

Antioxidant and antitumor activity of chloroform extract of *Alangium salvifolium* flowers

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Abstract

The present study was designed to investigate the antioxidant and antitumor activities of *Alangium salviifolium* (AS) wang flowers. Antioxidant potential of the AS extract was evaluated *in vitro* by DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay and reducing power assay method. AS extract showed scavenging activity in all the methods with IC₅₀ value of 182.31 ± 0.31 µg/ml for DPPH assay method. In reducing power assay, AS extract also showed significant (p<0.001) activity. In addition, total phenolic and total antioxidant capacity were also determined. The antitumor effect of the flowers of AS against Ehrlich ascites carcinoma (EAC) in mice at the doses of 10 mg/kg body weight intraperitoneally. Significant (p<0.001) increases of survival times 32.4 ± 0.77 days for chloroform extract of the (10 mg/kg) treated tumor bearing mice were confirmed with respect to the control group (22 ± 0.12 days). From the result it was showed that the extract has significant antioxidant as well as antitumor activity.

Keywords: Antitumor, Ehrlich ascites carcinoma, *Alangium salviifolium*, Cancer

Introduction

Cancer is the abnormal growth of cells in our bodies that can lead to death. Cancer cells usually invade and destroy normal cells. These cells are born due to imbalance in the body and by correcting this imbalance, the cancer may be treated. Billions of dollars have been spent on cancer research and yet we do not understand exactly what cancer is. Free radical, one of the major cause for the conversion of normal cell to cancerous cells, are generated as a consequences of a number of endogenous metabolic processes involving redox

enzymes and bioenergetics electron transfer and exposure to a plethora of exogenous chemicals (Rajkumar et al., 2011). However, overproduction of free radical and reactive oxygen species (ROS) would assault on important biological molecules such as DNA, protein or lipid leading to many degenerative diseases, such as cancer, Alzheimer's, arthritis and ischemic reperfusion (Suja et al., 2004). More and more evidence suggests that this potentially cancer-inducing oxidative damage might be prevented or limited by antioxidant. Antioxidant may mediate their effect by directly reacting with ROS, quenching them or chelating the catalytic metal ions (Sun et al., 2002). It has been shown that antioxidant rich diets can reduce oxidative damage to DNA, thus preventing a critical step in the onset of carcinogenesis and the impact of antioxidants on mutagenesis and carcinogenesis has been well established (Zhang et al., 2008; Meyskens et al., 2005). Plants have a long history of use in the treatment of cancer. Over 60% of currently used anti-cancer agents are derived in one-way or another from natural sources, including plants, marine organisms and microorganisms (Newman et al., 2003). A number of plants have been accepted currently as one of the main source of cancer chemoprevention drug discovery and development (Gonzales et al., 2006) due to their diverse pharmacological properties including cytotoxic and cancer chemo preventive effects (Gupta et al., 2004; Dahiru et al., 2008). Hence, the natural products now have been contemplated of exceptional value in the development of effective anticancer agents with minimum host cell toxicity.

Alangium salviifolium wang is a deciduous, rambling shrub or a tree belonging to the family Alangiaceae (Jubie et al., 2008). The different parts of this plant are used for a wide range of diseases. Root is used in diarrhoea, paralysis, piles, vomiting (Pandey et al., 2005) and is useful for external application in acute case of rheumatism, leprosy and inflammation (Anjaria et al., 2002). Antibacterial compound was isolated from the flower of *Alangium salvifolium* (Anjum et al 2002). The plant has been reported for its anti-tubercular, anti-spasmodic and anti-cholinesterase activity (Warrier et al., 2005). Anti-Fertility activity of the stem Bark of *Alangium salvifolium* (Linn.F) Wang in Wistar female rats has also been reported (Murugan et al., 2000). Previous phytochemical investigation revealed that it is a rich source of alkaloids including ipecac alkaloid and benzopyridoquinolizidine alkaloids. It is also known to produce alangiside, a tetrahydroisoquinoline monoterpene glucoside (Itoh et al., 1992). Recent phytochemical studies of this plant resulted in the isolation of several flavonoid, phenolic compound, irridoid glycosides and oxyoglucoside of some alcohol (Ramni et al., 2003). New alkaloid, ankorine was isolated from leaves (Jain et al., 2002). Plant is also rich in tetrahydroisoquinoline monoterpene glycoside. e.g. alangiside-1 or ipecoside-2 whose structures are closely related to the ipecac alkaloid (Itoh et al., 1994). Two sterol alangol and alengol were isolated from seed kernels (Pakrashi et al., 1968). The present study was carried out to evaluate the antioxidant and antitumor activity of the chloroform extract of the flowers of *Alangium salvifolium* against Ehrlich ascites carcinoma (EAC) in mice.

Materials and methods

Plant materials

The flowers of the *Alangium salvifolium* were collected from the adjoining area of Rajshahi University Campus, Bangladesh during February 2007 and were identified by

Taxonomist, Department of Botany and University of Rajshahi, Bangladesh where a voucher specimen number (Voucher No. 105) 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, 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 extracts

The flower material was shade-dried with occasional shifting and then powdered with a mechanical grinder, passing through sieve #40, and stored in a tight container. The powdered flower (750gm) was taken in large glass bottle and extracted with chloroform for 7 days. The procedure was repeated twice using same solvent system for next 3 days. The extract was decanted first through a cotton plug and finally filtered through filter paper to get clear filtrate. The filtrate obtained by repeated maceration was evaporated under reduced pressure at 40°C using Rotary evaporator. The net weight of dry extract was 8.25 gm.

Phytochemical screening

The extract was subjected to qualitative chemical investigation for the identification of different phytoconstituents like sterols, glycosides, saponins, carbohydrates, alkaloids, flavonoids, tannins, proteins and triterpenoids (Yarnalkar, 1991).

Animals

Albino mice (25-30g) and Wistar rats (175-250 g) of both sexes were used for assessing 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 five animals which were fasted overnight prior to the experiments. Experiments on animals were performed in accordance with guidelines of the Institutional Animal Ethics Committee, Rajshahi University, Rajshahi, Bangladesh.

Acute toxicity

The acute oral toxicity of plant in male Swiss albino mice was studied as per reported method (Lorke, 1983).

In vitro antioxidant activity

The amount of phenolic compounds

The total phenolic content of chloroform extract of AS was determined using Folin–Ciocalteu reagent (Yu et al., 2002). The content of total phenolics in the extract of AS was calculated from regression equation of the calibration curve ($y = 0.013x + 0.127$, $r^2 = 0.988$) and is expressed as Gallic acid equivalents (GAE).

Determination of total antioxidant capacity

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method (Prieto et al., 1999). 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-picryl-hydrazyl (DPPH)

The free radical scavenging activity of chloroform 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 (Braca et al., 2001). 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 ($\mu\text{g/ml}$) versus % inhibition.

Reducing power activity

The reducing power of AS was determined according to the method described by Oyaizu (Oyaizu, 1986). 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), Calcutta, 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 tumor bearing) of the tumor cells. Each animal received 0.1 ml of tumor cell suspension containing 2×10^6 tumor cells intraperitoneally.

Treatment schedule

48 Swiss albino mice were divided into four groups ($n = 12$) and given food and water *ad libitum*. All the animals in each groups except Group-I received EAC cells (2×10^6 cells/mouse i.p.). This was taken as day '0'. Group-I served as normal saline control (5 ml/kg i.p.) and Group-II served as EAC control. 24-h after EAC transplantation, Group-III received extract of *Alangium salvifolium* flower at a dose of 10 mg/kg i.p. for nine consecutive days,

respectively. Group-IV received reference drug Bleomycin (0.3 mg/kg i.p) for nine consecutive days (Mazumder et al., 1997). Twenty-four hours of last dose and 18 h of fasting, 6 animals of each group were sacrificed by cervical dislocation to measure antitumor activity and the rest were kept with food and water *ad libitum* to check percentage increase in life span of the tumor host. The antitumor activity of the extract of *Alangium salvifolium* flower was measured in EAC animals with respect to the following parameters.

Tumor cell count

The ascitic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubauer's counting chamber and the numbers of cells in the 64 small squares were counted.

Viable/nonviable tumor cell count

The viability and nonviability of the cell were checked by trypan blue assay. 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.

$$\text{Cell count} = (\text{Number of cells} \times \text{dilution factor}) / (\text{Area} \times \text{thickness of liquid film})$$

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) as per the following formula (Sur et al., 1994).

$$\text{Mean survival} = (\text{Day of First death} + \text{Day of last death})/2$$

$$\% \text{ ILS} = \left[\frac{\text{Mean survival time of the treated group}}{\text{Mean survival time of the treated group}} - 1 \right] \times 100$$

Statistical analysis

All data are expressed as mean \pm S.E.M. (n = 6 mice per groups). Statistical significance (p) calculated by Student's t test. $p < 0.001$, and < 0.05 were considered to be statistically significant.

Results

Phytochemical Screening

The phytoconstituents were identified by various chemical tests which showed the presence of alkaloids, tannins, phenolic and flavonoid compounds and steroid in chloroform extract of *Alangium salvifolium* (Table 1).

Acute toxicity studies

The acute toxicity studies mainly aims at establishing the therapeutic index, i.e., the ratio between the pharmacologically effective dose and the lethal dose on the same strain and species.

Table 1. Result of chemical group tests of the chloroform extract of *Alangium salvifolium* flower.

| Extract | Carbohydrate | Tannin | Flavonoid | Saponin | Phenol | Steroid | Alkaloid |
|-----------------------------|--------------|--------|-----------|---------|--------|---------|----------|
| <i>Alangium salvifolium</i> | - | ++ | ++ | - | ++ | + | +++ |

Ch: Chloroform extract; (+): Present; (-): Absent; (+++): Reaction intensity is high; (++): Reaction intensity is medium; (+): Reaction intensity is normal.

The extract of *A. salvifolium* was safe up to a dose of 1000 mg/kg (p.o.) body weight behavior of the animals was closely observed for the first 3h then at an interval of every 4h during the next 48h. The extract did not cause mortality in mice and rats during 48h observation but little behavioral changes, locomotor ataxia and diarrhea and weight loss were observed. Food and water intake had no significant difference among the group studied.

Total phenolic contents

The total phenols content was found to be 215.51 ± 0.05 mg/g plant extract (in GAE); presented in Table 2.

Total antioxidant capacity

Percentage yield of chloroform extract of AS and its total antioxidant capacity are given in Table 2. Total antioxidant capacity of AS flower extract is expressed as the number of equivalents of ascorbic acid and was found to be 129.10 ± 0.05 mg/g equivalent of ascorbic acid.

DPPH radical scavenging activity

The percentage (%) scavenging of DPPH radical was found to be concentration dependent i.e. concentration of the extract between 5-200 μ g/ml increasing the inhibition activity (Figure 1). The IC_{50} value of the extract of AS was 182.31 ± 0.31 μ g/ml, while ascorbic acid showed the value of 30.12 ± 0.11 μ g/ml.

Reducing power assay

For the measurement of the reductive ability, we investigated the Fe^{3+} to Fe^{2+} transformation in the presence of chloroform extract of AS. Like the antioxidant activity, the

Table 2. Total amount of plant phenolic compounds and total antioxidant capacity of chloroform extract of *Alangium salviifolium* flowers.

| Sample | Total phenols mg/g plant extract (in GAE) ^a | Total antioxidant capacity mg/g extract (in ASC) ^b |
|------------|--|---|
| AS extract | 215.51 ± 0.05 | 129.10 ± 0.05 |

^a Gallic acid equivalents (GAE, mg/g of each extract) for the total phenolic content.

^b Ascorbic acid equivalents (mg/g of each extract) for the total antioxidant capacity. The GAE and ASC values are expressed as means \pm SEM of triplicate experiments.

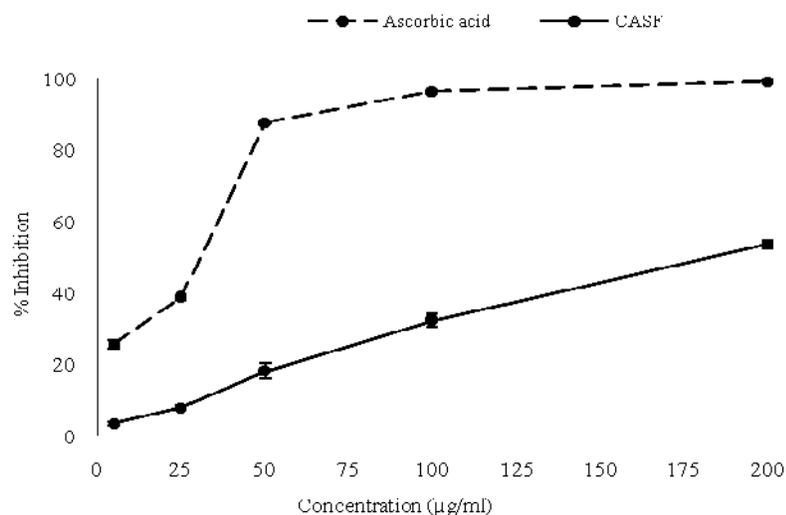


Figure 1: Free radical scavenging activity of different concentrations of chloroform extract of *Alangium salviifolium* flowers and ascorbic acid by DPPH radicals

reducing power of AS extract increased with increasing concentration of the sample and effect was statistically significant ($p < 0.001$). Figure 2 shows the reductive capabilities of the AS compared with quercetin, galic acid and ascorbic acid

Tumor growth and survival parameters

Antitumor activity of extract against EAC tumor bearing mice was assessed by the parameters such as cell count (viable and non viable), mean survival time and % increase of life span. The results are shown in Table 3. The viable cell count were found to be significan-

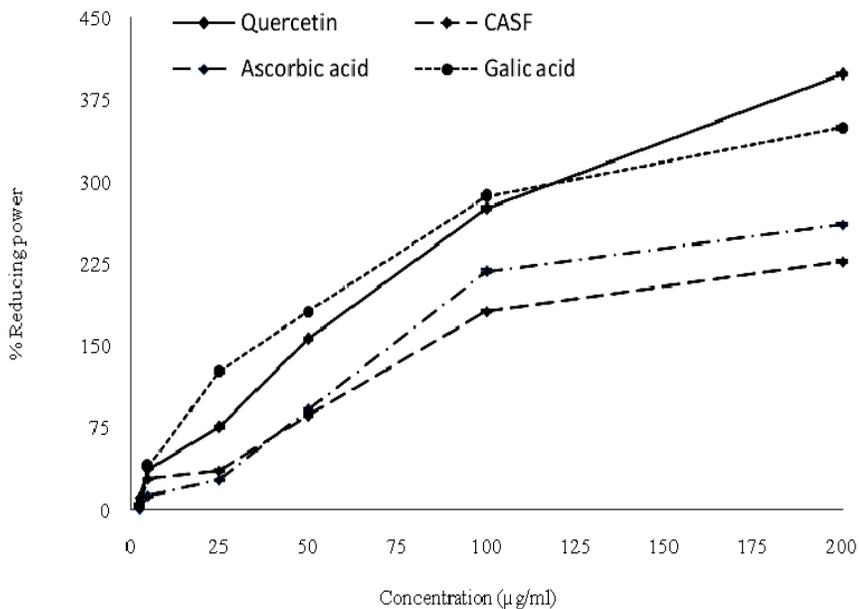


Figure 2: Reducing power of chloroform extract of *Alangium salviifolium* and standards (quercetin, ascorbic acid and galic acid) by spectrophotometric detection of Fe^{3+} to Fe^{2+} transformation.

Table 3. Effect of the chloroform extract of *Alangium salvifolium* flower on mean survival time (MST), percentage increase life span (% ILS), viable and non-viable tumor cell count in EAC bearing mice.

| Parameter | EAC control | Chloroform extract | Bleomycin |
|---|-------------|--------------------|-------------|
| MST (days) | 22 ± 0.12 | 32.4 ± 0.77 | 44.6 ± 0.12 |
| % ILS | 00.0 | 57.3 | 96.81 |
| Viable cell (x 10 ⁷ cell/ml) | 8.1 ± 0.22 | 0.23 ± 0.05* | 0.5 ± 0.05* |
| Non-viable cell (x 10 ⁷ cell/ml) | 0.5 ± 0.24 | 0.76 ± 0.54* | 3.3 ± 0.05* |
| Total cell (x 10 ⁷ cell/ml) | 8.6 ± 0.15 | 0.99 ± 0.21* | 3.8 ± 0.05* |
| Viable % | 94.18 | 23.7 | 13.15 |
| Non-viable % | 5.82 | 76.6 | 86.85 |

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

tly (*p*<0.001) increased and non-viable cell count was significantly (*p*<0.001) low in EAC control animals when compared with normal control animals. Administration of chloroform extract at a dose of 10mg/kg significantly (*p*<0.05) decreased viable cell count. Furthermore, the median survival time was increase to 32.40 ± 0.77 (% ILS = 57.3) on administration of chloroform extract at a dose of 10 mg/kg.

Discussion

To determine the efficacy of natural antioxidants either as pure compounds or as plant extract, a great number of *in vitro* methods have been developed in which antioxidant compounds act by several mechanisms. 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 (Prieto et al., 1999). DPPH, is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Nakayama, 1994) and is usually used as a substrate to evaluate the antioxidant activity of a compound (Chang et al., 2002). Based on the data obtained from this study, DPPH radical scavenging activity of AS extract showed IC₅₀ value 182.31 ± 0.31 µg/ml, while ascorbic acid showed the value of 30.12 ± 0.11µg/ml. It was revealed that AS extract did show the proton donating ability and could serve as free radical inhibitor or scavenger. In fact, the radical scavenging capability of phenolic compounds are due to their hydrogen donating ability/number of hydroxyl groups present, which in turn is closely related both to the chemical structure and spatial conformation, that can modify the reactivity of the molecules (Gorelik et al., 2008) .In the present study this possibility is supported by the estimation of total polyphenols (Gajula et al., 2009) which was found to be present in the *Alangium* sp. extracts.

The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Duh et al., 1999). 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 chelation potential (El-Seedi et al., 2002; Hsu, 2006). Our results suggest that the antioxidant activity of AS extract might be attributed to the phenolic and flavonoids, which detected by phytochemical analysis in our study.

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 means to meet the nutritional requirement of tumor cells (Prasad et al., 1994). Treatment with AS chloroform extract reduced the intraperitoneal tumor burden, thereby reducing the 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 (Clarkson et al., 1965). It can therefore be inferred that chloroform extract increased the life span of EAC bearing mice may be due to decrease the nutritional fluid volume and delay the cell division (Sur et al., 1997). This hypothesis is strongly supported by the previous study, wherein *Aristolochia indica* increase the life span 47% at a dose of 50 mg/kg body weight (Rana et al., 2002). In this study, chloroform extract increase the non viable cell count upto 72.92% at a dose of 10mg/kg, which agree with our previous study (Khatune et al., 2003) and suggested that 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 (Kennedy et al., 2001).

A number of scientific reports indicate certain terpenoids, steroids and phenolic compounds such as tannins, coumarins and flavonoids have a chemo preventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis (Blois, 2002). The anticancer activities of chloroform extract of *A. salvifolium* are probably due to the presence of alkaloid, phenolic compounds, flavonoids as well as terpenoids.

In present study, it was accomplished that chloroform extract of *Alangium salvifolium* have antioxidant activity as well as significantly reduced tumor growth, viability of tumor cells, raising life span as compared with those of EAC control mice. Now our next aim is to explore the isolation and characterization of lead compound liable for aforementioned activity from this plant.

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