

Investigation of some biologic activities of *Swertia longifolia* Boiss.

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Abstract

Swertia species are widespread in Eastern and Southern Asian countries and used in traditional medicine as anti-pyretic, analgesic, gastro and liver tonic. Among different species, only *Swertia longifolia* grows in Iran. In this investigation, antioxidant, cytotoxic and acetylcholinesterase inhibitory activities of *S. longifolia* have been studied. Aerial parts and roots of the plant were collected, dried and extracted with methanol 80% (total extract). Different extracts of the plant were obtained using hexane, chloroform, ethyl acetate, methanol, methanol:water (1:1) and water, respectively. Cytotoxic activity was determined by MTT assay on MDBK, HepG2, MCF7, HT29 and A549 cell lines. Antioxidant activity was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals and acetylcholinesterase inhibitory (AChEI) effect was evaluated based on Ellman's method in 96-well microplates. The results showed no cytotoxicity of the plant extracts on MDBK, HepG2, MCF7, HT29 and A549 cell lines up to 100 µg/ml. All samples showed radical scavenging activity but methanol extract of aerial parts and ethyl acetate extract of the roots showed the highest effects. Total extract of the roots showed higher AChEI activity than the aerial parts. Among different extracts, chloroform and ethyl acetate extracts of the roots and chloroform and methanol:water extracts of the aerial parts were more potent in AChEI assay. It is concluded that aerial parts and roots of the plant are rich in antioxidant agents with no cytotoxicity on selected cell lines up to 100 µg/ml. Moreover, since antioxidant and AChEI activity of compounds play an important role in the treatment of Alzheimer's disorder, this plant might be a potential candidate for isolation of antioxidant and AChEI compounds which could be used as supportive treatment of Alzheimer's disease.

Keywords: *Swertia longifolia*; Cytotoxic; MTT; Acetylcholinesterase inhibitor; Antioxidant; DPPH

INTRODUCTION

Swertia (Gentianaceae) is a large genus of plants distributed in the mountainous regions of tropical areas at an altitude of 1200-3600 m. They are used for treatment of variety of disorders. In Indian medical system, these plants are used as a remedy for liver disorders, bronchial asthma, chronic fevers, anaemia and diarrhoea. In Ayurveda, some species of *Swertia* are used as antipyretic, antihelmintic, laxative and in asthma and leucorrhoea. In Unani medicine, they are used as astringent, tonic and anti-inflammatory agents. While various parts of the plants of *Swertia* are

considered as active remedies, the roots are the most potent parts. Phytochemical studies have been shown the presence of xanthone derivatives, flavonoids, iridoid glycoside and triterpenoids in this genus. Simple polyoxygenated xanthenes have been isolated from most of the genus. Because of the presence of different components, various biological activities such as hepatoprotective, antimicrobial, anti-inflammatory, anticarcinogenic, hypoglycaemic, antimalarial, antioxidant and CNS depressant properties have been reported for these plants (1). Recently pharmacological activities of *Swertia* have raised great interest. Among different species of *Swertia*, only *S.*

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longifolia Boiss. grows wildly in Iran (2). Some phytochemical studies have been carried out on this plant and it has been demonstrated that the plant contains xanthenes and iridoid glycosides (3-5) of which one of the xanthenes has shown hepatoprotective effect (6). Since, various biologic activities are expected from *S. longifolia*, in this investigation some biologic effects of this plant were studied.

Cancers belong to the group of disorders with treatment difficulty and sometimes are incurable. Local healers have been treating various kinds of cancers for ages. Many plants have been reported to be useful in the management of such conditions. Plants have been one of the sources of well-known anticancer drugs such as camptothecin, podophyllotoxin and paclitaxel (7). Recently many investigations have been performed to find new anti-cancer agents from nature. In this research, cytotoxic activities of aerial parts and roots of *S. longifolia* against four tumor cell lines MCF7, HepG2, HT29 and A549 and one normal cell line MDBK were investigated, using MTT method which is usually used for preliminary evaluation of anti-tumor compounds (8-10).

Alzheimer's disease is one of the most widespread neurodegenerative diseases that involves dementia and mainly afflicts people over 65 years old. The therapy of early and moderate stages of Alzheimer is mainly based on acetylcholinesterase inhibitors (AChEIs) such as donepezil and galanthamine as synthetic drugs. However these drugs have severe peripheral and central side effects, for example gastrointestinal disturbances, insomnia, fatigue or depression. These side effects have encouraged researchers to investigate safer acetylcholinesterase inhibitors (11). The biologically active plant components may be considered as a source of new anti-cholinesterase drugs from different classes of compounds. The bioactive substances are mainly including indoli, steroidal-piperidine-alkaloids, furanocoumarins, xanthenes, flavonoids and diterpenes (12,13). Since *S. longifolia* contains xanthenes (3,5), it may have AChEI activity.

Antioxidant compounds play an important role as a health-protecting factor. Scientific evidences suggest that antioxidants reduce the

risk for chronic diseases including neurodegenerative disorders, cancers and heart diseases. Plants are primary sources of naturally occurring antioxidants. The plant antioxidants belong to various classes of compounds with a wide variety of physical and chemical properties. The main characteristic of an antioxidant is its ability to scavenge free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative diseases. Antioxidant compounds trap free radicals such as peroxide, hydroperoxide or lipid peroxide and inhibit the oxidative mechanisms that lead to degenerative diseases (14,15). Xanthenes are secondary metabolites of which many has been proven to have radical scavenging activity (16,17). In another part of this study, antioxidant effects of aerial parts and roots of *S. longifolia* which is rich in xanthenes (3,5) have been investigated.

MATERIALS AND METHODS

Plant materials

Swertia longifolia Boiss. (aerial parts and roots) was collected in July 2010 from the North of Iran, Mazandaran province, Pole-Zangooleh, Road to Yush, Lavashm mountains, No. 3058 (TMRC) and identified by Atefeh Pirani, Botanist, from Traditional Medicine and Materia Medica Research Center, SBMU, Tehran.

Chemicals

Acetylthiocholine iodide (ATCI), acetylcholinesterase enzyme (AChE) from bovine erythrocytes, 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB), [3-(4,5-dimethyl thiazol-2-yl)-2,4-diphenyl tetrazolium bromide] (MTT) and 2,2-diphenyl-1-picryl hydrazyl (DPPH) were obtained from Sigma (Germany). Methanol, DMSO and all other organic solvents (analytical grade) were purchased from Merck (Germany).

Plant extraction

Total plant extracts were obtained by extraction of dried and milled aerial parts and

roots of the plant with methanol 80% (1:10) using maceration method for 4 days. Every 24 h, the mixture was filtered and fresh solvent was added to the plant powder. The combined extracts were concentrated and dried using rotary evaporation and freeze drying methods.

In order to fractionate the plant aerial parts and roots, *S. longifolia* dried powder was macerated with hexane for 4 days and each day the solvent was replaced with the fresh solvent. After the fourth day, the residue of the plant was extracted with chloroform, ethyl acetate, methanol, methanol:water (1:1) and water, respectively with the same process. The same filtrates were combined and concentrated under reduced pressure and freeze drying method.

Cell lines

HepG2 (human hepatocellular liver carcinoma), MCF7 (human breast adenocarcinoma), HT29 (human colon adenocarcinoma), A549 (human lung adenocarcinoma) and MDBK (bovine kidney cells) cell lines were obtained from Pasture Institute of Iran. Each cell line was cultured in suitable medium for desired growth, plus 5 or 10% FBS in a humidified incubator at 37°C in an atmosphere of 5% CO₂. Then the growth curve of each cell line was plotted.

MTT assay

Cytotoxic properties were assessed by MTT assay using different concentrations of the plant extracts (10). The cells were seeded into 96-well microplates at 5×10^3 to 10^4 cells per well, depending on the cell lines. Three wells for each concentration were seeded and triplicate plates were used for each cell line. Then, the cells were incubated at 37°C. After 24 h the medium was replaced by fresh medium containing different concentrations of the plants extracts and incubated further for 72 h. The initial concentration of samples was 10 mg/ml in DMSO, which was serially diluted with medium to obtain six final concentrations from 100 to 3.125 µg/ml. The medium was replaced with fresh medium containing MTT with a final concentration of 0.5 mg/ml. The cells were incubated for another 4 h in a humidified atmosphere at 37°C and thereafter the medium containing

MTT was removed and remaining MTT-formazan crystals were dissolved in DMSO. The absorbance was measured at 570 nm using a microplate reader and viability of the cells was assessed relative to the negative control which was exposed to the solvent without extract. Tamoxifen was used as the positive control at concentrations of 25 to 0.781 µg/ml.

Acetylcholinesterase inhibitory assay

AChEI activity was determined using a 96-well microplate reader based on Ellman's method (18). The enzyme hydrolyses the substrate acetylthiocholine resulting in the formation of thiocholine which reacts with Ellman's reagent or DTNB to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitro-benzoate which can be detected at 405 nm. In the 96-well plates, 125 µl of 3 mM DTNB, 25 µl of 15 mM ATCI and 50 µl of phosphate buffer pH 8, and 25 µl of sample dissolved in methanol (3 mg/ml) were added to the wells. The absorbance was measured at 405 nm every 13 sec for 65 sec. 25 µl of 0.22 U/ml of AChE enzyme was then added and the absorbance was again read every 13 sec for 104 sec. Absorbance was plotted against time and enzyme activity was calculated from the slope of the line. Any increase in the absorbance due to the non-enzymatic hydrolysis of substrate was corrected by subtracting the rate of reaction before addition of the enzyme from the rate after addition of the enzyme. Percentage of enzyme inhibition was calculated by comparing the rates for the sample to the blank (using methanol without extract) (19). Because hexane extract was not soluble in methanol, further experiments were not performed in this study. Donepezil was used as the positive control.

2,2-Diphenyl-1-picryl hydrazyl radical scavenging assay

This method is one of the most extensively used antioxidant assay for plant samples. The method is based on scavenging of DPPH free radicals by antioxidant agents, which produces a decrease in absorbance at about 520 nm. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, the reduced form of the radical is generated accompanied by loss of color. This

delocalization is also responsible for the deep violet color, characterized by an absorption band at about 520 nm (14,20).

In the current study, in order to determine DPPH radical scavenging activity of *S. longifolia* samples, 2 ml of 100 μ M DPPH methanol solution was added to 2 ml of various concentrations of the extracts. The mixture was shaken vigorously and left at room temperature for 30 min. Then, the absorbances of the solutions were measured at 517 nm and antioxidant activity was calculated using the following equation:

Scavenging capacity % = $100 - [(ABS \text{ of sample} - ABS \text{ of blank}) \times 100 / ABS \text{ of control}]$.

Mixture of 2 ml methanol with 2 ml plant extract solution was used as the blank, while 2 ml DPPH solution plus 2 ml solvent corresponding to each extract was used as the negative control. Vitamin C was used as positive control with concentrations of 1, 1.5, 2, 2.5, 3 μ g/ml. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the plot of inhibition percentage against extract concentration (21,22). The tests were performed in triplicate.

RESULTS

Cytotoxic activity

This investigation showed no cytotoxicity of *S. longifolia* aerial parts and roots on MDBK, MCF7, HepG2, A549 and HT29 cell lines at concentrations of less than 100 μ g/ml (Table 1).

Acetylcholinesterase inhibitory activity

The results of AChEI properties of *S. longifolia* aerial part and root extracts at concentration of 300 μ g/ml have been shown in Fig. 1. All the plant extracts showed enzyme inhibition activity except water extract in the manner that the total extract of the plant root exhibited the highest effect (36.2%).

Antioxidant activity

Antioxidant activities of the tested extracts have been demonstrated in Fig. 2. The results showed that *S. longifolia* aerial parts and roots had considerable radical scavenging activity compared to vitamin C as the standard.

Table 1. The viability of cell lines at concentration of 100 μ g/ml of different extracts of *Swertia longifolia* aerial parts.

Aerial part extracts	Viability in different cell lines*				
	MCF7	HepG2	MDBK	A549	HT29
Total	102.08±0.95	105.81±3.45	100.68±2.07	98.26±3.05	96.98±3.62
Hexane	100.03±7.53	96.86±4.88	99.61±1.37	92.8±4.02	109.02±7.53
Chloroform	94.60±9.39	100.60±3.78	101.87±9.79	94.33±1.22	111.94±5.29
Ethyl acetate	90.90±3.60	100.55±3.95	88.74±7.13	102.34±9.29	114.25±2.89
Methanol	100.14±3.95	109.39±4.94	92.88±7.04	99.94±1.28	102.58±2.17
Methanol:Water	100.16±4.48	100.34±1.54	98.05±4.01	100.73±5.86	94.25±4.00
Water	100.55±3.20	103.70±5.27	94.76±6.96	99.13±4.13	96.77±2.46

* Values represent the mean \pm SD of three experiments.

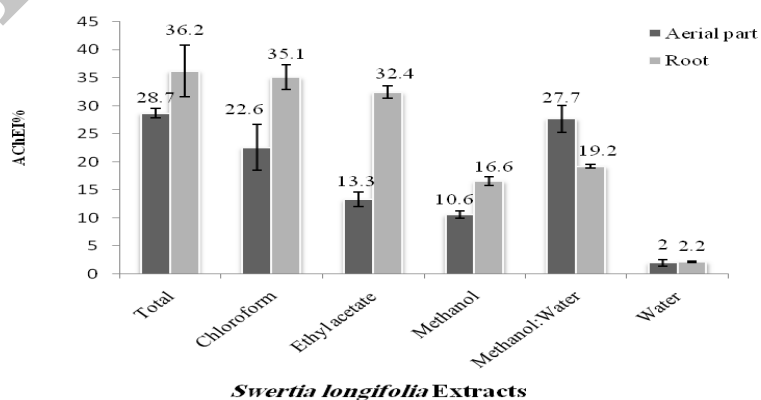


Fig 1. Acetylcholinesterase inhibitory activity of *Swertia longifolia* extracts

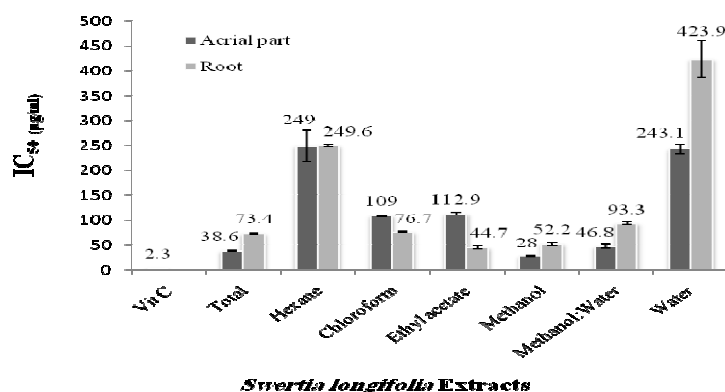


Fig 2. Radical scavenging activity of *Swertia longifolia* extracts by using DPPH radicals

DISCUSSION

Swertia species are used in traditional medicine in different countries, therefore seems essential to evaluate their toxicities on normal cell lines. Reports on the cytotoxic activity of *Swertia* spp. are scarce. An investigation on *S. chirata* demonstrated no toxicity of the plant extract on *Artemia salina* (23). Toxic effects of some xanthenes have been reported. It is proven that presence of methoxyl or hydroxyl group at C₈ in xanthone structure causes cytotoxicity such that methoxyl group on C₈ position showed more toxicity than hydroxyl group (24). Since, some xanthenes of *S. longifolia* contain hydroxyl or methoxyl group at C₈ (3,5), it is expected that *S. longifolia* extract shows at least some cytotoxic effects. In this study, however, no cytotoxicity was observed against MDBK, MCF7, HepG2, A549 and HT29 cell lines at concentrations of less than 100 µg/ml.

Among different extracts of the plant roots, chloroform and ethyl acetate extracts had more AChEI activity while chloroform and methanol:water extracts of aerial parts showed the highest effects. It seems that compounds responsible for AChEI activity have various structures and present in different extracts of the plant. Several investigations have demonstrated the AChEI effects of the xanthenes. A study on xanthenes of *Gentiana campestris* has proved the presence of four xanthenes including bellidine, bellidifolin, norswertianolin and swertianol in which are responsible for AChEI activity; in the manner that bellidifolin showed similar activity to

galanthamine which was used as a reference (25). In another study the role of non-alkaloidal compounds in inhibition of AChE enzyme has been discussed and it has been demonstrated that xanthenes are one of the secondary metabolites with AChEI activity (26). Since, *S. longifolia* is rich in xanthenes (3,5), these compounds may be responsible for AChEI activity in this plant.

Among different extracts of the plant aerial parts, methanol extract had the highest antioxidant effect (8% of vitamin C). Hexane and water extracts which contain lipophilic and hydrophilic compounds, respectively, showed the least activity. It is concluded that in aerial parts of the plant, relatively high polar compounds which could be extracted with methanol and methanol:water solvents have reasonable antioxidant activity. Moderately polar compounds which present in chloroform and ethyl acetate extracts have demonstrated moderate effect (about 2% of vitamin C). Other investigations on *Swertia* species have proved xanthenes are powerful antioxidant agents. It has been demonstrated that 1,7,8-trihydroxy-3-methoxyxanthone, a polyhydroxylated xanthone, had high radical scavenging activity (27). The compound 1,3,8-trihydroxy-5-methoxyxanthone isolated from *S. longifolia* (5) bears the same number of methoxyl and hydroxyl functional groups as 1,7,8-trihydroxy-3-methoxyxanthone though in different positions. With regard to the previous study that showed position of methoxylation has no effect on antioxidant activity (27), it could be suggested that the strong antioxidant activity might

be related to the presence of compound 1,3,8-trihydroxy-5-methoxyxanthone in the chloroform extract; although, other components of chloroform extract might also be involved in antioxidant activity.

Other studies have demonstrated free radical scavenging activity of gentiacauleine and swertiaperenine isolated from *Swertia* genus (1). In our previous study, these compounds were isolated and purified from *S. longifolia* as well (3). The results of the present study showed that methanol extract of the plant aerial parts had the highest antioxidant effects. This extract contains glycosidic compounds, however, in the literature reports on antioxidant activity of glycosidic xanthenes are limited and the effect cannot be attributed to these compounds. Thus, purification of the components of this extract and evaluation of their biologic activities seems imperative.

The results of the antioxidant assay of *S. longifolia* roots showed that radical scavenging activity of total extract of the plant roots is about half of the antioxidant effect of the aerial parts. Among different extracts of the plant roots, ethyl acetate extract had the least IC_{50} , thus it was considered the most potent antioxidant extract and methanol, chloroform, methanol:water, hexane and water extracts were in the next orders, respectively. It is concluded that in plant roots, compounds with moderate polarity which exist in ethyl acetate extract have more radical scavenging activity. In both aerial parts and roots of the plant, hexane and water extracts exhibited the lowest effects.

Antioxidants are considered as a group of important agents in prevention of cancers (28) thus radical scavenging may be considered as one of the mechanisms in cancer treatment. However, according to the recent investigation, there is no relationship between antioxidant activity of the plant extracts and cytotoxic properties. On the other hand, the plant is rich in antioxidant agents with no cytotoxic activity. Therefore, it seems that antioxidant agents play important role only in prevention of cancers and not in their treatment.

Due to decreasing acetylcholine of brain in Alzheimer's disease, many synthetic and natural AChEI agents have been used in the

treatment of the disorder (11-13). In addition, several investigations have demonstrated the effects of antioxidant agents in the prevention and treatment of Alzheimer's disorder because these compounds protect the brain from destructive effects of free radicals (29). In this study, good correlations were observed between antioxidant and AChEI activity of *S. longifolia* (correlation coefficient of -0.6940 for aerial parts and -0.7826 for roots). It has demonstrated that some components of the plant may have radical scavenging properties and also AChEI activity. In general, *S. longifolia* can be considered as a good candidate for supportive treatment of Alzheimer with no cytotoxicity but more complementary investigations are needed.

CONCLUSION

S. longifolia has no cytotoxic effect on HepG2, MCF7, A549 and HT29 cell lines at tested concentrations but it showed considerable radical scavenging activity and some acetylcholinesterase inhibitory properties without toxicity on MDBK normal cell line up to 100 $\mu\text{g/ml}$. Therefore, it may have the potential to be used as medicinal plant in supportive treatment of Alzheimer's disorder.

ACKNOWLEDGMENT

The results have been obtained from Pharm.D. student thesis (Taraneh Emrarian). The authors wish to thank Traditional Medicine and Materia Medica Research Center, Shahid Beheshti University of Medical Sciences for the grant (No. 116).

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