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Antibacterial Activity and Total Phenolic Content of the *Onopordon acanthium* L. Seeds

Khadijeh Zare[1*], Hossein Nazemyeh[2], Farzaneh Lotfipour[3], Somayeh Farabi[1], Mehdi Ghiamirad[1], Abolfazl Barzegari[2]

[1]*Departeman of biology, Ahar branch, Islamic Azad University, Ahar, Iran.*

[2]*Research Center for Pharmaceutical Nanotechnology, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.*

[3]*Hematology and Oncology Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Iran.*

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ABSTRACT

Background: *Onopordon acanthium* L. is an important medicinal plant which has been used in Iranian medicine. In the present study, total phenolic content (TPC) was evaluated and antibacterial properties of n-hexane and methanol extracts of *O. acanthium* L. seeds were screened. ***Methods:*** TPC was determined by Folin–Ciocalteu reagent method. Antibacterial activities of *O. acanthium* were investigated using disc diffusion method, minimum inhibitory concentration (MIC) and resazurin microtiter assay against some gram-positive bacteria including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus loteus* as well as gram-negative ones *Escherichia coli* and *Klebsiella pneumonia*. ***Results***: The TPC was found to be 168.56 ± 4.89 mg of gallic acid/g of extract. Plant methanol extract showed significant antibacterial activity against gram-positive bacteria; *Staphylococcus epidermidis* and *Micrococcus luteus* (with MIC= 0.612 mg/ml), while n-hexane extract demonstrated negligible to *no* inhibitory activity against gram-negative and gram-positive bacteria. ***Conclusion***: The results of the present study suggested the effect of *O. acanthium* methanol extract against the tested bacteria *in vitro*, may contribute to the *in vivo* efficacy of this extract.

Introduction

For centuries, the therapeutic properties of various medicinal plants have been extensively applied to combat human health disorders. It has been estimated that between 60-90% of the population in developing countries almost entirely utilize traditional and botanical remedies as normal part of their primary healthcare.[1]

Plant-derived products contain a vast combination of phytochemicals such as phenolic acids, flavonoids, tannins, lignans and other compounds.[2] These compounds show several health-related activities such as antibacterial, antimutagenic, anticarcinogenic, antithrombotic and vasodilatory activities.[3,4]

Some of the plants have an activating or inhibiting effect on microbial growth according to their constitution and concentration.[5] Phenolics are widely distributed in plants and used for defensive functions in many plants.[6] They are a class of plant secondary metabolites that contain one or more hydroxyl derivatives of benzene rings and may affect the growth and metabolism of bacteria.[7]

Considering the emergence of resistance microorganisms to the commercial antibiotics, the medicinal plants have been recognized as valuable alternative resources.[8] The natural antimicrobial compounds exist in plant products are widely studied as a potential solution to fight against resistant microorganisms.[9]

Onopordon acanthium (Scotch thistle) from *Compositae* family is a biennial plant growing in the Asia and Europe.[10] Seven of them are endemic to the semi-arid region of Iran.[11] The pink flowers, coarse inflorescence, spiny leaves and shoots, faveolate receptacle, and smooth achen, glabrous and almost tetrachen are typical characteristics of the genus.[12] *O. acanthium* a well-known valuable medicinal herb which is used to relief cancer and skin ulcers and diminish excessive mucous discharge as well.[13] Moreover, the genus is known to produce a variety of compounds, including flavonoids, sesquiterpenes, flavonolignans and phenylpropanoids.[14,17] To the best of our knowledge, few phytochemical investigation has been carried out on *O. acanthium*.[18] Although, the biological activities of many *Onopordon* species have not been investigated and there are few reports about the *O. acanthium*.[19] This work attempts to investigate

***Corresponding Author:** Khadijeh Zare, *Departeman of biology, Ahar branch, Islamic Azad University, Ahar, Iran.* Tel (+98) 411- 3367914, Fax (+98) 411- 3367929, E-mail: Kh-zare@iau-ahar.ac.ir

the antimicrobial effects and study of the TPC of *O. acanthium*.

As such the aim of this study was to estimate the TPC and antimicrobial properties of *O. acanthium* seeds. TPC of *O. acanthium* seeds was determined by Folin–Ciocalteu reagent method and antibacterial activities were investigated with disc diffusion method, minimum inhibitory concentration (MIC) and resazurin microtiter assay.

Materials and Methods

Plant material and extraction

Plant seeds were collected in june 2010 from Maragheh, East- Azerbaijan province, Iran and confirmed. A voucher specimen (voucher no. Tbz-Fph 539) representing this collection has been deposited in the Herbarium of the Tabriz University of Medical Sciences, Tabriz, Iran. The dried and powdered seeds of *O. acanthium* (30 g) were extracted successively with n-hexane and methanol (MeOH) by long maceration at room temperature. The extracts were filtered using Whatman filter paper (No:1) and concentrated using a rotary evaporator at a maximum temperature of 45°C to yield 0.5 and 0.9 g of dried n-hexane and methanol (an orange amorphous mass) extracts, respectively.

Determination of total phenolic content

The concentration of phenolics in plant extract was determined using modified Folin-Ciocalteu assay.[20] Methanolic extract in the final concentration of 0.05 mg/ml was used in the analysis. The reaction mixture was prepared by blending 1ml of methanolic solution of extract, 0.2 ml of Folin-Ciocalteu's reagent dissolved in water and 1ml of 0.2% aqueous $NaHCO_3$. The sample was thereafter incubated in a thermostat at room temperature for 60 min. The absorbance was determined using spectrophotometer at λ max of 760 nm. The samples were prepared in triplicate for each analysis. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of phenolics was recorded (mg/ml) from the calibration line. The content of phenolics in extract was expressed in terms of gallic acid equivalent (mg of GAE/g of extract).

Antibacterial activity

Antibacterial properties of the plant were investigated against five gram negative (g^-) and gram positive strains (g^+) including *Escherichia coli* (ATCC 8739) and *Klebsiella pneumonia* (ATTC 1053) as our g^- test strain and *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus aureus* (ATCC 6538) and *Micrococcus luteus* (ATCC 10240) as g^+ test organisms. The bacterial strains in lyophilized form were purchased from Persian Type Culture Collection, Tehran, Iran. After activating, the cultures of bacteria were maintained in their appropriate agar media at 4°C and used as stock cultures. A single colony from the stock plate was transferred into Mueller Hinton Broth and incubated over night at 37°C. After incubation time the cells were harvested by centrifugation at 3000 rpm for 15 min and washed twice and re-suspended in saline solution to provide an optical density equal to 0.5 McFarland or bacterial concentration around 10^8 CFU/ml.

The agar disc diffusion method

The agar disc diffusion method was used to evaluate the antimicrobial activity of *O. acanthium* n-hexane and methanol extract by measuring the inhibition zone diameter. The extracts were dissolved in 50% V/V aqueous dimethylsulfoxide (DMSO). One hundred microliters of the prepared inocolum were spread over the plates containing Mueller-Hinton agar using a sterile cotton swab in order to get a uniform microbial growth. The sterile blank discs (6 mm in diameter) were impregnated with 50 µL of *O. acanthium* extracts. A standard disc containing Amikacin (30 mg) was used as reference control. Another discs impregnated with 50% V/V aqueous DMSO was used as negative control. The plates were incubated for 30 min in refrigerator to allow the diffusion of extracts, and then they were incubated at 37°C for 24h. After the incubation period, the zone of inhibition was measured with a calliper. All experiments were performed in triplicate, and mean value was calculated.

Minimum inhibitory concentration (MIC) determination

The broth macro dilution method was employed to determine minimum inhibitory concentration (MIC) of the extracts according to CLSI guideline. The prepared inoculum was added to Muller Hinton broth to reach the final concentration of 10^6 CFU/ml. One millilitre of the inoculated medium was added to 8 sterile tubes including seven tubes containing serially diluted extracts in 50% aqueous DMSO and a control tube with 50% aqueous DMSO without the extract respectively. The tubes were incubated at 37°C for 24 hours. After that, from the content of the each tube streak culture was performed onto Mueller Hinton Agar plates. The first concentration with no sign of bacterial growth on plates considered as MIC. All experiments were performed in three separate occasions.

Furthermore, MIC values were determined by resazurin microtiter method. The resazurin microtiter assay

solution was prepared by dissolving a 270 mg tablet in 50 mL of sterile distilled water. A vortex mixer was used to ensure that it was a well-dissolved and homogenous solution.

Plates were prepared under aseptic conditions. A sterile 96 well plat each containing 200µL of nutrient broth with resazurin microtiter assay indicator solution was prepared. Accordingly, 50 µL of serially diluted extracts in 50% aqueous DMSO and finally, 10 µL of bacterial suspension (5×10^6 cfu/mL) was added to each plate. Also each plate had a set of controls: a column with a broad-spectrum antibiotic as positive control (Kanamycin serial dilution). The plates were placed in an incubator set at 37 °C for 24 h. The colour change was then assessed visually. Any colour changes from purple to pink or colourless were recorded as positive. The lowest concentration at which colour change occurred was taken as the MIC value.

Result

Total phenolic content

The total phenolic content in the examined plant using the Folin-Ciocalteu's reagent is expressed in terms of gallic acid equivalent (R^2 = 0.999). According to the present study the phenolic content of the methanol extract of *O. acanthium* seeds was determined 168.56 ± 4.89 mg GAE/g extract.

Antimicrobial activity

The antibacterial activities of *O. acanthium* n-hexane and methanol seed extracts were tested by the disc diffusion agar method and the picture of the related plates are presented in Figure 1 A and B. Also the inhibition zone diameters for positive extracts are shown in Table 1. The presence of an inhibition zone clearly indicated the antibacterial effect of the extracts and varied according to the type of bacteria. The highest activity was demonstrated against *M. luteus* (with inhibition zone diameter of 21±1 mm) while the lowest activity was demonstrated against *S. aureus, K. pneumonia* and *E.coli*.

The broth macro dilution method was employed to determine MIC values for positive samples and the results are shown in Table 2. The picture of plate related to streak culture from the serially diluted methanol extract of *O. acanthium* against *M. luteus* was demonstrated in Figure 1 C and D. The minimum concentration of extract which inhibited the growth of bacteria was 0.612 mg/ml against both *S.epidermidis* and *M. luteus*. This result was also confirmed by Resazurin microtiter assay.

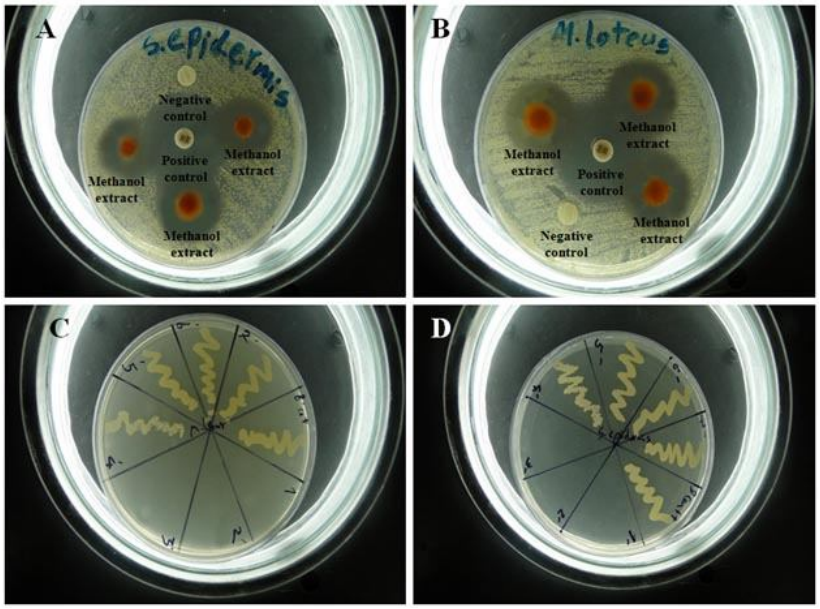

Figure 1. The inhibition zone of *O. acanthium* methanol extract for *S. epidermidis* (A) and *M. luteus* (B) and MIC determination of *O. acanthium* methanol extract for *S. epidermidis* (C) and *M. luteus* (D).

Discussion

It has been proven that medicinal plants contain diverse classes of bioactive compounds such as tannins, alkaloids and flavonoides, which in turn are responsible for various pharmacological properties.[21,22] Many reports are available on the antiviral, antibacterial, antifungal and anti-inflammatory properties of plants.[24,25]

Few studies about the chemical composition of *O. acanthium* have been reported.[18] The plants of

Onopordon genus contain compounds such as flavonoids, flavonolignans, phenylpropanoid and sesquiterpenes that may have the defence mechanisms for the plant against many microorganisms.[16] Furthermore, literature surveys indicated that plant phenolics comprise one of the compounds with multiple biological effects. [26,27]

Therefore, it is worthwhile to determine the TPC of the *Onopordon* genus selected for this study. This study for the first time has shown the TPC of the extracts; as estimated by the Folin-Ciocalteu reagent method.

Table 1. Zone of growth inhibition (mm) showing antimicrobial activity of *O. acanthium* seeds extract

	Diameter of inhibition zone (mm)				
	S. epidermidis	*M. luteus*	*E.coli*	*S. aureus*	*K. pneumonia*
n-hexane	-	-	-	-	-
Methanol extract	18.66±1.53	21±1	ND	ND	ND
DMSO	0	0	0	0	0

ND: no detection

Table 2. Minimal inhibitory concentration (mg/ml) of methanol extract of *O. acanthium* seeds

	Microorganism				
	S. epidermidis	*M. luteus*	*E.coli*	*S. aureus*	*K. pneumonia*
Methanol extract	0.612	0.612	-	-	-

Previous investigations showed the antimicrobial activity of several species of *Onopordon*.[29] However, Salehi and coworkers reported that methanol extracts of aerial parts of *O. acanthium* not to be active against seven g^- as well as two g^+ strains. [19] In good agreement with this study, we also did not get any antibacterial effect against tested g^- strains. It is certainly due to the lipopolysaccharide structure of these bacteria which notably restricts the penetration of external polar and hydrophilic materials through cell wall.[30,31] On the other hand, our experiments revealed remarkable antibacterial effect of methanol seed extract of *O. acanthium* against *S. epidermidis* and *M. luteus*. G^- bacteria possess an outer membrane and a unique periplasmic space not found in g^+ bacteria.[32]

Among gram-positive bacteria *S. epidermidis* and *M. luteus* are usually an innocuous commensal microorganism on human skin. *S. epidermidis* can cause severe infection after penetration of epidermal and mucosal barriers.[33]

According to the finding, it can be concluded that existence of phenolic compounds in the methanolic extracts, such as phenylethanoids and flavonoids (found in other species of *Onopordon*), is probably the main cause of antibacterial activity. The antimicrobial activities of phenolic compounds may involve multiple modes of action. For example, essential oils degrade the cell wall, interact with the composition and disrupt cytoplasmic membrane,[34,35] damage membrane protein, interfere with membrane integrated enzymes,[36] cause leakage of cellular components, coagulate cytoplasm, deplete the proton motive force, change fatty acid and phospholipid constituents, impair enzymatic mechanisms for energy production and metabolism, alter nutrient uptake and electron transport,[37] influence the synthesis of DNA and RNA and destroy protein translocation and the function of the mitochondrion in eukaryotes.[36]

Nevertheless, antibacterial activity of methanolic extracts supports further studies like isolation and purification of phenolic compounds, which will be carried out in further investigations and the relationship between biological activities of the extracts with isolated compounds will be revealed.

Conclusions

Collectively, it was concluded that the inhibitory effect of the methanol extract of *O. acanthium* seeds against two important bacterial strains is valuable and the plant can be considered as a potential candidate for further investigation for isolation and characterization of the active fractions and to know the exact mechanism of such action of *O. acanthium* seeds extract.

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Conflict of interests

The authors declare that they have no competing interests.

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