

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. officinalis* 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 ($\leq 45^{\circ}\text{C}$) 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 <i>S. officinalis</i>
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 ichthyotoxicity, 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).

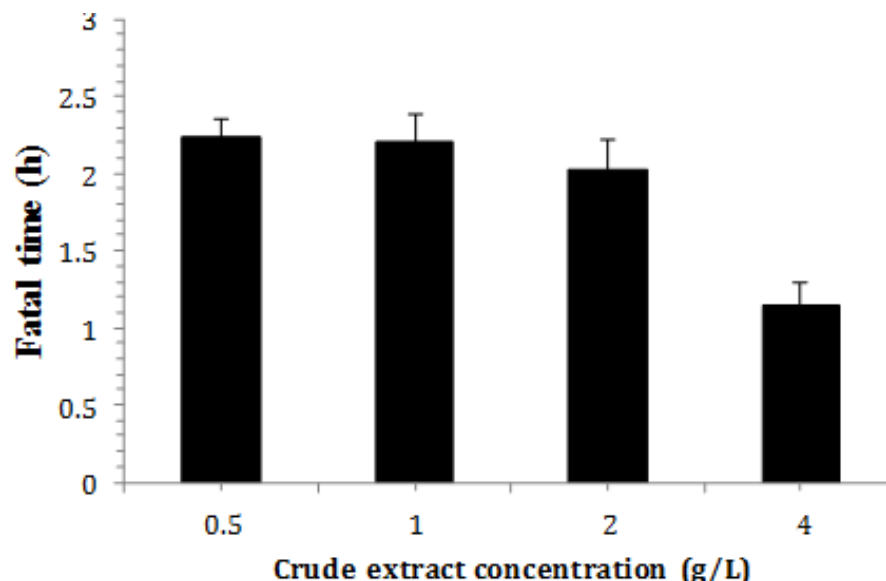


Figure 1. Relationship between the ichthyotoxic fatal time and the concentration of the ethanolic extract.

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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