Exploration of Antioxidant and Anticancer Activity of Stephania japonica Leaves Extract

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Authors’ contributions

This work was carried out in collaboration among all authors. Author MDI designed the study, performed the statistical analysis and wrote the first draft of the manuscript. Authors AI, NT and SFA managed the literature searches and carried out the tests. Author MSU managed the analysis of the study and reviewed the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The demand for antioxidants from the natural source has drawn promising attention to outturn desired pharmacological effect by subsidizing the adverse effect for treating cancer. This study evaluated the antioxidant activity of Stephania japonica leaves extracts to explore the anticancer activity.

Methods: Antioxidant potential of crude extracts were evaluated using various methods which include total antioxidant activity, ferric reducing antioxidant assay, DPPH free radical, ABTS free radical, nitric oxide and superoxide anion radical scavenging assay. Anticancer activity was determined in vitro by MTT assay and in vivo on mice against Ehrlich ascites carcinoma (EAC) cell.

Results: Phytoconstituents with free radical scavenging capacity were quantified in terms of inhibitory concentration (IC\(_{50}\)) with the values of 17.00±3.22 µg/mL, 33.30 ± 5.45 µg/mL, 43.70±5.26 µg/mL and 52.30±1.07 µg/mL in DPPH, ABTS, superoxide anion radical and nitric oxide free radical scavenging assay, respectively as the highest quencher, acetone extract of S. japonica leaves (ASJL). ASJL and methanol extract (MSJL) showed low lethal dose (LD\(_{50}\)) values of 21.76 and 26.63, respectively indicating higher toxicity. In vitro anti-proliferative activity (MTT

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1. INTRODUCTION

Malignant neoplastic disease, cancer is one of the foremost among the grievous diseases within which deregulating proliferation of abnormal cells invades and disrupts encompassing tissues due to the pernicious change of genetic materials through the mutation [1]. In recent years, it constitutes serious public health issues in each developed and developing nation. From the initiation step to the progression level, various cellular changes occurred that resist the normal regulatory mechanism of the existing cellular system leading to the malignant formation. The metabolic processes in aerobic stress conditions, as well as normal cellular conditions, continuously produces dangerous by-product substances referred to as free radicals. Highly reactive organic molecules can be formed when oxygen interacts with certain molecules. As a result, unstable and unpaired electrons bearing organic metabolites can initiate a chain reaction once they take shape in the body just like the domino effect. Due to the unstable electron, the radicals tend to bond with different molecules to capture the electron to become stable, thereby destroying healthy tissue through damaging the proteins, DNA and lipids that are associated with chronic degenerative diseases, including cancer, coronary artery diseases, hypertension, neurodegeneration and diabetes [2,3]. Currently used artificial synthetic antineoplastic medicines are suspected to possess adverse effects like excretory organ toxicity, hepatotoxicity, gastrointestinal disturbances, myelosuppression and alopecia (hair loss), etc. [4]. In most cases, the desired outcomes for cancer treatment were not achieved due to the poor drug delivery system of those synthetic agents. As part of ongoing finding medicinal plants content, abundant quantities of chemical species including the secondary metabolites have the promising synergetic outcome to control the oxidative stress and management of transformed cells in pathophysiological conditions. Therefore, numerous efforts with systematic identification proceeded to search the novel antineoplastic phytoconstituents from natural sources that prevent the transformed cell formation or delay the tumor cell progression.

Considering the conventional approach to screening antioxidant potentiality and drawing the neutralization potentiality of oxidative stress, Stephania japonica under the Menispermaceae family has been studied in the view of searching the antineoplastic agent. Plants of the genus Stephania have miscellaneous types of bioactive secondary metabolites and traditionally have been used in the treatment of asthma, inflammation, tuberculosis, dysentery, hyperglycemia, cuts, and wounds [5,6]. Among the different types of secondary metabolites, diverse types of alkaloids such as tertiary phenolic bisococlaurine type stepholine and hasubanone are found in roots of S. japonica [7,8]. Nitrogen atom and a heterocyclic ring containing bioactive chemical compounds, alkaloids exhibit antiproliferation and antimetastasis effects on various types of cancers [9,10]. To get the desired novel anticancer drug this plant originated secondary metabolite may promisingly use a structural scaffold for developing the chemically modified more efficient anticancer agents. Conventional extraction of phytoconstituent from biological material significantly depends on solvent properties as well as on the chemical nature of phytoconstituents. Therefore, the natural product derived antineoplastic remedies from S. japonica leaves extracts obtained by polar, nonpolar and bipolar solvents were used to evaluate antioxidant scavenger through the diverse view of neutralizing capacity as like a broad range of

Keywords: Stephania japonica; antioxidant activity; anticancer activity; free-radical scavenging, apoptosis; cytotoxicity.
unpaired electron balancer. The toxicity and exponential cell growth inhibition were evaluated with brine shrimp lethality bioassay and MTT cell viability assay, respectively. An alternative promoting mode of rapidly proliferating cancer cell death was investigated through the morphological change under the microscopic view.

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

Green leaves of *S. japonica* were collected from the area of Rajshahi University campus (northwestern part of Bangladesh). The plant was authenticated by Prof. Dr. A. H. M. Mahbubur Rahman, Taxonomist, Department of Botany, University of Rajshahi, Bangladesh. Collected leaves were washed with sufficient running tap water to remove the adhering dirt and air-dried under shade for several days. The dried materials were ground into coarse powder by a grinding machine (FFC-15, China) and stored in airtight containers at 4 °C for further use.

2.2 Preparation of Extract

Approximately, 200 gm of dried powder of *S. japonica* leaves were merged with 500 mL of each non-polar (chloroform, acetone, n-hexane), bipolar (methanol, ethanol), and one polar (water) solvents into the separate round-bottomed glass bottle. The glass bottles were sealed by cotton plug and aluminum foil and kept stored in airtight containers at 4 °C for further use.

2.3 Chemicals

Analytical grade of research chemicals such as potassium acetate, phosphate buffer, catechin, trichloroacetic acid (TCA), potassium ferricyanide, sodium phosphate, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ammonium molybdate, FeCl₃, gallic acid (GA), trypan blue, 4,6-diamidino-2-phenylindole (DAPI) 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, RPMI 1640 media were obtained from Merck (Darmstadt, Germany), Sigma Chemical Co. (St. Louis, MO, USA) and Lobachemie Pvt. Ltd. (Mumbai, India). Ehrlich ascites carcinoma (EAC) cells were obtained from the Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi-6205, Bangladesh.

2.4 Determination of Antioxidant Activity

2.4.1 Total antioxidant capacity

Total antioxidant capacity (TAC) was determined colorimetrically by the formation of green color through the reduction of phosphomolybdate in presence of phytoconstituents under the acidic condition according to Prieto et al. [11] with slight modification. Exactly, 500 µL of extract at different concentrations (10-200 µg/mL) was mixed with 3 ml of reaction mixture containing 0.6 M sulphuric acid, 28 mM sodium phosphate, and 1% ammonium molybdate into the test tubes. The test tubes were incubated at 95 °C for 10 min to complete the reaction. The absorbance was measured at 695 nm using a spectrophotometer against blank (without extract) after cooling at room temperature. Ascorbic acid was used as a reference standard. The antioxidant activity was expressed as the number of gram equivalent of ascorbic acid.

2.4.2 Ferric reducing antioxidant power assay

The ferric-reducing antioxidant capacity of leaves extracts was evaluated according to Oyaiuz [12]. Formation of Perl’s Prussian blue spectra proportionally correlated to the conversion of Fe³⁺ to Fe²⁺ in presence of antioxidants. In brief, 250 µL of a sample at various concentrations (10-200 µg/mL) were added to a solution containing a mixture solution of 625 µL of 0.2 M potassium buffer and 625 µL of 1% potassium ferricyanide [K₃Fe(CN)₆]. The reaction mixture was incubated for 20 min at 50 °C to complete the reaction and then 625 µL of 10% TCA solution was added. The total mixture was centrifuged at 3000 rpm for 10 min. Clear supernatant (1.8 µL) mixed with an equal volume of distilled water and reducing color probe chemical compound was detected at 700 nm after the addition of 360 µL of ferric chloride (0.1% w/v) solution. The absorbance was recorded immediately against the blank and the calculated result was expressed as gram equivalent of ascorbic acid.
2.4.3 DPPH free radical scavenging assay

Free radical scavenging activity of the plant extracts was tested by 2,2-diphenyl-1-picryl-hydrazyl (DPPH) according to Braca et al. [13] with slight modification. A purple-colored stable free radical, DPPH was reduced into yellow-colored diphenylpicryl hydrazine. Various concentrations (10 to 200 µg/mL) of extracts were mixed with 3 mL of 0.1 mM DPPH solution and the reaction aliquot was homogenized immediately. The mixture was kept for 30 min to complete the reaction under the dark conditions at room temperature and then the absorbance was recorded at 517 nm by a spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. Percentage of DPPH radical scavenging activity was calculated by the following equation:

\[
\% \text{ of Inhibition} = \frac{[A_0 - A_1]}{A_0} \times 100
\]

Where \(A_0\) is the absorbance of the control without sample and \(A_1\) is the absorbance of the extracts/standard. Ascorbic acid (AA) was used as standard. The percentage of DPPH radical scavenging activity was plotted against concentration and from the graph, \(IC_{50}\) was calculated.

2.4.4 ABTS free radical scavenging assay

Based on the hydrogen donation capacity, the antioxidant potentiality of crude extracts was evaluated through the scavenging of preformed 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS●) monocation radical [14]. The ABTS cation radical was obtained by adding 7 mM ABTS to 2.45 mM potassium persulfate solution, and the mixture was left in the dark at room temperature for 12-16 h. The absorbance of ABTS cation radicals was adjusted to 0.70±0.02 at 734 nm by adding deionized water. ABTS● solution (3 mL) was added to 1 mL of the test sample with various concentrations (10-200 µg/mL) and mixed vigorously. The absorbances were measured at 734 nm after incubation for 6 min. The percentage of ABTS free radical scavenging activity was calculated as described above for the DPPH method.

2.4.5 Superoxide anion radical scavenging assay

Non-enzymatic aerobic superoxide radicals were generated through the reduction of riboflavin which can directly facilitate the reduction of nitro blue tetrazolium to form chromophore formazan. Inhibiting the formazan formation by slinking the superoxide through antioxidants and absorbance of testing reaction mixture were decreased proportionally with the increasing of extract concentration. In this assay, nitro-blue-tetrazolium (NBT) is converted into NBT diformazan via superoxide radical [15]. In brief, 100 µL of the plant extract was added to the reaction mixture containing 20 µg/mL riboflavin, 12 mM EDTA, and 0.1 mM NBT on 50 mM phosphate buffer (pH 7.6). The radical formation was initiated by illuminating the reaction solution upon reducing the riboflavin. The reaction was initiated by illuminating the reaction mixture for 40 min at 25 °C and the absorbance was measured at 590 nm. Catechin was used as a reference standard. The percent of inhibition was calculated by the following equation:

\[
\% \text{ Inhibition} = \frac{[A_0 - A_1]}{A_0} \times 100
\]

Where \(A_0\) is the absorbance of the control and \(A_1\) is the absorbance of the extracts/standard. Then percent inhibition was plotted against concentration and from the graph, \(IC_{50}\) was calculated.

2.4.6 Nitric oxide radical scavenging assay

Nitric oxide radical scavenging activity of leaves extracts was evaluated according to Marcocci et al. [16]. Stable nitric oxide (NO) free radicals are generated upon decomposition of sodium nitroprusside in presence of phosphate buffer saline that is neutralized by examined extracts. The color of an azo dye is developed through the Griess diazotization reaction. Briefly, 2 mL of 10 mM sodium nitroprusside dissolved in 500 µL phosphate buffer saline (pH 7.4) was mixed with 500 µL of a sample at various concentrations. The mixture was incubated at 25°C for 150 min and then mixed with 500 µL of Griess reagent. The mixture was again incubated at room temperature for 30 min and absorbance was measured at 546 nm. Catechin was used as a reference standard. The amount of nitric oxide radical inhibition is calculated from the following equation:

\[
\% \text{ Inhibition of NO radical} = \frac{[A_0 - A_1]}{A_0} \times 100
\]

Where \(A_0\) (control) was the absorbance of the NO radical solution without a test sample and \(A_1\) is the absorbance of the test sample. The percent of inhibition was plotted against concentration and \(IC_{50}\) was calculated from the graph.
2.5 Brine Shrimp Lethality Bioassay

Evaluation of cytotoxic properties of the plant crude extracts was determined by brine shrimp (Artemia salina L) nauplii lethality bioassay [17]. Crude extracts samples of different concentrations (10, 25, 50, 100, 200 µg/µL) were prepared in different test tubes with artificial seawater (38 g NaCl/L, pH 7.0). Ten brine shrimp nauplii were added to each test tube and the final volume of solution was adjusted up to 5 mL with artificial seawater. After keeping one day at room temperature (~30°C) under light conditions the percentages of mortality of the nauplii were calculated for each concentration and the LD50 values were determined using probit analyzing software.

2.6 MTT Assay for in vitro Anticancer Study

Based on the antioxidant and free radical scavenging capacity, two extracts of S. japonica leaves (ASJL and MSJL) were used to evaluate anti-proliferative activity as well as anticancer activity. Cytotoxicity of the crude extract was evaluated by measuring the reduction capacity of mitochondrial NADP dehydrogenase on yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan formation on the EAC cancer cell line. Cells were plated onto 96 well plates at a cell density of 5×10³ in 100 µL RPMI 1640 media solution and allowed to grow in a CO2 incubator for 24 h (37°C, 5% CO2). The medium was replaced by a fresh medium containing different concentrations (10.28 µg/mL to 833.33 µg/mL) of two crude extracts of S. japonica leaves and then cells were incubated for 24 h (37°C, 5% CO2) to executing the pernicious effect of crude extracts. After removal of the aliquot, 10 mM of PBS (180 µL) and MTT (20 µL, 5 mg/mL MTT in PBS) were added and incubated for 8 h at 37°C. The aliquot was discarded again and 200 µL of acidic isopropanol was added into every well and incubated again at 37°C for 30 min. Cell viability was measured by recording the absorbance at 570 nm using a titer plate reader. The proliferation inhibition ratio of EAC cells was determined by the following equation:

\[
Proliferation\% = \frac{(A_0 - A_i) \times 100}{A_0}
\]

Where A0 is the absorbance of the cellular homogenate (control) without S. japonica leaves extract and A1 is the absorbance of the cellular homogenate with the extract.

2.7 In vivo Anticancer Assay

2.7.1 Test animals

Male Swiss albino mice average weighing 25-30 gm were purchased from the Department of Pharmacy, University of Jahangir Nagar, Saver, Dhaka, Bangladesh. The animals were housed in polypropylene cages in well-ventilated rooms under standard conditions (temperature 22±2 °C, humidity 50±5% with 12 h light/dark cycle) and were allowed free access to standard dry pellet diet and water.

2.7.2 Experimental design

After one week acclimatization of Swiss albino male mice (6 mice in each group) were randomly divided into seven different groups: Group-I, the normal control mice fed with standard pellet diet and water; Group-II, the EAC tumor-bearing mice without treatment of extract; Group-III, the bleomycin-treated EAC tumor-bearing group at the doses of 0.3 mg/kg; Group-IV, Group-V and Group-VI, Group-VII, the EAC tumor-bearing mice treated with ASJL and MSJL at the dose of 100 and 200 mg/kg body weight. In each case, the volume of the test solution injected (i.p.) was 100 µL/day/mouse.

2.7.3 Collection and transplantation of EAC cells

EAC cells were collected according to Kabir et al. [18]. Intraperitoneally proliferated EAC cells were collected weekly from donor Swiss albino mice bearing 6-7 days old ascites tumors. The cells were adjusted to approximately 3×10⁶ cells/mL by diluted with normal saline (0.9%) and were counted by hemocytometer. The trypan blue (0.4%) exclusion assay was used to check the viability of tumor cells. Cell samples (100 µL saline) showing above 99% viability were used for transplantation through strict aseptic condition into intraperitoneal of Swiss albino mice. After 24 h of EAC cell—transplantation, mice were randomly divided into respective groups with at least six mice per group.

2.7.4 Inhibition of cell growth

In vivo tumor cell growth inhibition was carried out by the method of Sur et al. [19]. For this study, seven groups of mice were used. For
therapeutic evaluation 1.6 × 10^6 EAC cells/mouse were inoculated into each group and treatment was started after 24 h of tumor inoculation and it was continued for 5 days. At the end of treatment (6th day), each group of mice was sacrificed and the total intraperitoneal tumor cells were harvested by normal saline and were counted by a hemocytometer. The total numbers of viable cells in each mouse of the treated groups were compared with those of controls (without extracts treatment). Cell growth inhibition was calculated by the following formula:

Percent of cell growth inhibition = \(\frac{(T_0 - T_f) \times 100}{T_0}\)

Where \(T_0\) is the number of EAC cells of the untreated (control) group and \(T_f\) is the number of EAC cells of \(S. japonica\) leaves extracts treated group.

### 2.7.5 Mean survival time and hematological profile studies

Effect of \(S. japonica\) leaves extracts on hematological parameters were determined by intraperitoneally injected EAC cells on Swiss albino mice with the comparison of seven groups (n=6) of mice on the 14th day after transplantation. Group-I and group-II were served as normal, control mice were treated with normal saline only and group-III were considered as positive control treated with bleomycin of 0.3 mg/day/kg body weight. On the other hand group-IV, V, VI, and VII were treated with ASJL and MSJL at the dose of 100 and 200 mg/day/kg body weight respectively. Treatments were given 24 h after the tumor inoculation at the respective dose with 100 µL solution. Blood was collected from each mouse through a freely flowing tail vein under sterilized conditions and hematological parameters (RBC, hemoglobin and WBC) were determined [20]. Mean survival time (MST) and the percent of expending lifetime of each EAC cell inoculated groups were calculated as the following formula:

Mean survival time (MST) = \(\sum\) (Survival days of each mouse in a group)/Total number of mice

Increased life span (ILS)\% = \(\frac{(MST \text{ of treated group} - MST \text{ of control group}) \times 100}{MST \text{ of control group}}\)

### 2.7.6 Cell morphological change and nuclear damage

Cellular morphological alteration of EAC cells was observed under fluorescence microscopy (Olympus IX71, Korea) after the treatment of crude extract of \(S. japonica\) leaves by the method described by Kabir et al. [18]. Briefly, the EAC cells from the group-II, group-IV, group-V, group-VI, and group-VII of mice were collected and stained with 0.1 mg/mL of Hoechst 33342 at 37°C for 10 min in the dark condition. The cells were then washed thrice with phosphate buffer saline (PBS). Finally, morphological appearances of the cells were observed under the fluorescence microscope.

### 2.8 Statistical Analysis

The experimental data were expressed as mean± standard deviation. SPSS software (version 16) was used to perform a one-way analysis of variance (ANOVA) followed by Dunnett’s ‘t’ test and IC_{50} was calculated using Graph Pad Prism 6. P<0.05 was considered to be statistically significant.

### 3. RESULTS AND DISCUSSION

#### 3.1 Determination of Antioxidant Activity

##### 3.1.1 Antioxidant capacity through reduction of phosphomolybdenum

The hydrogen donation capacity or electron transferring potency of phytoconstituents present in crude extracts may be evaluated through different chemical reactions using various synthetic oxidants or stable free radicals. Most of the phytochemicals have aromatic ring structures that may inhibit oxidation reactions even in presence of oxygen. The single-electron transferring capacity of \(S. japonica\) leaves extracts showed dose-dependent phosphomolybdate consumption (Fig.1) which indicated that electron-rich chemical species present in the extract had strong antioxidant capacity as compared to the ascorbic acid reducing capacity. Although the reduction potentiality of all extracts varied very less at initial concentration ASJL, ESJL, and MSJL subsequently generated higher antioxidant activity than that of CSJL, NSJL, and WSJL with rising concentration up to 200 µg/mL. Thus, the oxidant reducing capacity of extracts might be ameliorated oxidative stress leading to the molecular damage and prevention of free radical leading cascade reaction [21]. The extraction of bioactive phytoconstituents marginally depends on the chemical properties of solvent and phytochemicals. The various types of secondary metabolites such as flavonoids, flavonols, and
related polyphenolic components present in plant extract contributed significantly to the phosphomolybdate quenching capability [22].

3.1.2 Ferric reducing antioxidant power assay

The ferric reducing activities of different extracts were analyzed by evaluating the reduction of Fe$^{3+}$ to Fe$^{2+}$ in the presence of extracts. Reduction of Fe$^{3+}$ to Fe$^{2+}$ in presence of plant extract materials significantly implies the electron-donating capacity of the structural component of phenolic constituents containing at least one electron-rich aromatic ring and more than one hydroxyl group in their architect [21,23].

**Fig. 1.** Total antioxidant capacity of different extracts of *S. japonica* leaves

*Values are expressed as mean ± SD (n=3)*

**Fig. 2.** The ferric reducing antioxidant capacity of different extracts of *S. japonica* leaves

*Values are expressed as mean ± SD (n=3)*
3.1.3 DPPH free radical scavenging assay

Stable nitrogen centered DPPH free radical scavenging propensity indicate a strong correlation with antioxidant propagation by accepting hydrogen atom to form a decolorization hydrazine product. DPPH free radical scavenging activities of different extracts were shown in Fig. 3. Significant hydrogen transferring capacities were recorded on 10 to 200 μg/mL extract compositions. The highest scavenging propagation were 88.43%, 73.86%, 51.13%, 58.55%, 51.44%, 41.52% and 55.38% for the extracts of AA, ASJL, CSJL, ESJL, MSJL, NSJL and WSJL, respectively at the concentration of 200 μg/mL extract. The IC_{50} values of extracts in the DPPH scavenging assay were recorded as the order of AA<ASJL<ESJL<NSJL<CSJL<WSJL and the low IC_{50} value had the higher radical scavenging potentiality depending on their extract phytoconstituents. The nature of the phytoconstituents would be related to the radical scavenging capacity where polyhydroxyl group present in the phytoconstituents thus contributed to their hydrogen donation capacity. The presence of a high concentration of flavonoids and phenolics in ASJL might be responsible for the highest scavenging activity. The linear relationship of radial quenching ability depends on the polyphenolic contents [25,26].

3.1.4 ABTS free radical scavenging assay

The ABTS• radicals quenching ability of different extracts of S. japonica leaves at various concentrations are given in Fig 4. ASJL showed strong antioxidant activity with a lower IC_{50} of 33.30 μg/mL (Table 1). Although ESJL and MSJL captured higher free radicals in an early concentration of extracts they generated relatively moderated scavenging activity at 200 μg/mL extract composition with the IC_{50} values of 78.56 and 49.81μg/mL, respectively. The results indicated that ESJL and MSJL had moderate oxidant scavenging activity as compared to ascorbic acid (IC_{50}=27.38 μg/mL). The other extracts CSJL, NSJL, and WSJL possessed almost similar activity higher IC_{50} values of 119 μg/mL, 131 μg/mL and 111.6 μg/mL, respectively indicating their lower free radical neutralizing capacity. Hydrogen ions capturing the potentiality of ABTS• radical strongly fluctuated with the quantity of polyphenolic components including tannins, flavonoids and flavonols due to their high conjugation patterns and free 3-hydroxyl group in their structural skeleton [27]. Aromatic hydro-skeleton, as well as polyhydroxyl feature, give predominate feature to terminate the chain termination reaction and act as a direct radical scavenger by providing the electron.

3.1.5 Nitric oxide free radical scavenging assay

Nitric oxide (NO) is an essential, gaseous, and reactive oxygen species of the bioregulatory molecule required for several physiological processes like neural signal transmission, immune response, control vasodilation and control of blood pressure. NO also plays an important role in various types of an inflammatory processes in the animal body. Inhibition of azo dye formation through neutralization of NO that chemically generated on the decomposition of nitroprusside marked a strong relationship to decrease the absorbance than control at 546 nm. The values of NO free radical inhibition activities of different extracts of S. japonica are shown in Fig. 5. All extracts neutralized the radical relatively uprising within 100 μg/mL indicating strong electron donation sensitivity but only ASJL holding more strength forward quenching potentiality extending to 200 μg/mL. Synchronize combination of solvent properties and antioxidants chemical nature ASJL extracted more functional active antioxidant with impressive (IC_{50} = 52.30±1.07 μg/mL) (Table 1) nitric oxide scavenging properties. Under the life-leading cellular condition vast amount of NO that was generated was lead to cell injury and imposed higher strength toxicity through infusion with superoxide radical [28]. The experiment data suggested that S. japonica leaf with acetone solvent might be significantly buffering the NO leading to harmful cascade reactions under the malignant neoplastic cellular conditions.

3.1.6 Superoxide anion radical scavenging assay

Weak superoxide anion generated under the pathophysiological conditions leads to a couple of formation of peroxynitrite, hydrogen peroxide and singlet oxygen. Fig. 6 shows the superoxide anion radical scavenging activities of different extracts at various concentrations. Inhibitions of superoxide radical leading chromophore formation were recorded markedly at the concentration of 200 μg/mL as 79.22±0.97%, 65.53±2.14%, 76.41±2.26%, 69.20±2.62%, 53.70±2.52% and 58.55±1.88% on corresponding of ASJL, CSJL, ESJL, MSJL, NSJL, and WSJL, respectively. Comparative
higher inhibition propensity with marginal IC₅₀ values of 43.70 µg/mL and 52.21 µg/mL (Table 1) were recorded in ASJL and MSJL, respectively and it indicated that phytoconstituents of these extract showed more sensitivity to neutralize the superoxide anions. Evaluated finding imposed positive argument of polyphenolic and flavonoid component to uprisingsuperoxide neutralizing capacity. Ardestani and Yazdanparast [29] the rising reduced propensity of oxidant in presence of phenolic components into the reaction solution.

3.2 Comparative Evaluation of IC₅₀ Values

Synthetic stable free radical neutralizing potency of S. japonica leaves crude extracts quantitatively were estimated by IC₅₀ values (Table 1) where antioxidant substance partially stabilized the unpaired free radical. The diversity of phytoconstituents with their theoretical chemical nature as antioxidants had been evaluated by solvent-dependent extracted crude component neutralizing potency against diverse free radicals. In vitro, various free radical inhibition capacities make strong evidence to the diversity of hydrogen-rich phytoconstituents that can make inhibitory reaction on free radical leading cascade reactions in the biological system. S. japonica leaves extracts possessed the diverse type of photochemical that were associated with strong free radical scavenging effect with lower IC₅₀ values. Acetone extract process the highest antioxidant effect at inhibition of characteristic diamagnetic component formation in DPPH, azinomonocation decolorization in ABTS, chromophore formation in superoxide and azo dye generation in nitric oxide assay considerably higher than the other extracts with recorded IC₅₀ values of 17.00±3.22 µg/mL, 33.30±5.45 µg/mL, 52.30±1.07 µg/mL and 43.70±5.26 µg/mL, respectively. The inhibition capacities of other extracts were strongly related to the active phytoconstituents that significantly towered to the intermediate metabolite compositions. However, diverse secondary metabolites such as flavonoids and tannins are highly operative free radical scavengers among most of the oxidizing molecules that are produced in various normal and pathological metabolic processes [30]. Moreover, flavonoids have also been reported to overwhelm reactive oxygen formation, scavenge reactive species, chelated trace elements involved in free-radical production, and up-regulate protection of antioxidant defenses [31].

3.3 Brine Shrimp Lethality Bioassay

Concentration-dependent cytotoxicity of S. japonica leaves extracts with a median lethal dose (LD₅₀) against brine shrimp (Artemia salina L) nauplii are given in Table 2. Through the probit statistical analysis mortality rate (LD₅₀) of extracts are found to 8.38, 21.76, 26.63, 30.32, 33.79, 41.78, 62.73 μg/mL from gallic acid, ASJL, MSJL, ESJL, CSJL, WSJL, and NSJL, respectively, The lower value indicate higher toxicity and higher value indicate vice versa. The high cytotoxic potentiality of ASJL extract might be attributed to the higher quantity of polyphenolic components that was confirmed in the antioxidant assay.

![Fig. 3. DPPH free radical scavenging ability of different extracts of S. japonica leaves](image)

Values are expressed as mean ± SD (n=3)
Fig. 4. ABTS free radical scavenging ability of different extracts of *S. japonica* leaves
Values are expressed as mean ± SD (n=3)

Fig. 5. Nitric oxide free radical inhibition properties of *S. japonica* leaves extracts
Values are expressed as mean ± SD (n=3)

Fig. 6. Superoxide free radical scavenging activities of *S. japonica* leaves extracts
Data are expressed as mean ± SD (n=3)
Table 1. IC\textsubscript{50} values of free radical scavenging assay from different extracts of S. japonica leaves

| Extracts & Standards | DPPH assay | ABTS assay | Nitric oxide assay | Superoxide assay |
|----------------------|------------|------------|-------------------|-----------------|
| ASJL                 | 17.00±3.22 | 33.30±5.45 | 52.30±1.07        | 43.70±5.26      |
| CSJL                 | 84.82±2.55 | 119.0±8.14 | 122.0±3.12        | 80.76±5.20      |
| ESJL                 | 18.27±2.58 | 78.56±3.12 | 59.17±3.55        | 70.80±4.21      |
| MSJL                 | 17.12±2.55 | 49.81±5.36 | 56.09±1.95        | 52.21±4.55      |
| NSJL                 | 54.06±1.49 | 119.0±8.14 | 122.0±3.12        | 142.9±3.48      |
| WSJL                 | 159.9±1.09 | 119.0±8.14 | 122.0±3.12        | 142.9±3.48      |
| AA                   | 15.53±1.43 | 27.38±5.65 | -                 | -               |
| CA                   | 22.73±2.41 | 22.73±2.41 | 19.51±1.44        | -               |

Table 2. Cytotoxic potentiality of different extracts of S. japonica leaves on brine shrimp nauplii. Data are expressed as mean ± SD (n=3)

| Conc. (µg/mL) | Percentage (%) of mortality |
|--------------|-----------------------------|
|              | ASJL | CSJL | ESJL | MSJL | NSJL | WSJL | GA |
| 10           | 27   | 20   | 20   | 23   | 13   | 20   | 57 |
| 25           | 50   | 40   | 40   | 43   | 30   | 33   | 80 |
| 50           | 77   | 63   | 67   | 67   | 43   | 53   | 94 |
| 100          | 97   | 74   | 83   | 93   | 57   | 70   | 100|
| 200          | 100  | 97   | 100  | 100  | 80   | 90   | 100|
| LD\textsubscript{50}(µg/mL) | 21.76 | 33.79 | 30.32 | 26.63 | 62.73 | 41.78 | 8.38 |

3.4 MTT Assay for Cytotoxicity

MTT assay performs the preliminary screening involving proliferation studies and indirectly readout measurement of cellular metabolism. Cell viability associated with the intercellular reducing potentiality of yellow tetrazolium to purple formazan markedly depends on mitochondrial interior enzyme succinate dehydrogenase. Indirectly readout measurement of cellular metabolism of EAC cell in exposure to serially diluted extracts of S. japonica leaves (10.28 µg/mL to 833.33 µg/mL), ASJL and MSJL showed the mortality of EAC cell with 15.44±2.6% to 80.94±2.87% and 11.67±1.58% to 74.25±4.33%, respectively (Fig. 7). ASJL and MSJL also showed significant cytotoxicity against EAC cells with half inhibitory concentration (IC\textsubscript{50}) values of 135.9 µg/mL and 251.3 µg/mL, respectively. Different active phytoconstituents mainly polyphenols, flavonoids and tannins that show cytotoxicity work alone as an inhibitor or combining to arrest the cell cycle and leading to cell death program [32].

3.5 In vivo Anticancer Assay

3.5.1 Inhibition of cell growth

S. japonica leaves extract, ASJL and MSJL potentially inhibited EAC cell growth of 58.25±4.24% to 79.09±2.45% and 46.26±2.68% to 61.74±4.41% at the dose of 100 mg/kg (i.p.) and 200 mg/kg (i.p.), respectively while results were compared with a control group (Table 3). Treatment with bleomycin at the dose of 0.3 mg/kg (i.p.) showed cell growth inhibition at 88.23±1.33%. Uncontrolled cell proliferation with a breakdown regulatory mechanism characterized in EAC was controlled through the inhibitory action of protein kinase inhibitors [33]. Plant-derived flavonoids and their derivative functioned as cell growth interceptors by intermolecular interaction with nitrogen base containing basic nucleosides [34]. Polyphenols present in S. japonica leaves extracts might be responsible for cell growth inhibition through the flavonoid interacting with kinase leading to cell growth inhibition. In the chemical nature of antioxidants, they have the potency to meet the demand for generating therapeutic efficacy and buffering the cellular free radical misbalancing conditions through stabilizing the unstable conditions of reactive oxygen species.

3.5.2 Mean survival time and increase the life span

Administration of ASJL at the doses of 100 and 200 mg/kg/day in EAC cells induced mice showed the increase mean survival time significantly (P<0.05 to P<0.001) by 29.4±1.16 and 35.8±1.14 days whereas MSJL showed 27.6±1.14 and 31.2±0.84 days, respectively (Fig. 8). The increased life span of intraperitoneal induced EAC cell bearing mice was recorded as
29.05% and 57.02% on the treatment of ASJL with 100 and 200 mg/kg/day, respectively (Fig. 9). On the other hand, MSJL increased the life span by 21.84% and 39.47% at the dose of 100 and 200 mg/kg/day, respectively. The reference drug bleomycin at 0.3 mg/kg showed 92.45% increased life span. The potency of EAC cell growth retardation in mentioning to increase mean survival time and life span were correlated to the dose of extracts. Combining chemotherapeutic effect with antioxidant properties expressed the conventional strategy to avoid the resistance of cancer drug as well as a side effect of host internal mechanism. Recently, flavonoid based antioxidant marginally draws the attention to control the cancer cell growth by inducing the chemotherapeutic effect along with threshold level of toxicity [35]. The flavonoid-rich antioxidant present in extracts of S. japonica leaves might be responsible for generating potent cytotoxicity against EAC cells.

3.5.3 Hematological profile studies

Transplanted tumor model through Ehrlich adenocarcinoma responding host immune Fig. 7. Inhibition of EAC cells after treatment of S. japonica leaves extracts

Fig. 8. Effects of S. japonica leaves extracts on survival time on EAC cell-induced mice. Values are expressed as mean ± SD (n = 6). *P<0.05, **P<0.01 and ***P<0.001 when compared with control.
confirmed through comparing the hematological parameters and widely used to screening the conventional anticancer numerous substances. Table 4 showed the significant change of hematological parameters after the 14 days treatment of ASJL and MSJL extracts on EAC cell-induced mice. In EAC tumor-bearing mice, RBC, hemoglobin, and WBC were remarkably interrupted as compared to controls while these blood parameters restore to normal when treated with ASJL and MSJL extracts at the dose of 100 and 200 mg/kg body weight (i.p.). Moreover, at the same time interval tumor burden was almost reduced by the treatment of reference drug bleomycin (0.3 mg/kg) whereas maximum and significance alteration occurred in the ASJL treatment at the dose of 200 mg/kg body weight. In the control mice group without extract treatment, RBC and hemoglobin levels reduced significantly as compared to other groups. Previous studies reported that alteration of blood parameters and the hepatoprotective effect occurred by polyphenolic component flavonoids in the study of Eucalyptus camaldulensis against EAC cells induced transplanted carcinoma [36].

3.5.4 Cell morphological change and nuclear damage

Hallmark signs of cell damaged were observed through the fluorescent microscopy with Hoechst 33342 staining after the treatment and control of extracts in intra-peritoneal induced EAC cells. Control cells remained round and homogeneous stained with Hoechst 33342 whereas extract-treated cells exhibited characteristic morphological alteration such as blebbing, membrane alteration, and nuclear fragmentation (Fig. 10). Through the study of nuclear components and cell morphology, it was imposed that apoptosis of EAC cells occurred by the treatment of ASJL and MSJL. Cytotoxic compounds trigger the cell existing intracellular signaling mechanism to initiate the extrinsic or intrinsic cascade leading to the cell death program. Plant-derived secondary bioactive metabolites act as an anticancer agent and induced selective cytotoxicity in vigorous dividing cells. Polyphenolic component, flavonoids inhibit the cell growth through the kinase enzyme.

Fig. 9. Effects of S. japonica leaves extracts on the life span of EAC cell-induced mice. Values are expressed as mean ± SD (n = 6). *P<0.05, **P<0.01 and ***P<0.001 while compared with control.
### Table 3. Effect of *S. japonica* leaves extracts on EAC cell growth inhibition

| Treatment groups | Dose (mg/kg/day, i.p.) | No. of viable EAC cells on day 6 after tumor cells inoculation ($\times 10^7$ cells/mL) | Cell growth inhibition |
|------------------|------------------------|---------------------------------------------------------------------------------|-----------------------|
| **ASJL**         |                        |                                                                                  |                       |
| 100              |                        | 2.55±0.25                                                                        | 58.25±4.24**          |
| 200              |                        | 1.28±0.15                                                                        | 79.09±2.45***         |
| **MSJL**         |                        |                                                                                  |                       |
| 100              |                        | 3.28±0.17                                                                        | 46.26±2.68*           |
| 200              |                        | 2.33±0.27                                                                        | 61.74±4.41**          |
| **Bleomycin**    | 0.3                    | 0.72±0.06                                                                        | 88.23±1.33***         |
| **Control**      |                        | 6.09±0.25                                                                        | -                     |

### Table 4. Effect of *S. japonica* leaves extracts on hematological parameters of experimental mice

| Groups       | Hemoglobin (gm/dL) | WBC (cells/mL) | RBC (cells/mL) |
|--------------|--------------------|----------------|----------------|
| Normal mice  | 15.25              | 7.73×10⁶       | 5.89×10⁹       |
| EAC Control  | 7.8                | 16.14×10⁶      | 2.68×10⁹       |
| Bleomycin    | 14.8               | 9.68×10⁶       | 4.26×10⁹       |
| **ASJL**     | 100                | 9.8            | 13.26×10⁶      | 3.19×10⁹       |
| (mg/kg/day)  |                    |                |                |
|              | 200                | 11.6           | 12.57×10⁶      | 3.75×10⁹       |
| **MSJL**     | 100                | 7.8            | 14.86×10⁶      | 2.86×10⁹       |
| (mg/kg/day)  |                    |                |                |
|              | 200                | 8.6            | 13.22×10⁹      | 3.26×10⁹       |
inhibition to form the intermolecular bond with a nitrogenous base of basic nucleosides [34,37]. Potent and selective killing ability of bioactive component against neoplastic cells by minimizing the adverse effect of normal cells through a cell death program is a highly desired aspect of anticancer drugs. As part of the conventional screening, S. japonica leaves extract had anticancer agents that might promisingly initiate cancer cell death through a cell death program. To understanding the trigger mechanism of apoptotic signaling mechanism it suggests a further advanced study to isolate and purify the bioactive medicinal constitutes to their corresponding intracellular mechanism.

4. CONCLUSIONS

Antioxidant potent plant-derived extracts in different organic solvents possess strong antioxidant and free radical scavenging activity. The hydrophilicity of polyphenolic component gives the accessibility to diffuse the cell membrane and interact with the cell compartment to neutralize the oxidative stress leading to unpaired free radicals along with intercepting the kinase leading cell growth inhibition. The restoring tendency of hematological parameters with increasing life span and non-viable cell counting explored the working strategy against rapidly proliferating EAC cells. S. japonica leaves might be promising natural sources for regulating oxidative stress and restoring the host immunity as a robust anticancer potency through the simultaneous effect of phytoconstituents. Regarding the host safety profile and better stimulating effect of host immunity it’s a demand to advance research to identify the anticancer component and confirming the mechanism of action using multiple cell lines.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Approval and permission of using mice model were obtained from Institute of Biological Sciences (Memo no: 97/320/IAMEBBC/IBSc), University of Rajshahi, Bangladesh.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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