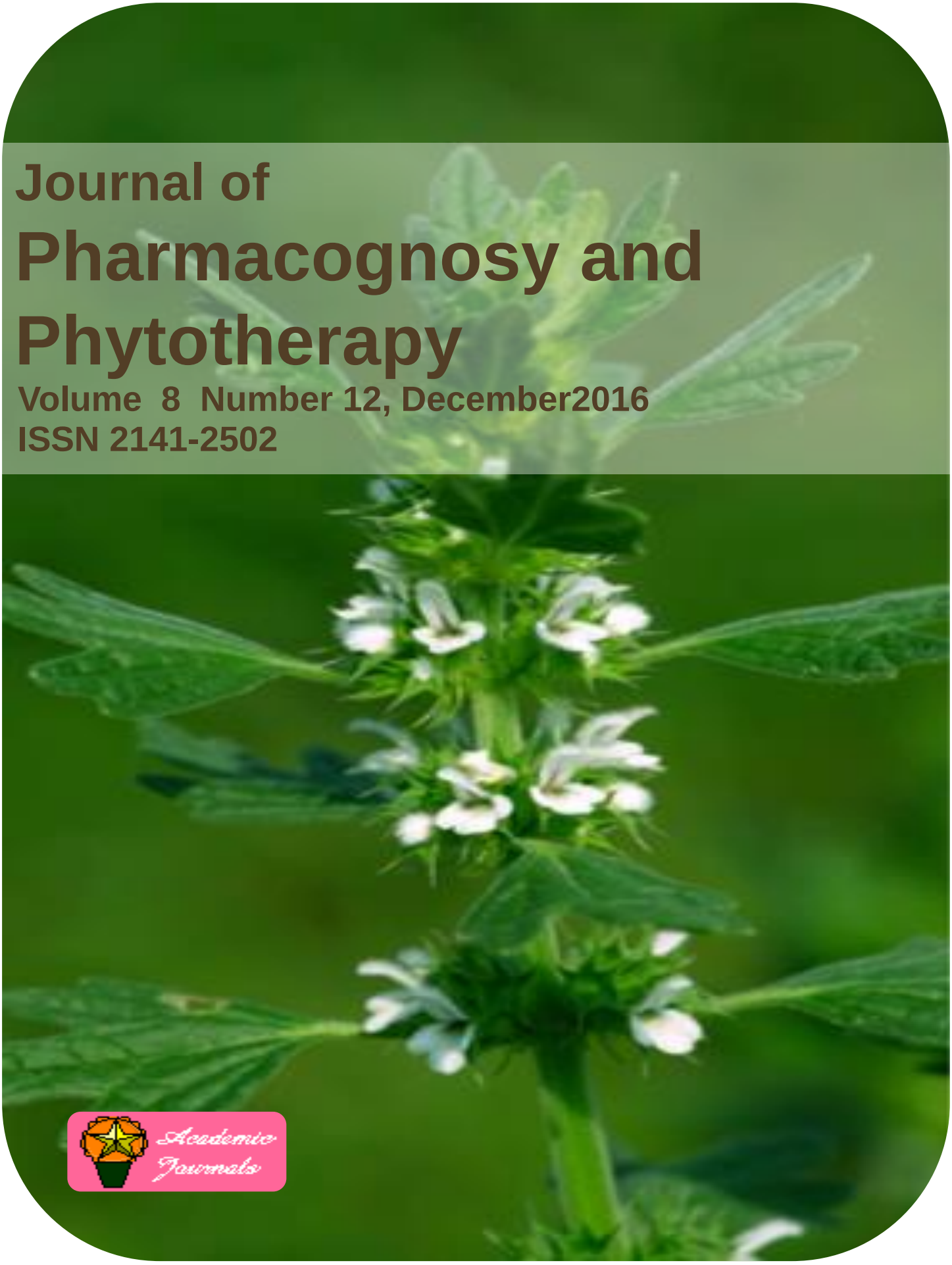

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Journal of Pharmacognosy and Phytotherapy

Full Length Research Paper

Phytochemical and antioxidant studies of methanol and chloroform extract from leaves of *Azadirachta indica* A. Juss. in Tropical region of Nepal

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This research was carried out with the aim of phytochemical analysis and determining antioxidant activity present in methanol and chloroform leaf extracts of *Azadirachta indica*. Due to its potential in curing various ailments as well as wide spread application of antioxidant activity such as in the field of cosmetology, the plant was selected for the study. The total phenolics contained in the plant extracts were also studied which are responsible for the antioxidant activity. Antioxidant activity of the extracts were evaluated by diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging method using ascorbic acid as standard in the concentration of 100, 50, 25 and 12.5 µg/ml. Phytochemical analysis were done with the established procedure and total phenolic content (TPC) was determined by using Folin-Ciocalteu colorimetric method. Phytochemical screening revealed the presence of similar constituents in both methanol and chloroform extracts such as alkaloids, glycosides, carbohydrate, phenol, flavonoid, steroids, protein, and amino acids. Total phenolic content in methanol and chloroform extracts were 207.39 ± 8.77 and 58.08 ± 4.41 mg gallic acid equivalent (GAE)/g, respectively. The inhibitory concentration (IC_{50}) value for methanol and chloroform extracts of *A. indica* were calculated and found to be 80.28 and 439.60 µg/ml, respectively. The finding suggests that methanol extract of the plant has significantly more antioxidant activity than the chloroform extract as clarified by total phenolics contained in the plant.

Key words: Phytochemical screening, antioxidant activity, total phenolic content, *Azadirachta indica*.

INTRODUCTION

Among various sources of medicine, plants have been known to contribute a crucial role in the health service as three quarters of world population relies on it and its extract for health care (Kunwar et al., 2006; Thomson, 2010). Although, Nepal is a small country, it has got many plants with medicinal and aromatic values due to geographical diversity. Most of the plants are being used in traditional medicine; however, some are not explored scientifically for their medicinal value yet (Lin et al., 2007).

Preliminary screening of phytochemicals is a valuable step for detecting various bioactive principles present in

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the plants which paved the way for drug discovery (Yadav et al., 2014). The presence or absence of such bioactive principles depends largely on the extent of accumulation, geographical location, method of collection, extraction procedure, amount of plant material used, and the analytical method employed (Yusuf et al., 2014). *Azadirachta indica* primarily comprised of several secondary metabolites including steroids, triterpinoids, reducing sugars, alkaloids, phenolic compounds, flavonoids, and tannins (Vinoth et al., 2012). Free radicals which have one or more unpaired electrons are produced as a result of metabolism in normal or pathological cell which role is crucial in cell injury accompanied by ageing and wide range of degenerative diseases including inflammation, cancer, atherosclerosis, diabetes, liver injury, Alzheimer, Parkinson and coronary heart pathologies (Halliwell, 1995; Erdemoglu et al., 2006; Gutteridge, 1994). Antioxidants prevent occurrence of these diseases by inhibiting the oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress (Durackova, 2010; Reuter et al., 2010). Thus, there is a need for isolation natural antioxidants having less or no side effects in order to displace synthetic antioxidants which are possible promoter of carcinogenesis (Kaur and Arora, 2009; Newman and Cragg, 2007).

MATERIALS AND METHODS

Study species

A. indica A. Juss (widely known as Neem in vernacular name), is a versatile plant belonging to the family Meliaceae which is inhabitant to tropical and subtropical parts of the world (NEEM, 2016; Sombatsiri et al., 2005). Mainly, two species of *Azadirachta* have been reported, *A. indica* A. Juss, native to Indian subcontinent and *Azadirachta excelsa* kack, confined to Philippines and Indonesia (Pankaj et al., 2011). The leaves of *A. indica* are imparipinnate, alternate, exstipulate; leaflets are alternate or opposite, very shortly stalked, ovate-lanceolate, attenuate at the apex, unequal at the base and are medium to dark green in colour (Ali, 2012). Almost every parts of neem is being used in traditional medicine for treating variety of human ailments as it possess manifold of biological activities such as antiallergenic, antidermatic, antifeedant, antifungal, anti-inflammatory, antioxidant, antipyorrhoeic, antiscabic, diuretic, etc (Biswas et al., 2002).

Plant and chemicals

The leaves of *A. indica* were collected from Paklihawa, Siddharthanagar Municipality, Rupandehi district of Nepal in the month of August, 2015. The leaves were identified from Department of Environmental Sciences, Institute of Agriculture and Animal Sciences, Tribhuvan University. All the chemicals used in the experiment were of analytical grade and were purchased from S.d. Fine-Chem Ltd; Himedia Laboratories Pvt. Ltd. and Qualigens Fine Chemicals.

Preparation of extracts

The leaves of the plant were washed with distilled water, dried at room temperature in the laboratory for 3 weeks to obtain consistent weight and were powdered using mechanical grinder. About 200 g of the crushed leaves were extracted by maceration using pure methanol as solvent and other 200 g crushed leaves via chloroform for 7 days with frequent agitation. The extracts were filtered using Buckner Funnel and Whatmann No. 1 filter paper. Each filtrate was concentrated to dryness under reduced pressure by Rotary vacuum evaporator below 40°C. Extract was stored at 4°C in air tight container with proper labeling.

Phytochemical screening

Phytochemical analysis was carried out for alkaloids, glycoside, saponin, steroid, phenol, flavonoid, tannin, protein, and amino acids and performed as mentioned by the authors (Yusuf et al., 2014; Tiwari et al., 2011). Mayer's and Hager's reagents were used to detect the presence of alkaloids; Molish's and Fehling's reagents were used for carbohydrate; Legal's test was used for glycosides; Froth and foam test were used for saponin; Salkowski's test was used for steroid; Ferric chloride test was used for phenol; Alkaline reagent and Lead acetate test were used for flavonoid; Ferric chloride and bromine water test were used for tannins and xanthoproteic test was used for the proteins and amino acids.

Total phenolic content

Preparation of standard

The total phenolic content in plant extracts was determined by using spectrophotometric method based on oxidation-reduction reaction with some modifications (Stanković, 2011). Various concentrations of gallic acid solutions in methanol (0.5, 0.4, 0.3, 0.2, 0.1, 0.05, 0.025, and 0.0125 mg/ml) were prepared. In a 20 ml test tube, 1 ml gallic acid of each concentration was added, 5 ml of Folin-Ciocalteu reagent (10%) and 4 ml of 7% Na_2CO_3 were added to get a total volume of 10 ml. The blue coloured mixture was shaken well and incubated for 40 min at 40°C in a water bath. Then, the absorbance was measured at 760 nm against blank. All the experiments were carried out in triplicate. The average absorbance values obtained at different concentrations of gallic acid were used to plot the calibration curve.

Preparation of sample

Two different concentrations of the extracts (1, 0.1 mg/ml) were prepared. Following the procedure described for standard, absorbance for each concentration of extract was recorded. Total phenolics content of the extracts was expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g). The total phenolic contents in all samples were calculated the using the formula $C = c \times V/m$; where C = total phenolic content mg GAE/g dry extract, c =concentration of gallic acid obtained from calibration curve in mg/ml, V = volume of extract in ml, and m = mass of extract in gram.

Antioxidant activity by diphenyl-β-picrylhydrazyl (DPPH) scavenging

The antioxidant activity was accessed by standard protocol, that is, spectrophotometric method (Subedi et al., 2012). Stock solution of 100 µM DPPH in methanol was made. Test sample of the extract were made at different concentration (12.5, 25, 50, and 100 µg/ml) in methanol. Similarly, reference sample of ascorbic acid were made at similar concentration. Two milliliters of 100 µM DPPH was

Table 1. Phytochemical screening of methanol and chloroform extract of *A. indica*.

S/N	phytochemical tests	Reagents used/Test performed	Result	
			Methanol	Chloroform
1	Alkaloid test	Mayer's reagent	+	-
		Hager's reagent	+	+
2	Carbohydrate test	Molish's reagent	+	+
		Fehling's reagent	-	+
3	Glycoside test	Legal's test	+	-
4	Saponin test	Froth test	-	-
		Foam test	+	-
5	Steroid test	Salkowski's test	+	+
6	Phenol test	Ferric chloride test	+	+
7	Flavonoid test	Alkaline reagent test	+	-
		Lead acetate test	+	+
8	Tannin test	Ferric chloride test	+	+
		Bromine water test	-	+
9	Protein and amino acid	Xanthoproteic test	+	-

+: Present; -: Absent.

added to 2.0 ml of each methanol and chloroform extract of *A. indica* at different concentration and kept in dark. Similarly, 2.0 ml of 100 µM DPPH was mixed with 2.0 ml of methanol and ascorbic acid and kept in dark for 30 min in incubator at 37°C. The absorbance was measured at 517 nm by UV spectrophotometer after 30 min and % scavenging was calculated by the following equation:

Percentage scavenging = $(Ao-A_T) / A_0 \times 100\%$

where AO = Absorbance of DPPH solution and AT = Absorbance of test or reference sample. The % scavenging was then plotted against concentration and regression equation was obtained to calculate IC_{50} (micromolar concentration required to inhibit DPPH radical formation by 50%) values.

Statistical analysis

All the data were expressed as mean value ± standard error of mean (SEM) of the number of experiments (n=3). Microsoft EXCEL program 2010 and Statistical Package for Social Sciences, Version 16.0 (SPSS V.16.0) were used for data analysis.

RESULTS

Phytochemical screening

The preliminary phytochemical screening of the extracts in methanol and chloroform extract revealed the presence of different phytochemicals which are presented in Table 1. The two extracts of plant showed the presence of similar phytochemicals such as alkaloids, glycosides, carbohydrate, phenol, flavonoid, steroids, protein, and amino acids.

Total phenolic content

The content of total phenol (TPC) was determined by using Folin-Ciocalteu reagent in terms of gallic acid equivalent (standard curve equation: $y=0.013x+0.252R^2$, 0.991). Total phenolic content in methanol extract was 207.39 ± 8.77 mg GAE/g which is significantly higher than chloroform extract and was found to be 58.08 ± 4.41 mg GAE/g of sample of dry weight as shown in Figure 1.

Antioxidant activity

Free radical scavenging activity of all the extracts and standard ascorbic acid increased with the increase in concentration. The maximum percentage inhibition of DPPH free radical at 517 nm is exhibited by standard ascorbic acid followed by methanol extract and chloroform extract of *A. indica* as shown in Figures 2 to 4.

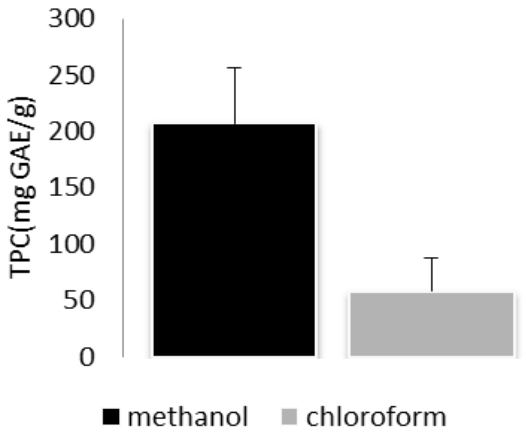

Figure 1. Total phenolic content (TPC) in different neem extract.

Figure 3. Antioxidant activity before absorbance measurement in UV spectrophotometry.

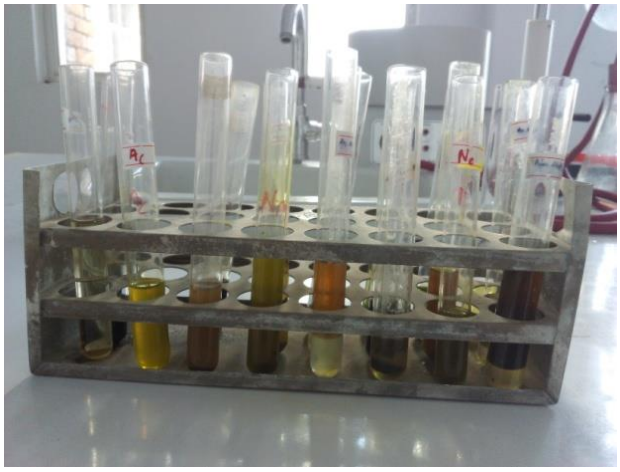

Figure 2. Phytochemical screening of extracts.

The inhibitory concentration IC_{50} value was compared which was found to be 80.28 and 439.60 µg/ml for methanol and chloroform extract of *A. indica*, respectively.

DISCUSSION

In recent years, the use of plants in herbal medicine possessing antioxidant property has been on the rise due to its potential in ameliorating various diseases. Various experimental studies have showed oxidative cellular damage arising due to imbalance between free radical generating and scavenging systems ultimately being the cause of cardiovascular diseases, cancer, aging, etc. Methanol and chloroform were the solvent system used for extraction as both the polar and non- polar components present in the plant can be extracted.

Phytochemicals such as phenol, flavonoid, alkaloid, glycoside, tannin, etc., were present in the extract which may be responsible for antioxidant activity as per the previous similar study conducted (Govindappa and Poojashri, 2011). Total phenolic content on methanol and chloroform extract is 207.39 ± 8.77 mg and 58.08 ± 4.41 mg GAE/g, respectively which is proportional to the antioxidant activity, that is, TPC exhibits positive correlation with the antioxidant activity (Karamian and Ghasemlou, 2013).

It is implied that plant extract contain compounds such as phenols, flavonoids, etc., which can donate hydrogen to a free radical in order to remove odd electron indicating its usefulness in various radical related pathological condition. Antioxidant activity test based on measurement of absorbance at 517 nm where all the extracts showed positive radical scavenging activity suggesting plant selected for the study was potently active (Aiyegoro and Okoh, 2010). Ascorbic acid is used as positive control which showed high percentage inhibition of free radicals about 95.65 to 96.66%; pattern of inhibition being similar at variable concentration due to the fact that it possesses high radical scavenging activity, that is, this concentration is sufficiently high to scavenge free radicals. Methanol extract of *A. indica* has shown the highest activity followed by its chloroform extract which coincide with the previous study (Sri et al., 2012). The antioxidant activity shown by methanol extract was much higher than that of chloroform extract which may be due to the presence of polar compounds like phenols, flavonoids, etc., that are soluble in methanol (Bhusal et al., 2014).

Conclusion

Thus, it can be concluded that methanol extract of neem possess better antioxidant activity than that of the

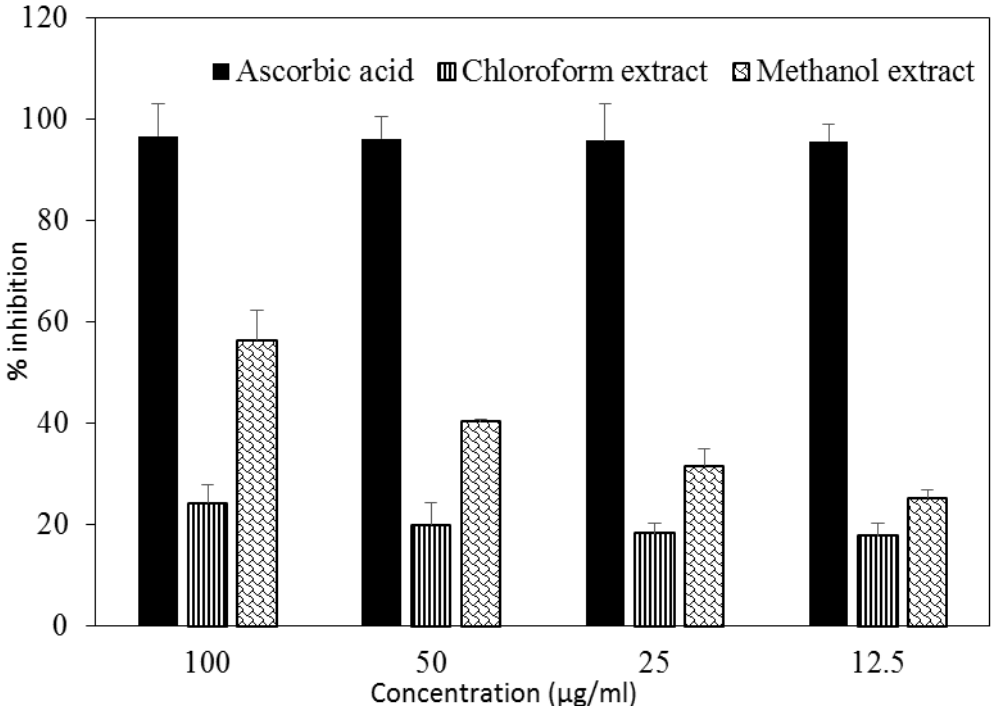

Figure 4. Percentage inhibition of DPPH free radical by Standard and extracts at 517 nm.

chloroform extract under similar condition, due to the presence of variable type and quantity of phytochemicals supporting its medicinal and cosmetic use in Nepal and globally. Further studies are required to evaluate *in vivo* antioxidant potential and also isolation and characterization of active compounds for commercialization in the field of pharmaceuticals.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Preliminary bioactivity investigation of *Styrax officinalis* fruit extract as potential biopesticide

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Styrax officinalis **is a deciduous large shrub that grows in many Mediterranean countries. Active ingredients are localized in the fruit pericarp. Primary phytochemical analysis showed the presence of saponins, tannins and triterpenes and absence of alkaloids and flavonoids. No antimicrobial, but strong ichthyotoxic and molluscicidal effects were observed in the saponin-rich extract .Toxicity tests with the snail *Cornu aspersum* proved to be a potent contact molluscicide. Allowing the snail to creep on leaves and surfaces sprayed with a 1% (w/v) of pericarp extract resulted in severe dehydration and foaming through their membranes, which resulted in their death after 30 min. When the snails were fed lettuce leaves treated with the same extract solution, it did not cause any observable effect. These findings show that *S. officinalis* is a promising natural source of a potent contact molluscicide with no visible effect on the snail upon ingestion.**

Key words: Styracaceae, *Styrax officinalis*, saponins, ichthyotoxic, molluscicidal, antimicrobial.

INTRODUCTION

Styrax is the largest genus in the 11 genera family Styracaceae constituting 80% of its species; 130 species have been identified to belong to the styrax genus (Pauletti et al., 2006; Jones, 1995). *Styrax officinalis L.* grows in southern Europe and the eastern Mediterranean region in Cyprus, Israel, Jordan, Lebanon, Syria, and Turkey (Huang et al., 2003; Fritsch, 1999). In Lebanon, the fruit of this tree is traditionally used for fishing in fresh water streams. Its saponins-rich fruits, once ripe, are crushed and thrown in water pools which stunt fish and make them rise to the surface. Previous studies on *S. officinalis L.* fruits have reported the presence of benzofurans (Anil, 1980; Akgul and Anil, 2003), lipids (Ulubelen et al., 1976) and saponins (Yayla et al., 2002; Zehavi et al., 2008). Furthermore, a recent phytochemical investigation conducted on the endocarps of *S. offcinalis L.* revealed the presence of five different compounds: Americanin A, egonololeat, egonol- 2‴- metil butanoat, egonolgentiobiside and homoegonolgentiobiside (Pazar and Akgül, 2015).

Saponins are bioactive compounds found abundantly in a variety of plants. They have an amphiphilic nature that results from the presence of a hydrophilic sugar moiety called glycone and a hydrophobic genin called sapogenin or aglycone. The amphiphilic nature of saponins is responsible for the main characteristics of these

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compounds, that is, the marked ability to form foam in water. They are classified as triterpenoid or steroidal glycosides depending on the structure of the aglycone moiety.

There have been many investigations related to the biological activity of saponins (Francis et al., 2002; Sparg et al., 2004; Moses et al., 2014). Seeman et al. (1973) observed a haemolytical activity resulting from the affinity of saponins for membranes sterols evidenced by electronic microscopy. Some saponins have also been described to be highly toxic to fish because of their damaging effect on the respiratory epithelia (Roy et al., 1990). They are considered to be the active components of many traditionally used fish poisons, like mahua oil cake (Francis et al., 2001). This ichthyotoxic effect was also reported in a study conducted on a powder prepared from the seeds of *S. officinalis L.* and tubers of common cyclamen, both species growing wild in Lebanon (Nigel Hepper, 2004). Saponins extracted from fenugreek were reported to induce hypoglycemia by increasing insulin levels through β-cells stimulation in rats (Petit et al., 1993). Some studies show the hypocholesterolemic role of saponins, due to their interaction with bile acids, therefore enhancing the metabolism in the liver (Oakenfull and Sidhu, 1990; Al-Habori and Raman, 1998). Additionally, saponins were recorded to have an anticarcinogenic effect especially in breast cancer, leukemia, and prostate cancer (Bachran et al., 2008; Guo and Gao, 2013; Yildirim and Kutlu, 2015). They are capable of stimulating the cytochrome c–caspase 9–caspase 3 pathway in human cancer and other cell lines thus inducing apoptosis (Liu et al., 2000).

Much attention has been drawn lately on the use of biopesticides as alternatives to conventional pesticides. They have many potential advantages in terms of lower toxicity, little or no impact on non-target organisms, and biodegradability. The molluscicidal properties of saponins were first observed in Ethiopia (Lemma, 1965). Research was targeted for molluscicidal effects of saponins to control diseases such as schistosomiasis transmitted by freshwater snails (Hostettmann, 1980). Their activity may be due to their damaging effect on the soft body wall of the mollusks (Chaieb, 2010; Winder et al., 1995). This study aims at investigating some bioactivity properties and identifying the active ingredients of *S. officinalis* fruits. The molluscicidal effect of the fruit pericarp extract against the terrestrial gastropod *Cornu Aspersum* is also described.

MATERIALS AND METHODS

Bioactivity-guided localization of the ichthyotoxic ingredients

S. officinalis fruits were collected during September and October from Akkar, north Lebanon. The fruits were cleaned, air dried in the shade, and stored at -10°C. The ichthyotoxic material was localized as follows: The seeds and pericarps of 6 six *S. officinalis* fruits were respectively ground with a pestle and mortar in 15 mL distilled water. The resulting suspension was filtered through cheesecloth and the filtrate was transferred into a crystallizer containing 500 mL water. A crystallizer containing distilled water was used as control. The localization of the active ingredients (pericarp vs. seed) was tested based on the ichthyotoxic effect of the respective filtrates on goldfish *(Carassius auratus)*.

Crude extracts separation

Five grams of the dried pericarp powder of *S. officinalis* was extracted with 70% ethanol solution for 4 h at 60°C on a mechanical orbital shaker. After vacuum filtration, the liquid extract was mixed with 0.4 M ammonium sulfate in a 1:1 ratio and left to stand overnight at 20°C. The precipitate was removed after centrifugation at 8000 rpm for 30 min. The filtrate was evaporated in a rotary vacuum evaporator (≤ 45°C) and the residue was stored at 4°C for further use.

Foam test for saponins

In order to check if the active fraction contains saponins, 1 ml solution of extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 min. Development of stable foam suggests the presence of saponins.

Phytochemical screening

Phytochemical tests were performed on the gummy residue obtained after extraction. Phytochemical tests were carried out for all the extracts following standard methods (Singh, 2012; Tiwari et al., 2011):

1. Test for saponins: 300 mg of extract was boiled with 5 ml water for two minutes. The mixture was cooled and mixed vigorously and left for three minutes. The formation of froth indicates the presence of saponins.
2. Test for Triterpenes: 300 mg of extract was mixed with 5 ml chloroform and warmed for 30 min. The chloroform solution was mixed with a small volume of concentrated sulfuric acid and mixed properly. The appearance of red color indicates the presence of triterpenes.
3. Test for alkaloids: 300 mg of extract was digested with 2 M HCl. The acidic filtrate was mixed with amyl alcohol at room temperature. A pink color in the alcoholic layer indicates the presence of alkaloids.
4. Test for flavonoids: Extracts were treated with a few drops of sodium hydroxide solution. The formation of intense yellow color, which becomes colorless on addition of dilute acid, indicates the presence of flavonoids.
5. Test for phenolic compounds: The extract (50 mg) was dissolved in 5 ml of distilled water. To this, a few drops of neutral 5% ferric chloride solution were added. A dark green color indicates the presence of phenolic compounds.

Thermal stability

A 100 mL aqueous crude extract of the saponins was divided into 25 mL portions. Three portions were heated under reflux at a temperature of 95°C for 30 min, 1 h and 1 h 30 min respectively. These solutions were then transferred into a 100 mL evaporating flask and dried in a rotary evaporator at 45°C. The resulting residues were mixed with water to a total volume of 250 mL. A control sample was prepared in a similar way but without being heated. The thermal stability of saponins was investigated based on

Table 1. Preliminary phytochemical screening of the ethanolic extract of *Styrax officinalis* plant.

Phytoconstituents	Ethanolic extract of *S. officinalis*
Saponins	+
Tannins	+
Triterpenes	+
Alkaloids	-
Flavonoids	-

+/-: Presence or absence of the component tested.

the ichthyotoxic effect of the different heated samples vs. the control.

Ichthyotoxic effect

Goldfish were exposed to different concentrations of the ethanolic crude extract of the pericarp of *S. officinalis* in order to determine the lethal ichthyotoxic dose. Gold fish in pure water were used as controls.

Antibacterial effect

Bacteria employed in this study were *Escherichia Coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Pseudomonas aeruginosa* (ATCC 27853), *Enterobacter aerogenes* (ATCC 35029), and *Proteus vulgaris* (ATCC 8427). The bacteria were cultured overnight at 37°C on 15 mL Muller Hinton Agar gel plates. 2.5 mL of 50 g/L crude extract were added to each plate. The bacterial plates were incubated for 48 h at 37°C. The antimicrobial activity was evaluated by measuring the diameter of inhibition zone.

Molluscicidal effect

Wild snails (*Cornu aspersum*) were collected locally and adapted to laboratory condition for 1 week before being tested. To study whether the molluscicidal effect is by contact and/or by ingestion, the following experiments were performed:

1. Ingestion: A lettuce leaf fed the mollusk was soaked in 10 g/L of the ethanolic extract solution containing saponins and left to dry before given it in small portions as food to the mollusk.
2. Contact*:* The container and the lettuce leaf were pulverized by mean of 250 mL spray bottle with a solution of 10 g/L of the ethanolic extract before introducing the mollusk pest.

RESULTS AND DISCUSSION

The localization of the ichthyotoxic ingredients in the seeds vs the pericarps of the *S. officinalis* fruit was determined against goldfish. As a first approach, an aqueous extraction was performed. Goldfish subjected to seed and pericarp extracts respectively showed normal behavior the first 15 min. However, after this period, only goldfish exposed to the pericarp extract exhibited marked distress signs revealed by the erratic swimming performance and loss of balance followed by death after 45 min of exposure. These results provide good evidence for saponins toxicity as a causative factor. Therefore, it is suggested that the active ingredients are localized in the pulp of the *S. officinalis* plant.

Phytochemical characterization showed that the ethanolic extraction is more effective than aqueous extraction to isolate more compounds from the fruits (Oenning et al., 1994; Arya et al., 2012). Thus, a primary phytochemical analysis was carried out on the ethanolic extract of the pericarp of *S. officinalis* in order to determine the groups of active ingredients. The analysis reveals the presence of saponins, tannins and triterpenes. However, the pericarp extracts tested negative for alkaloids and flavonoids. The results are summarized in Table 1. Furthermore, the observed persistent foam obtained after shaking the crude extract confirms further the presence of saponins. This is consistent with the literature reporting the presence of saponins in *S. officinalis* (Pauletti et al., 2006). Among the three phytochemical groups (saponins, tannins, and triterpenes) found in the pericarp of *Styrax* fruits, only saponins of some plant species are known to be ichthyotoxic. All these provide good evidence that saponins are the bioactive components of *S. officinalis* and they are localized in the pericarp of the fruit.

In order to determine the relation between the concentration of the ethanolic crude extract and its ichthyotoxic effect, goldfish were exposed to 4, 2, 1 and 0.5 g/L of the ethanolic crude extract. Figure 1 shows that the lethal time decreased with increase in the crude extract concentration. Fish controls showed normal behavior. Up to 10 ppm concentration of crude extract was perceived to be fatal for goldfish.

Moreover, it was shown from the thermal stability test that saponins retained their functionality, at least in relation to their ichtiotoxicity, despite prolonged exposure to high temperature. In fact, it was observed that goldfish exposed to the crude extract of saponins heated at 100°C for 30 min, 1 h and 1 h 30 min died within 1 h 15 min to 1 h 30 min of exposure. This time was comparable to the fatal time of the non-heated control crude extract. Earlier research shows that saponins are relatively heat stable components (Oenning et al., 1994).

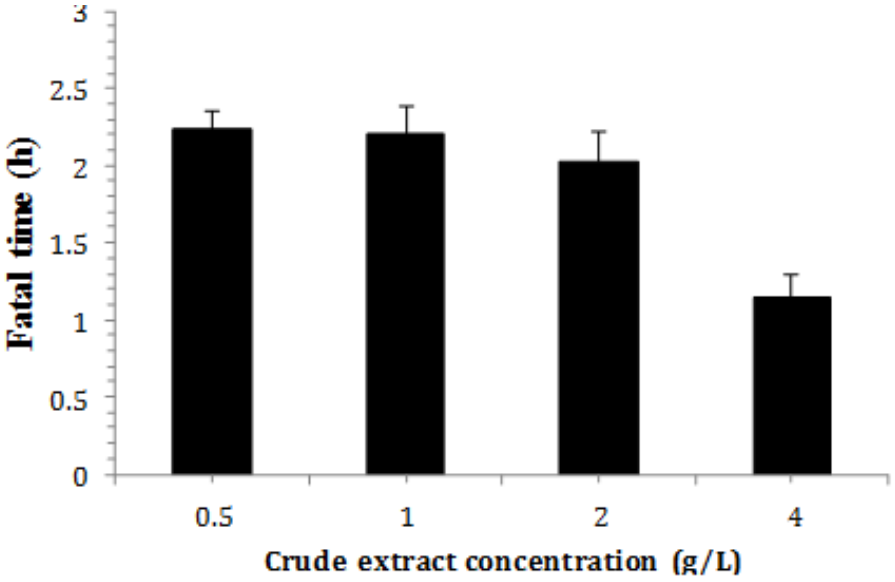

Figure 1. Relationship between the ichtyotoxic fatal time and the concentration of the ethanolic extract.

Beside the ichthyotoxic effect, other bioactivity effects such as the antibacterial and the molluscicidal behaviors were tested using the ethanolic extract.

In classifying the antibacterial activity, most antibacterial medicinal plants are more active against Gram-positive strains than Gram-negative strains (McCutcheon et al., 1992; Srinivasan et al., 2001). However, in this study the saponin extract did not show any activity against all the strains tested as observed by the absence of an inhibition zone of growth on the plates of bacteria cultured.

The molluscicidal activity of saponins from many plant species is well documented. However the vast majority of saponins have been tested for their effects on fresh water mollusks which are vectors of some epidemic disease such as malaria and Schistosomiasis (Diab et al., 2012; Winder et al., 1995; Akinpelu et al., 2012; Aladesanmi et al., 2007; Abdel-Gawad et al., 1999). Gonzalez-Cruz and San Martin (2013) investigated the molluscicidal effects of saponin-rich plant extracts via forced oral injection on the grey field slug. Little research has been conducted so far on the effects of saponins on terrestrial mollusks and a literature survey showed no report of molluscicidal effect of saponins upon contact.

The lettuce leaf treated with 1% (w/v) solution of the pericarp extract and used to feed the mollusk did not cause any observable effect on them. However, it was observed that mollusks creeping on leaves and supports sprayed with a 1% (w/v) of the pericarp extract had their soft membrane damaged and died after 30 min of exposure. It was also noted that the gastropod body shrank and contracted by losing its fluid. These results are in line with the disintegrating action of the saponins on the membrane tissue due to their amphiphilic nature. Furthermore, it was noticed that saponins cause shortening and ulceration of the gastric epithelium upon ingestion by slugs (Gonzales-Cruz and San Martin, 2013). Most of the biological effects of saponins have been related to their permeabilisation of membranes and ability to form pores via interaction with specific membrane constituents such as cholesterol (Francis et al., 2002). It is probable that *S. officinalis* saponins alter the external membrane of the soft-bodied gastropod that makes them loose their fluids and die by dehydration. The interesting finding in this work is that the saponin dose that caused the molluscicidal effect by contact did not affect the snail by oral ingestion. These results show that *S. officinalis* saponins or extract could be a promising biological molluscicide.

Conclusion

The active ingredients in the saponin-rich extract of the *S. officinalis* fruit pericarp showed a strong ichthyotoxic and molluscicidal effects. To our knowledge, this is the first investigation and use of *S. officinalis* fruits as a natural source of a potential potent molluscicide with no visible effect upon ingestion. This work provides the basis for further characterization of the biopesticidal properties of these plant active ingredients.

Conflict of Interests

The authors have not declared any conflict of interests.

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