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Study on the antioxidant activity and *in vitro* antifungal activity of *Verbascum speciosum* methanolic extract

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Abstract:

The present study was conducted to determine the antioxidant and antifungal effects of methanol extract from aerial part of *Verbascum speciosum.* The total phenolic and flavonoid content and the antioxidant activity of the plant extract were determined by using the Folin-Ciocalteau and the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assays. The total phenols and flavonoid content of leaves were determined as 82±6.43 mg GAE/g extract and 30.79±0.5 mg RE/g extract, respectively. The extract exhibited significant radical scavenging activity with IC_{50} value of 32.35µg/ml. The methanolic extract was investigated for its antifungal activity against some fungal reference strains which include: *Candida albicans, Candida tropicalis, Candida parapsilosis, Candida krusei, Candida dubliniensis, A. flavus, A.niger, Penicillium* and *Alternaria*. For minimum inhibitory concentrations (MICs) determination, a broth microdilution method according to CLSI M27-A and M38-A for yeasts and filamentous species was performed. The results showed that the highest *in vitro* antifungal activities were against *C. parapsilosis* and *Alternaria.* However, there were no significant differences between different fungal species. These results indicate a high potential of antifungal properties of this methanolic extract. Therefore, it can be suggested to be an effective replacement treatment for fungal infections or for the preservation of foods against pathogenic and toxigenic microorganisms.

Keywords: DPPH, methanolic extract, phenolic component, *Verbascum speciosum*.

Introduction

For a very long time now, plants have been widely used for their useful natural products such as antioxidants. Excessive production of free radicals in the body causes numerous problems such as coronary heart disease, cancer and acceleration of age (Karimian and Ghasemlou, 2013). Phenolic components of plants are considered as an excellent antioxidant agent with useful properties.

The genus *Verbascum* L. (*Scrophulariaceae*) comprises of about 323 species distributed throughout the world (Saltan *et al.*, 2011).The drug, prepared from several parts of the plant has been used as an expectorant and for the treatment of cough and chills (Tatli *et al.*, 2004). GC-mass analysis and total phenol and flavonoids contents of *V. phlomoides* flower extracts were investigated (Armatu *et al.*, 2011).

Antimicrobial and antioxidant potential of the aerial parts of four new *Verbascum* species which include *V. bellum, V. detersile, V. myriocarpum* and *V. pestalozzae* from Turkey origin were determined previously (Saltan *et al.*, 2011). Some *Verbascum* species have been found to have antibacterial activity against methicillin-resistant *Staphylococcus aureus* (Duglar and Hacioglu, 2009). In a recent study in Iran, total phenolic content, antioxidant and antibacterial activities of three *Verbascum* species including *V. speciosum, V. nudicaule and V. sinuatum* were investigated (Karimian and Ghasemlou, 2013). In the present study, total phenolic and flavonoid content, antioxidant and antifungal activity of *V. speciosum* of Mahabad origin were investigated.

Materials and Methods

Plant material

The *V. speciosum* were collected from Mahabad (Northwest of Iran). The plant identity was confirmed by herbarium of the Faculty of Agriculture, University of Tabriz, under code number 16696 Tbz-Fph.

Preparation of the methanolic extract

The dried and powdered plant materials (15g) were extracted successively with 150 mL of methanol (99%) by Soxhlet extractor for 72 h at a temperature not exceeding the boiling point of the solvent (Sokmen *et al.*, 1999). The methanolic extracts were filtered using Whatman filter paper (No. 1) and then concentrated *in vacuo* at 40°C using a rotary evaporator. The extract was black and very sticky. The extract yield obtained was 20.66%. Dried extract was stored in labeled sterile screw-capped bottle at 4°C and later used for *in vitro* study (Tambeker and Dahikar, 2011).

Antioxidant activity

The DPPH assay

In this assay, hydrogen atom or electron donation ability of the essential oil was evaluated by measuring the bleaching of the purple-colored methanol solution of DPPH (Sigma, Aldrich). Five milliliters of a 0.004% methanol solution of DPPH radical was mixed with 50 µl of various concentrations of the extract. The mixture was incubated at room temperature for 30 min, and then the absorbance was read against a blank at 517 nm using a spectrophotometer (Pharmacia, Uppsala, Sweden). Inhibition of the free radical DPPH in percent (I %) was calculated using the following equation:

$$I\ \% = (A_{blank} - A_{sample} / A_{blank}) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. The IC_{50} value is defined as the concentration of the extract that resulted in 50% inhibition (Mahmoudi *et al.*, 2013). Vitamin C and rutin were used as standard controls.

Total phenols assay

The total phenolic content of *V. speciosum* extract was determined by employing the method described by Chandler & Dodds (1983). Folin-Ciocalteu reagent and gallic acid (both Sigma–Aldrich) were used as standard. Briefly, 0.1 ml of extract solution containing 1 mg of extract was mixed with 46 ml distilled water, and then 1 ml Folin-Ciocalteu reagent was added to the flask and was shaken thoroughly. After 3 min, 3 ml of 2% Na_2CO_3 solution was added and the mixture was incubated for 2 h with intermittent shaking. Absorbance was determined at 760 nm. The same procedure

was performed for all standard gallic acid solutions (0–1000 mg in 0.1 ml). The standard curve was obtained according to the following equation:

Absorbance = 0.0012 × Gallic acid (µg) + 0.0033

Estimation of total flavonoid concentration

The flavonoids content in extract was determined by using a method based on the formation of a complex flavonoid–aluminum using aluminum chloride and rutin solutions. One milliliter of the diluted sample was mixed with 1 ml of 2% aluminum chloride methanolic solution. The absorbance of the reaction mixture was measured against a blank at 430 nm with a spectrophotometer, after incubation at room temperature for 15 min. The total flavonoid was determined as mg rutin equivalent (RE)/g extract.

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentrations (MFC)

The tested fungi, including *C. albicans, C. tropicalis, C. parapsilosis, C. krusei, C. dubliniensis, A. flavus, A. niger*, *Penicillium* species and *Alternaria* species were cultured on Sabouraud dextrose agar (Merck, Germany) at 30°C for 48 h before the experiment. The correct concentrations of prepared suspension of the fungi were adjusted by heamocytometer count. Broth microdilution method (M27-A) as described by CLSI was used to determine *in vitro* MIC of methanolic extract against fungi strains. The test was performed in 96-well flat bottomed microtiter plates, using RPMI 1640 which had been buffered to pH 7.0 with 0.165 M morpholinopropanesul-fonic acid (Sigma). 100 µl extract (20%) was added to first well containing 100 µl medium, then serially diluted twofold. The yeast inoculum was adjusted to a concentration of 0.5×10^{3} to 2.5×10^{3} CFU/ml and an aliquot of 100µl was added to each well of the microdilution plate. The filamentous fungi inoculums were adjusted to a concentration of 0.5×10^{4} to 1×10^{4} CFU/ml and an aliquot of 100µl was added to each well of the microdilution plate. The plates were incubated at 35°C for 48 h. MIC was determined as the lowest concentration of extract which inhibited the visual growth of fungi and MFC, the lowest concentration resulting in total growth inhibition. The test was performed twice on separate plates.

Results

Antifungal activity. The antifungalactivities of *V. speciosum*extract assayed against the microorganisms in the current study were quantitatively assessed by evaluating MIC values. The results are given in Table 1. The results showed that methanol extract of *V. speciosum* had antifungal activity against 10 fungi. The methanolic extract showed highest antifungal activity against *C. parapsilosis*, followed by *Alternaria*. These fungi were followed by *C. albicans, A. flavus, A. niger* and *Penicillium*. As shown in the results of the present study, the highest MFC value was determined for *C. parapsilosis* followed by *C. albicans*. The lowest MICs and MFCs of the extract were 0.09 (0.39) µg/mL against *C. parapsilosis*, followed by *C. albicans* (MIC values is 1.56 (3.12) µg/mL).

Table 1. MIC and MFC of *Verbascum speciosum* methanolic leaf extract against fungal strains

Verbascum speciosum methanolic extract	MIC(µg/ml)	MFC (µg/ml)
C.albicans	1.56	3.12
C.tropicalis	3.1	6.25
C.parapsilosis	0.09	0.39
C.krusei	6.25	12.5
A.flavus	1.56	25
A.niger	1.56	50
Penicillium	1.56	25
Alternaria	0.78	12.5
C.dubliniensis	2.125	12.5

Antioxidant activity: The hydrogen atoms or electron donating ability of *V. speciosum* methanolic extract was determined by measurement of DPPH radical scavenging activity. Methanol extract was able to reduce the stable radical, DPPH the yellow-colored diphenylpicryhydrazine with an IC_{50} value of 32.35 µg/mL. The IC_{50} value for ascorbic acid was 5.18 µg/mL.

Total phenolic and flavonoid contents

In the present study, total phenolic content was determined by using the Folin-Ciocalteu reagent. Total phenolic content of *V. speciosum* was solvent dependent and expressed as milligrams of gallic acid equivalents (GAE). The content of flavonoid was expressed as rutin equivalent (Table 2).

Table 2. Flavonoids and Phenol content in *Verbascum speciosum*

Plant name	Flavonoids (mg/g)	Phenol (mg/g)
Verbascum speciosum	30.79 ± 0.5*	82 ± 6.43*

Discussion

Several techniques have been used to determine the antioxidant activity *in vitro* in order to allow rapid screening of substances since substances that have low antioxidant activity *in vitro*, will probably show little activity *in vivo* (Saeed *et al.*, 2012).Natural antioxidants present in spices are responsible for inhibition or prevention of the deleterious consequences of oxidative stress. Spices and herbs contain free radical scavengers like polyphenols, flavonoids and phenolic compounds. In the present study, the free radical scavenging activity of methanolic extract of *V. speciosum* was evaluated. The results indicate that the antioxidant activity of the crude extract of *V. speciosum* is lower than that of ascorbic acid, since their IC_{50}

value was found to be higher. A previous study revealed that extract of *V. pestalozzae* was the most active, providing an IC_{50} value at 15 µg/mL. The IC_{50} values of other studied species of *Verbascum* were 27 µg/mL for *V. detersile*, 130 µg/mL for *V. bellum* and 220 µg/mL for *V. myriocarpum* (Saltan *et al.*, 2011). Another study showed that methanol and water extracts of three *Verbascum* species exhibited greater antioxidant activity than those of other plant extracts with an IC value of 130.8, 309.3, 65.5, 235.6, 123.8 and 132.8 µg/mL for *V. leptocladum, V. mucronatum* and *V. davisianum*, respectively (Alan *et al.*, 2009). According to previous studies, leaves of *V. speciosum* contain boric acid, phenol and pyrrolidine. Therefore, it can be assumed that these components are involved in antioxidant activity of methanolic extract. Based on the results of the present work, the *V. speciosum* methanolic extract has antioxidant activity and remarkable phenolic content, thus, it can serve as an excellent natural source of antioxidant agents.

Free radicals have wide adverse effects on cells, resulting in many disease conditions such as coronary disease or cancer. Phenolic components act as free radical scavengers and cause delay or prevent oxidative stress caused by free radicals (Katiraee *et al.*, 2015). In the present study, total phenolic content was determined by using the Folin-Ciocalteu reagent. It was 82±6.43mg/g, presented as gallic acid equivalent in milligrams per gram of methanolic extract. In a previous study, total phenolic contents of methanolic extract of *V. speciosum* was investigated and found to be 95.83±1.39 mg/g (Karamian and Ghasemlou, 2013). Phenolic compounds may contribute directly to desirable and undesirable aromas and tastes of food. Phenolic compounds contribute to the aroma and taste of certain spices and plant extracts. Considerable variation was found in phenolic compounds of different species. As a result of the diversity and complexity of natural mixtures of phenolic compounds in hundreds of herb extracts, it is rather difficult to characterize every compound and elucidate its structure, but it is not difficult to identify major groups and important aglycones of phenolic compounds. The contents of phenolic compounds, measured by HPLC after enzymatic hydrolysis, were different from those values measured using the Folin-Ciocalteu method. The amount of polyphenols was also dependent on the extraction method. The samples for HPLC were subjected to enzymatic hydrolysis which in contrast to methanol extraction, resulted in specific disruption of linkages and deglycosylation of phenolic compounds (Wojdylo *et al.*, 2007).In a study in Hatay, Turkey the phenolic component of *V. antiochium* was determined to be 92.71 mg of GAE/g (Ozcan *et al.*, 2010).

The results of general screening for antifungal activity are shown in Table 1. There was antifungal activity of *V. speciosum* methanolic extract against all the tested fungi. The methanolic extract showed highest antifungal activity against *C. parapsilosis*, followed by *Alternaria*. These two fungi were followed by *C. albicans, A. flavus, A. niger* and *Penicillium* which were affected at the same level by methanolic extract. According to the results of the present study, the highest MFC value was determined for *C. parapsilosis* followed by *C. albicans*. Antimicrobial activities of *V. speciosum* have been investigated previously by the disc diffusion method (zone size, mm) and showed its ability to inhibit *Bacillus anthracis* (15.3±0.33), *S.aureus* (12.6±0.33), *Salmonella* (11.3±0.33), *Bacillus cereus* (9.6±1.2) and *Listeria monocytogen*

(9.3±0.33), yet methanolic extract had no effect on *Escherichia coli* (Nofouzi, 2015). In terms of their antimicrobial activity, *Verbascum* species have a broad variation. For instance, the methanolic extract of *V. antiochium* had no antifungal effect against *C. albicans* by disc diffusion method (Ozcan *et al.*, 2010). *Verbascum* L. species owe their antimicrobial activities to a wide range of compounds such as glycosides, alkaloids and saponins (Kalpoutzakis *et al.*, 1999). There are many reports suggesting that phenolic compounds have antimicrobial activity. In conclusion, phytochemicals of *Verbascum* can be used against some pathogenic bacteria and fungi (Saltan *et al.*, 2011; Ozcan, *et al.*, 2010; Karimian and Ghasemlou, 2013). The results of this study revealed that *V. speciosum* methanolic extract with its potent antifungal activity can serve as an excellent natural source for use in traditional medicine.

In this study, methanolic extract of aerial part of *V. speciosum* showed effective *in vitro* antifungal activity against all the studied species. Further investigations on the components which are mainly responsible for these effects and their mechanism of action would be valuable.

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