, regression of tumor size and increase of survival time.

**Cell growth inhibition:** *In vivo* tumor cell growth inhibition was carried out by the method as described by Sur and Ganguly (1994). For this study, 5 groups of mice (5 in each group) were used. For therapeutic evaluation $14\times10^5$ cells/mouse were inoculated into each group of mice on the first day. Treatment was started after 24 h of tumor inoculation and continued for 5 days. Group 1 to 3 received the test compound (effective dose selected on the basis of 1/10 of $LD_{50}$ value) at the doses of 20 mg $kg^{-1}$ (i.p.), 30 mg $kg^{-1}$ (i.p.) and 40 mg $kg^{-1}$ (i.p.), respectively per day per mouse. In each case the volume of the test solution injected (i.p.) were 0.2 mL per day per mouse. Group 4 received bleomycin (0.3 mg $kg^{-1}$, 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. The ascitic fluid was taken in a hematocrit (micro) tube and diluted 1000 times. Then a drop of the diluted cell suspension was placed on the neubauer counting chamber and the cells in 64 small squares were counted. 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:

$$\% \text{ Cell growth inhibition} = (1-\frac{Tw}{Cw})\times100$$

where, Tw is mean of number of tumor cells of the treated group of mice and Cw is mean of number of tumor cells of the control group of mice.

**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 (Abbot, 1976) as follows:

$$\text{Mean survival time (MST)} = \frac{\sum \text{Survival time in days of each mouse group}}{\text{Total number}}$$

$$\text{Percent increase of life span (ILS) \%} = \left(\frac{\text{MST of treated group}}{\text{MST of control group}}\right) \times 100$$

**Antioxidant capacity determination:** Antioxidant potentiality of methanol extract of the aerial parts of *P. zeylanica* 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 (Hasan *et al.*, 2006; Alam, 2008). A methanolic solution of DPPH (0.004% w/v) was mixed with solutions of different concentrations (0 to 500 μg) of *P. zeylanica* extracts and after 10 min the absorbance was read at 515 nm 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 (Singleton *et al.*, 1999). 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 760 nm after 60 min 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 (2007) using quercetin as a reference compound. One milligram of plant extract in methanol was mixed with 1 mL aluminium trichloride in Ethanol (20 mg $mL^{-1}$) and a drop of acetic acid and then diluted with Ethanol. The absorption at 415 nm was read after 40 min. The absorption of blank samples and standard quercetin solution (0.5 mg $mL^{-1}$) in methanol was measured under the same conditions.

**Reducing power:** The reducing power of *P. zeylanica* extractives was determined according to the method described by Oyaizu (1986). Different concentrations of *P. zeylanica* extracts in 1 mL of distilled water was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) and the absorbance was measured at 700 nm. Ascorbic acid was used as a reference standard. Phosphate buffer was used as blank solution.

**Brine shrimp lethality bioassay:** Brine shrimp lethality bioassay was carried out to investigate the cytoxicity of plant extracts (Meyer *et al.*, 1982). Fifty milligram of *Artemia salina* (Leach) eggs were added to a hatching chamber containing sea water (75 mL). The hatching chamber was kept

under an inflorescent bulb for 48 h for the eggs to hatch into shrimp larvae. The matured nauplii were then used in the experiment. For the experiment 3 mg of the compound was dissolved in 0.6 mL (600 μL) of distilled water to get a concentration of 5 μg $\mu L^{-1}$ and by serial dilution technique, solutions of varying concentrations such as 5, 10, 20, 40, 80 and 100.0 μg $mL^{-1}$ were obtained. After 24 h 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 (Clarkson and Burichenal, 1965).

**Statistical analysis:** The experimental results have been expressed as the Mean±SEM (Standard Error of Mean). Data have been calculated by one way ANOVA followed by Dunnett 't' test using SPSS software (version 10). P values<0.05 were considered significant.

## RESULT

**Acute toxicity study ($LD_{50}$):** No mortality was noticed up to 300 mg $kg^{-1}$ body weight (i.p.), whereas, 100% mortality was noticed at the dose of 500 mg $kg^{-1}$ (i.p.). The $LD_{50}$ of the extracts was found to be 400 mg $kg^{-1}$ body weight (i.p.). One-tenth of this dose was selected as the therapeutic dose (Jalalpure *et al*., 2003) for the evaluation of antiproliferative activity.

**Cell growth inhibition:** The effects of the different doses of methanol extract of the aerial parts of *P. zeylanica* at the dose of 20 mg $kg^{-1}$ (i.p), 30 mg $kg^{-1}$ (i.p) and 40 mg $kg^{-1}$ (i.p) and bleomycin at 0.3 mg $kg^{-1}$ (i.p.) per mouse per day on EAC cell growth inhibition (*In vivo*) was observed. Methanol extract at the dose of 40 mg $kg^{-1}$ body weight showed maximum antiproliferative activity with 81.90% inhibition of EAC cell growth. The activity was comparable to that of standard drug bleomycin, which showed 89.81% cell growth inhibition when administered similarly at a dose of 0.3 mg $kg^{-1}$ (i.p). The same extract at the dose of 20 mg $kg^{-}$ and 30 mg $kg^{-}$ body weight also showed significant activity in a dose dependent manner with 72.85 and 73.82% cell growth inhibition, respectively (Fig. 1).

**Average tumor weight and survival time:** *In vivo* tumor weight of EAC cell bearing mice after treatment with methanol extract of *P. zeylanica* at the dose of 20, 30 and 40 mg $kg^{-1}$ for 20 days was calculated. It was found that tumor weight decreases approximately in a similar manner with

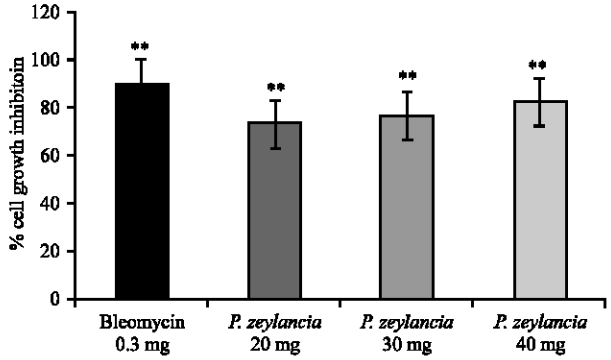


Fig. 1: Effect of methanol extract of the roots of *P. zeylanica* on cell growth inhibition EAC cell bearing mice (*in vivo*). Values are Mean±SEM, (n = 5), where significant values are, **p<0.001, as compared to control

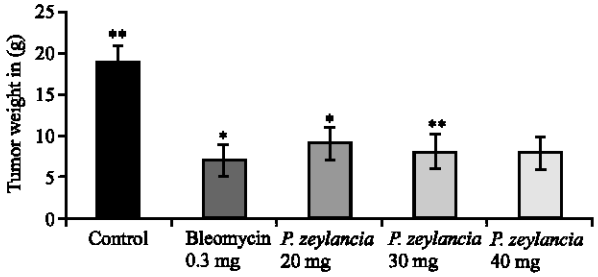


Fig. 2: Effect of methanol extract of the roots of *P. zeylanica* on tumor weight of EAC cell bearing mice. Values are Mean±SEM, (n = 5), where significant values are,*p<0.01 and **p<0.001, as compared to control

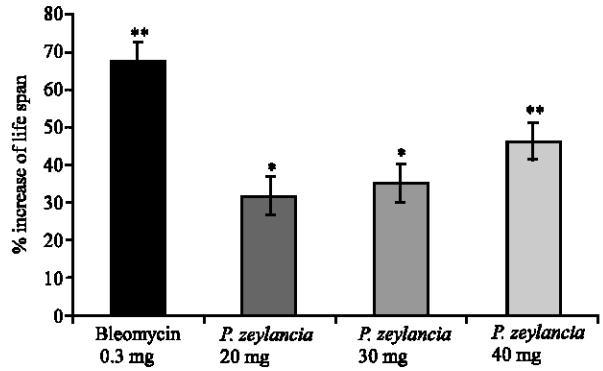


Fig. 3: Effect of methanol extract of the roots of *P. zeylanica* on survival time of EAC cell bearing mice. Values are Mean±SEM, (n = 5), where significant values are,*p<0.01 and **p<0.001, as compared to control

bleomycin (0.3 mg $kg^{-1}$). Highest tumor weight reduction (7.83 g) was observed at the dose of 40 mg $kg^{-1}$ (i.p.) compared to the standard drug bleomycin (7.05 g). Whereas, same extract at the dose of 20 and 30 mg $kg^{-1}$ body weight showed moderate reduction of tumor cell weight (Fig. 2).

Mean Survival Time (MST) of the untreated tumor bearing mice was 17.80 days. With the treatment of the three different doses of methanol extract of *P. zeylanica*, this value increased remarkably. Maximum of 46.06% enhancement of life span was found at the dose of 40 mg $kg^{-1}$ (i.p.) while at the dose of 20 mg $kg^{-1}$ (i.p.) and 30 mg $kg^{-1}$ (i.p.) the plant extract showed moderate effect having enhancement of life span of 31.46 and 34.83%, respectively (Fig. 3). Under the same experimental condition, bleomycin at the dose of 0.3 mg $kg^{-1}$ (i.p.) increased the MST value to 37.82% ($p<0.01$).

**DPPH radical scavenging activity:** The DPPH radical scavenging activity of *P. zeylanica* is shown in Fig. 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 515-517 nm and also for a visible deep purple color. The $IC_{50}$ value of the methanol extract was 393.66 µg $mL^{-1}$ while the $IC_{50}$ value of ascorbic acid was 15.04 µg $mL^{-1}$.

**Total phenol and flavonoid content:** Table 1 shows the results of total phenol and flavonoid content of methanol extracts of the aerial parts of *P. zeylanica*. The total phenol and total flavonoid

Table 1: Total amount of plant phenolics and flavonoid content of *P. zeylanica*

| Extract | Total phenol (mg $g^{-1}$ gallic acid equivalents) | Total flavonoid (mg $g^{-1}$ quercetin equivalents) |
|---|---|---|
| Methanol extract of the roots of *P. zeylanica* | 191.01±0.361 | 200.44±0.097 |

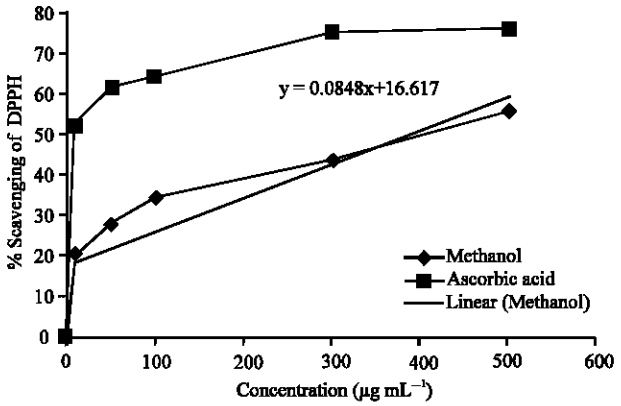


Fig. 4: DPPH radical scavenging activity of methanol extract of the roots of *P. zeylanica*

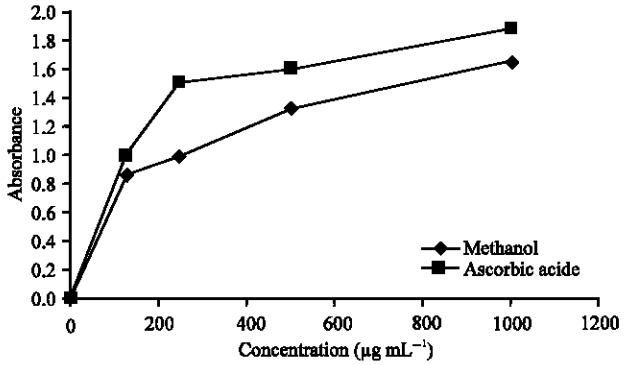


Fig. 5: Reducing power activity of the methanol extracts of the roots of *P. zeylanica* and ascorbic acid

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 (191.01 mg $g^{-1}$ GAE). Flavonoid content of the extract was also found significant (200.44 mg $g^{-1}$ quercetin equivalent).

**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.

**Brine shrimp lethality bioassay ($LC_{50}$):** The brine shrimp lethality bioassay was done to assess the *in vitro* cytotoxic effect of the compound. Median Lethal Concentration ($LC_{50}$) of brine shrimp lethality was found to be 27.87 µg $mL^{-1}$ (Fig. 6).

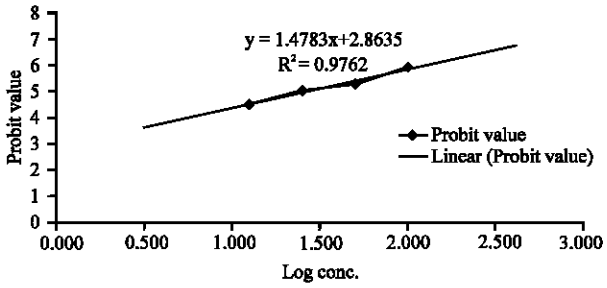


Fig. 6: Effects of methanol extract of the roots of *P. zeylanica* against brine shrimp nauplii after 24 h of incubation

## DISCUSSION

Apoptosis is a form of cell death characterized by active suicide of cells. Our studies have shown that *P. zeylanica* root was effective in imparting growth inhibition, cell cycle deregulation and apoptosis in EAC cells. The reliable criterion for judging the value of any antiproliferative drug is the reduction of tumor cell growth, prolongation of lifespan of the treated animal and decreased tumor weight (Clarkson and Burchenal, 1965). Investigational results presented above proved that the methanol extract of the root of *P. zeylanica* at its different doses (maximum effect observed at the dose of 40 mg $kg^{-1}$) can slow down the growth of tumor satisfactorily ($p<0.05$), reduce tumor weight markedly ($p<0.05$) and increase life span considerably ($p<0.05$). Present study results also indicated that the same extract of *P. zeylanica* showed significant antioxidant and cytotoxic activity. All these are measured as very important aspects in justifying the effectiveness of a compound in cancer chemotherapy (Price and Greenfield, 1958). Traditional screening models for antiproliferative agents are geared toward the selection of antioxidant and cytotoxic drugs (Islam *et al*., 2011; Ali *et al*., 2010; Khorshid, 2011). Antioxidants have been extensively studied for their ability to prevent cancer in human (Singh and Lippman, 1998). Several plant species rich in antioxidant flavonoids are reported to reduce disease risk and have high therapeutic values for the treatment of cancer (Ferguson *et al*., 2004; Abd-Elhady, 2012). Literature data also proved that flavonoids are biologically active against different strains of bacteria and many human cancer cell lines (Havsteen, 2002; Cibin *et al*., 2006). In addition, 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 (Jin and Russell, 2010). Notable cytotoxic activity of the root extract of *P. zeylanica* could be attributed mainly to phenol, flavonoids and alkaloid plumbagin. Alkaloids are derived from plants and block cell division by preventing microtubule function. There is increasing evidence showing that even minor alteration of microtubule dynamics can engage the spindle checkpoint, arresting cell cycle progression at mitosis and eventually leading to apoptotic cell death. Furthermore, cytotoxic compounds trigger 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 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 (Chinery *et al*., 1997). Antiproliferative 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 alkaloid "plumbagin" in the root extract of *P. zeylanica*. From literature review it was found that the methanol extract of the roots of *P. zeylanica* possesses alkaloid, carbohydrate, flavonoid, tannin, saponin and glycoside compounds (Devender *et al.*, 2011). New findings within the past few years have also revealed that the binding of selective glycosides compound to Na+, K+-ATPase results in complex but well-documented changes in cell signaling events (Newman *et al.*, 2008). This "signalosome" complex includes the enzyme, Na+, K+-ATPase as well as Src, Phosphoinositide-3 Kinase (PI3K) and phospholipase C each of which, in turn, sets into action complex signaling events that can result in tumor cell death through either apoptosis or autophagy-related mechanisms (Xie and Cai, 2003). Since the findings obtained from the present investigations demonstrated that the root extract of *P. zeylanica* could be a best example of antiproliferative drugs against EAC cell, So, much more analysis with all individual isolated compounds from the roots have to be carried out using higher animal models, in order to authenticate it as a potent antiproliferative drugs source against EAC cell.

**ACKNOWLEDGMENT**

The authors thank to Prof. Dr. Jahanara Khanom (Department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh) for providing the EAC cells and also to Prof. Dr. Shaikh Bokhtear Uddin (Department of Botany, University of Chittagong, Bangladesh) for identification of the plant.

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