



Evaluation of Phytochemical, Total Phenolic and Flavonoid content, Antioxidant Activities, and Repelling Property of *Sambucus ebulus*

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Abstract

The therapeutic and anti-infectious effects of *Sambucus ebulus* L. have been well documented in the traditional medicine. The purpose of this study was to identify the domestic herbal plant with insect repellent property. Leaf and fruit of *Sambucus ebulus* L. was collected from Kordkail Kola Sofla rural district and dried. To assess the repellent property, the concentrations of 50 mg/kg, 100 mg/kg, and 250 mg/kg) were prepared. Then 0.4 mL of each concentration was applied on an area of 4 × 6 cm on the shaved Albino (Indian pig) skin and exposed to the mosquito bite. After 30 minutes, the number of adult mosquito bites (three replications) was recorded and analyzed by ANOVA test at a probable level of 5%. The highest phenolic content in the leaf and fruit methanol extract was 155.925 mg/g and of 92.777 mg/g, respectively. Based on IC₅₀, the highest level of antioxidant activity in the leaf and fruit methanol extract were 60.09 μg/mL and 417.846 μg/mL, respectively. The highest repelling property was correlated with the concentration of 250 mg/kg of *Sambucus ebulus*. The repelling effects were 73.4% for the leaf and 78% for fruit extracts. Leaf and fruit extracts of *Sambucus ebulus* contained phenolic and flavonoid with strong antioxidant effects. The fruit hexane and methanol extracts were better repellent than the leaves, and the methanol extracts effect of these organs was greater.

Keywords: Phytochemical, Phenolic Compounds, Antioxidant, Insect repellent, *Culex pipiens*, *Sambucus ebulus*.

Introduction

Sambucus ebulus especially native species of Mazandaran province, Iran and are traditionally used in medicine. The importance of medicinal herbs is for the production of flavonoids, tannins, alkaloids, saponins, tripenoids and phenolic compounds. Therefore, the preliminary phytochemical study of leaf and fruit is important, show in Fig. 1[1-3]. Antioxidants are effective compounds that can prevent oxidation of the proteins, nucleic acids, and lipids [4,5]. This is done by inhibiting the start-up of oxidative chain reactions or the production of free radicals. The

production of nitrogen oxide derivatives and active forms of oxygen are one of the causes of scheduled death, cancers and cardiovascular diseases [6,7].

Biochemical reactions produce many active oxygen's in the body that can destroy biomolecules. The role of free radicals in the development of many diseases has been well documented. This harmful free radical effect can be blocked by antioxidants. The antioxidants damage free radicals. Some of these antioxidants that are occurring naturally in plants, are compounds such as phenols, flavonoids, steroids and terpenes. Flavonoids are extensively present in the fruits and vegetables that have anticancer effects [8,9].

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Phytochemical composition differs in the different parts of the *Sambucus ebulus*. root, leaves and fruit of this herbaceous plant, contain different groups of compounds. Many compounds such as flavanoids, anthocyanins, vitamin C, steroids, tannins, glycosides, cardiac glycosides, caffeic acid derivatives, chlorogenic acid, volatile substances, ursolic acid and phenols were detected and quantified in the extracts of the root, leaves and fruit of *Sambucus ebulus* [10-12].

The mosquitoes in the family of Colicidaceae produces diseases such as, Malaria, Filaryasis, and deno fever, deno bleeding, yellow fever, and encephalitis and transmit to the tropical countries. Based on the report of World Health Organization, the East Mediterranean coastal countries such as, Iran are the main centers of the diseases transmitted by the other arthropods. Considering the vector diversity, particularly of mosquito species, individuals would be at risk of acquiring various diseases [13, 14]. Mosquitoes control and protecting individuals from bite is one of the main ways of preventing disease transmitted by the mosquitoes. Insect bites in addition to the local complication (itching, burning, erythema), could lead to the spread of the lethal diseases, particularly in the tropical regions. Using repellent is one of the vector bite preventing methods, particularly the herbal repellent [15]. Long term using of pesticides for the prevention, and eradication of the

arthropods are harmful to the environment and human health.

Therefore, the herbal materials have been tried which are harmless to man and the environment. Chemical repellants are available, but many of them contain harmful ingredients. On this basis, efforts have been made to introduce absolutely herbal base chemical repellants [16]. Different studies have been done on the context of preparing chemical insect repellent from the beginning of the second war world [17], but complications due to long term application of chemical insect repellent made the researchers to prepare herbal base repellent [15]. Up to now many used mosquito repellent plants such as, Eucalyptus, basil (*Ocimum basilicum* L., *O.basilicam* L. Fa. Citratum Bach O. A zadriachtaindica A. *gratissimum* L. have been tested [18]. One of the aims of the present study was to identify and use the harmless and nontoxic plants base repellents from the plant being cultivable in Iran. In some studies, the herbal base chemicals as repellants and in activation of the larva and ovum of the insects (*Culex quinquefasciatus*, *Aedes aegypti*, and *Anopheles stephensi*) have been investigated. Finally, the ginger (*Zinngiber* L) and Rosemary (*Rosmarinus* L) essential have been introduced as inactivate and repellent of the mosquitoes, respectively, Fig. 2 [19, 20].



Fig. 1 *Sambucus ebulus* is characterized by opposite and pinnate leaves with 5-9 lobes, 15–30 cm long, and a finely toothed margin. The fruit is a small glossy black berry 5–6 mm diameter. *Sambucus ebulus*: A = berry; B = leaves.



Fig. 2 Structure of adult *Culex* Mosquito

In the present study, the *Sambucus ebulus*, from the family of Caprifoliaceae growing naturally in the northern Iran has been investigated to find its insect repellent property. This plant has substances such as, steroids, glycosides, tannins, cardiac glycosides, caffeic acid derivatives and flavonoids [21]. In the traditional medicine, the leaf, fruits and rhizome of this plant have been used orally or locally for the treatment of rheumatoid arthritis, insect bite inflammation, sore throat, etc [22]. Some reports indicated that this plant has anti hemorrhoid and anti-helicobacter properties and anti-edema due to the presence of glycosides Koercetin substances useful in treatment of various wounds, and yorsolic acid in treatment of inflammation and rheumatism, eczema and edema [23, 24]. Considering the pharmaceutical properties of plant products [25]. Since there are reports indicating the biologic and antioxidant properties of this plants extracts growing, being harmless and nontoxic has been selected for the study. *Cx.pipiens* mosquito living in most regions in Mazandaran province, as the dominant species has been used for investigation. The phytochemical and chemical assessment of phenolic compounds, a and antioxidant potentials were evaluated [26].

Material and Methods

Plant Collection and Preparation of Plant Extracts

The *Sambucus ebulus* was collected from Sari Township areas (central, shorab district, Galehkolahsofla and kordkhail), and identified by an expert in botany systematic. The fruit and leaf of plant were kept for drying in shadow, at the

ambient temperature and humidity for 14 to 18 days [27]. The material was dried and grinded into the powder and the phytochemicals present in the powdered samples were extracted by the percolation method. Extraction was done in three successive stages. At first, 600 mL Hexane was added to 400 g of dried fruit and kept for 24 hours, the next day the solvent was removed and new solvent was added. This procedure was repeated three times. Then the solvent in the rotary at 40°C was removed and the hexane extraction was obtained. The methanol extract was done similar to the hexane procedure. In this method, to certain amount of the prepared powder, methanol was added and kept for three days, then the extract was concentrated by the distillation in vacuum condition at 50°C [28].

Methods for Phytochemical Study and Qualitative Analysis of Secondary Compounds

This study was conducted to investigate the presence of alkaloids, saponins and tannins in leaf and fruit of *Sambucus ebulus*. 8 mL of chloroform, and 10 mL of ammonium chloroform was added to the 2 g of dried powder. This solution was filtered and transferred it to the test tube, then 0.5 mL of sulfuric acid (2 normal) added to the test tube and shake. Finally, 3 drops of Meyer (Mir) indicator was added. Milky sediment represents the alkaloids in the plant specimen [29]. In this study, 0.5 g of powdered plant materials was dissolved in 20 mL of distilled water and heated indirectly, the solution was filtered. Then a few drops of ferric chloride (0.1%) added to the solution. Greenish- brown (dark) color and dark blue indicate the presence of

catshins tannins, pyrogulla and tannins respectively [30]. The powdered (0.5 g) was placed in a test tube and 10 ml of distilled water was added and shaken vigorously. It was then allowed to stand for 30 min and observed. Formation of froth indicates the presence of saponins [31].

Methods for Flavonoids Measurement:

Aluminum chloride was used to measure flavonoids content. To the methanol extract, aluminum chloride solution 3, 10% in ethanol, 1 molar potassium acetate solution and distilled water was added. The absorbance of the mixture was read at half an hour later by spectrophotometric method at 420 nm vs. Blanc. Quercetin was used as the standard for drawing the calibration curve. The amount of flavonoids was reported based on the mg equivalent of Quercetin per g of extract [32].

Methods for Total Phenolic Content

The total phenol content was measured using Folin-Ciocalteu reagent. 0.5 mL of each extract (10 mg/mL) was mixed with 0.5 mL of Folin-Ciocalteu solution and 0.05 mL of 10% sodium carbonate solution, one hour later, absorbance at 760 nm was read vs. Blanc. Gallic acid was used as standard for drawing calibration curve and total phenolic content based on equivalent mg of Gallic acid per g of extract was reported. The experiments were repeated 3 times and their mean was reported [33].

Methods for antioxidant activity investigation

In the evaluation of DPPH free radical activity, 4 mL of each extract (1-20 mg / mL) was mixed with 1 mL of 10³M DPPH radical solution, (final concentration of 0.1 mM DPPH) and after mixing for 30 minutes incubated at dark then absorbance at 517 nm vs. blank was read. On this basis, the reducing ability of the extract was calculated. The experiments were repeated 3 times and their mean was reported. Based on the obtained data, the inhibitory power of 50% (IC₅₀) of the extract was obtained by using the software on the percent

control curve. Vitamin C, BHA and quercetin were used as a positive control for comparison [34].

Methods For insect Repellant Test

The 3 to 5 days mature culex mosquitoes were collected from a mosquito breeding center located in Amol city affiliated to the Tehran Environment Health Faculty, and immediately transferred to the Sari Environment Health Faculty. The mosquitoes were transported in plastic cage covered by net and with cotton wetted in Saccharose solution for nutrition of the mosquitoes. In the laboratory the female mosquitoes were used for testing. In this experiment, 24 hours prior to the examination the nutrition was restricted to keep them hunger at the time of the examination. Finally, for each test, in each cage with 25 of the 3 to 5 days matured mosquitoes were kept. The cage was made of polyethylene to see the inside of the cage. Also the roof of the cage was covered by net, let the mosquitoes biting.

The 400 to 450 g male Albino guinea pig was selected for the experiment. First the lateral abdomen area of the testing animal was shaved by a clean blade, and then disinfected with ethanol. The other area of the experimenting animal was covered to prevent the mosquito bite. At first, the animals that repellent was not applied on their skin considered as control were kept in the cage, the mosquitoes rushed to bite them. At the next stage, 0.4 mL of the extract prepared in 10% Dimethyl sulfoxide (DMSO) with concentrations of 50 and 100 and 250 mg/kg were spread on the skin area of 4×6 cm, after 30 minutes, numbers of the bites on the area were recorded. Also this experiment was done with the 0.4 mL repellent of N, N diethyl-3 methyl Benz amide as positive control by the same procedure. The experiment was repeated thrice and the obtained results were analyzed statistically considering the complete random base experimental design, and doing ANOVA at the probable level of 5% [35].

Results

Table 1The Alkaloids, Saponins and Tannins were found in the *Sambucus ebulus* L.

Number	<i>Sambucusebulus</i>	Saponin	Tanin	Alkaloid
1	Leaf	++	+	+++
2	Fruit	-	+	+++

- :nil+ little ++ medium +++ very much

Table 2 Total Phenol and Flavonoid content of leaf and fruit extracts of the *Sambucus ebulus* L.

Number	Type of extract	Total phenolic content(mg/g)	Total flavonoid content(mg/g)
1	Metanolic extract of the leaf	155.925±2.1	81.111±0.6
2	Hexane extract of the leaf	50±1.7	140.317±2.4
3	Metanolic extract of the fruit	92.777±2.6	82.063±0.5
4	Hexane extract of the fruit	77.037±1.5	125.555±2.3

Table 3 DPPH radical scavenging by leaf and fruit extracts of *Sambucus ebulus*L.

Number	Extract of <i>Sambucus ebulus</i>	DPPH Free Radical Scavenging IC ₅₀ (mg/mL ⁻¹) ^a
1	Leaf (methanol)	60.09±4.6
2	Leaf (hexane	883.4±13.4
3	Fruit (methanol)	417.846±11.9
4	Fruit (hexane)	1265.659±15.1

a: IC₅₀ off BHA was 29.3±5.9, Vitamin C 3.7±0.1, Quercetin 3.9±0.2 µg/mL⁻¹, respectively.

* Concentration in µg per milliliter

* Each of the figures of the table is obtained from the mean of three readings ± standard deviation.

Absorption rate of blank: 0.741 the total rate IC₅₀ was equal to 656.743 µg / mL.

Table 4 The comparison of the mean effect of *Sambucus ebulus* L. leaf & fruit extracts (with & hexanic solvent) on the rate of repelling the *Culex pipiens* in the guinea pig

Extract of <i>Sambucus ebulus</i>	Repeling mean%				
	C 50	C 150	C 250	F	P
Leaf (methanolic)	30	57/16	80	35/48	0.0001
Leaf(hexanic)	13/36	46/68	66/8	37/121	0.0001
Fruit (methanolic)	38/68	62/68	84	19/747	0.002
Fruit (hexanic)	18.68	53.36	72	43/033	0.0001

C= Concentration P= P.Value F= Degree of Freedom

The results of the *Sambucus ebulus* extract were as follow: leaf methanol extraction 17.8%, leaf hexane 2.7%, fruit methanol 27.1% and fruit hexane 3%. Alkaloids, saponins and tannins were found in the leaf of *Sambucus ebulus* while in the fruit, alkaloids and tannins were observed (Table 1).

Total phenol content was calculated by Folin Siocaltive method as gallic acid equivalent per g of extract based on the standard curve line equation ($y = 0.0054x + 0.0628$, $r^2 = 0.987$). The total phenolic content for methanolic extract of leaf and fruit, hexane fruit and leaf extract of *Sambucus ebulus* were stronger respectively. (Table 2 showed the organs used for extracts and total phenol and flavonoid content). Flavonoids content was calculated based on the standard curve line equation ($y = 0.0063x$, $r^2 = 0.999$). Flavonoids

content for the fruit and leaf hexane extract of *Sambucus ebulus* was stronger than methanol extracts. (Table 2 showed the organs used for extracts and total phenol and flavonoid content).

In evaluating the antioxidant activity of leaf and fruit of *Sambucus ebulus*, DPPH method showed better effects at higher concentrations and yellow turned to purple and darker but declined in low concentrations. The antioxidant activity of methanol extracts was better than hexane extracts and for the leaf was better than the fruit. The inhibition percentage and IC₅₀ are given in Table 3.

The means of comparison related to the repelling effect of the methanol leaf extract on the culex mosquito showed the highest repelling effect of 80% for the concentration of 250 mg/kg.

Comparison of the means attributed to the repelling effects of the hexane leaf extract on the culex mosquito showed the highest repelling effect of 66.8% for the concentration of 250 mg/kg. Comparison of the means related to the repelling effect of methanol fruit extract on the culex mosquito showed the highest repelling effect of 84% for the concentration of is 250 mg/kg. Comparison of the means related to the repelling effect of the methanol fruit extract on the culex mosquito showed the highest repelling effect of 72% related for the concentration of 250 mg/kg.

The data in the table 4 show the highest repelling rate of *Sambucus ebulus* fruit extract related to the concentration of 250 mg/kg. It worth mentioning that, the means at the statistical level of 5% differs significantly from each other.

The total mean repelling property of different concentrations of the leaf and fruit extracts of the *Sambucus ebulus*. are as follow: methanol leaf 55.72%, Hexane leaf 42.28%, methanol fruit 61.78% and Hexane fruit 58.9%. Finding shows the average repelling effect of the plant extracts on the Culex mosquito in the higher concentrations. The highest mean of effects for the leaf and fruit hexane and methanol extracts was 73.4% and 78%, respectively. The results show the highest rate of repelling attributed to the concentration of 250 mg / kg / mg / kg, and the mean at the level of 5% shows significant difference with each other. Branch leaf with methanol solvent 55.72%, leaf hexane 42.28%, fruit methane 61.78% and fruit hexane 48.01% (table 4).

Discussion

The results of this study showed that methanol and hexane extracts of leaf and fruit of *Sambucosebulus* contain tannin, saponin and alkaloids. Insects that feed on plant material containing tannin, a large amount of tannic acid passes through the membrane and can damage gastric epithelial cells. Tannins, especially tannic acid, act as toxin and can be used to exacerbate the effects of microbial agents such as *Bacillus thuringiensis* [36].

In a quantitative study on *Sambucus ebulus*, high total phenol levels were observed. In the methanol and hexane extracts the phenol content was 155.925 ± 2.1 mg, and 50 ± 1.7 mg respectively; on fruit the phenol content of methanol and hexane extracts was 92.777 ± 2.6 mg and 77.037 ± 1.5 mg respectively; the total flavonoid contents

were 81.111 ± 0.6 , 140.317 ± 2.4 , 82.063 ± 0.5 and 125.555 ± 2.3 mg respectively, with good antioxidant properties. Study of Fathiet *al.* on *Achillea wilhemsii*, showed the contents of Gallic acid 37.04/mg per g of extract and quercetin 2.5 mg per g of extract [31]. Fruits and leaves of *Sambucus ebulus* used in this study showed higher and stronger phenolic content than *Achillea wilhemsii*. We found that phenol and flavonoids content in comparison with the study of Ebrahimzadeh *et al.* study on the comparison of these properties in some plants, it can be said that the fruit of *Sambucus ebulus* compared with the fruit of *Hyoscyamus squarrosus griff*, which has the total phenol was reported to be 178.9 ± 8.94 and had a flavonoid content of 16.4 ± 0.82 , and the leaf had a phenol content of 98.95 ± 4.94 which contained 44.94 ± 2.24 flavonoid content. The leaf of the plant had a higher phenolic and flavonoid content and the fruit of *Pyrus boissieriana* Buhse which had a content of phenol 15.8 ± 0.19 and a flavonoid of $\pm 3.6 \pm 0.07$ and fruit of *Sambucus ebulus*. In this study, the phenol and flavonoids were stronger. *Phytolacca Americana* L. fruit content was 102.11 ± 5.1 and flavonoid content was 7.24 ± 0.36 the fruit of this study showed lower phenol content and higher flavonoid content than the above plants. The aerial part of *Salvia glutinosa* L. showed a phenolic content of 187.8 ± 11.3 and a flavonoid of 44.6 ± 1.2 , which was found to be lower than phenol and flavonoids in comparison with the phenol and flavonoid content of the leaf [35].

DPPH is a stable free radical with a central nitrogen atom, which is converted from purple to yellow by the process of taking hydrogen or electrons, and compounds with this capability are considered antioxidants. Based on IC50

The antioxidant property of *Sambucus ebulus* extract in comparison with ascorbic acid and quercetin and plants such as *Achillea wilhemsii* was lower than 58.9 ± 2.7 $\mu\text{g} / \text{mL}$ [29] and its leaf was more potent than *Hypericum perforatum* (96.0 ± 3.7 $\mu\text{g} / \text{mL}$) and of *Crocus caspius* (906.1 ± 21.3 $\mu\text{g} / \text{mL}$).

A study on phytochemical property of *Stachys byzantine* C. in north of Iran showed the presence of tannin, saponin, flavonoids, alkaloids, tripenoyds, glycosides and steroids .In the quantitative study, the mean of total phenol and tannin was 74.56 ± 4.343 mg GAE g-1 DW and 183.84 ± 3.353 mg TAE g-1 DW, respectively, and

the mean flavonoids and alkaloids were 0.396 ± 8.19 and 0.020 ± 0.69 , respectively in dry weight percent of the extract [37]. This corresponds with the results of this study. Plants with different defense mechanisms, including the production of effective chemical compounds against various pathogens, show that the quality and quantity of these materials varies according to different species and methods of extraction. The major antitumor natural oxidants are phenolic compounds and polyphenols, which are major components of secondary metabolites in plants [38]. In all, using of the chemical pesticides is a serious threatening, followed by development of genetic resistance insects and remaining of the toxin in the environment effects on the living beings [39]. The medicinal plant, *Sambucus ebulus* has many medicinal properties. It has been used in traditional medicine, in the biological and anti-nausea and anti-stress drugs and is an effective oxidative [40]. In a study done on blackberry infected with *Maconellicoccus hirsutus* (Green) pink flour, they tested the effects of the Henna plant extract *Lawsonia inermis* L. on this pest [41].

Studies on the insects repelling effects of the herbal extracts have been done on the German and American cockroach [42]. *Anopheles stephensi*, *Ixodes ricinus* L, showed the high potential repelling effect on the insects [43]. The results indicate the high repelling effect of the leaf and fruit extract of *Sambucus ebulus*, which agrees with studies of Rajkumar and Jebanesan [44] and Oshaghi [45]. The dimethyl-3 methyl benzamide (DEET) which is an insect repellent has been used as positive control [46]. Identification of the plant extract for evaluation of the effectiveness is very important. The effectiveness of the extract in repelling the insects varies depending the variety of the mosquitoes. There are different factors involved in the repelling insects such as, number of mosquito in the cage, using different plant extracts and also the percentage of extract [47]. Compounds of plants play a significant role in the protection of natural products and, on the other hand, in the fight against the viruses, bacteria, fungi and in particular against insect [48], which can be related to the role of *Sambucus ebulus* and its active ingredients. It can also be pointed out that tannin play an important role in destroying gastrointestinal epithelium [49] and, on the other hand, it plays a synergistic role in increasing the efficiency of biological products of *B. thuringiensis*. It has been

proved that tannic acid and tannin delay the development of larvae. Seth [50]. In the presence study tannins have been identified in the plant under study.

Leaf and fruit extracts of *Sambucus ebulus* have antioxidants, flavonoid and phenolic contents. Hexane extracts showed more phenolic compound while the methanolic extract recovered more flavonoids and antioxidants materials. The highest repelling effect of the *Sambucus ebulus* extract belonged to the methanolic fruits and the lowest to the hexane leaf extract. Considering the results, it was clear that the methanol extract has higher effect than the hexane extract. The difference could be due to the composition and the percentage of the components present in the extract. Also the fruit extracts had better effect compared to the leaf extracts. The effect of *Sambucus ebulus* extract in the experimental animal was 70%. Therefore, extract of this plant in repelling *Culex* of the mosquito in comparison with the other plant extract better. Hence, could be used as insect repellent.

Conclusion

Given the importance of identifying plant ingredients and their effectiveness in repelling insects, it is recommended that in order to improve the methods used for the collection of the plant, the variety of the plant the growing period should be considered. Also considering the fact that the repelling effect of the extract was tried on one type of the mosquito, the effectiveness of the extract should be tried on the other mosquito species. Knowing the chemical composition of the plant and their pharmacologic properties, (such as tannin, alkaloids, flavonoids) they can be used in other fields of medicine.

Conflict of interests

The authors declare that there are no conflicts of interest.

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