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Anti-microbial and anti-oxidant effects of *Campanula involucrata* Aucher ex A.DC. and *Nepeta menthoides* Boiss & Buhse from Iran

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ABSTRACT

Considering the global efforts against anti-microbial drug resistance and the need to find new natural sources with anti-microbial effects, the anti-microbial and anti-oxidant activities of two Iranian plants, *Campanula involucrata* and *Nepeta menthoides*, were evaluated. Aerial parts of both species were extracted using a Soxhlet apparatus and three solvents with different polarities (n-hexane, methylene chloride, and methanol), respectively. The methanol extracts of both species as the most potent parts were fractionalized using a solid-phase extraction (SPE) method. The anti-microbial activities were determined against two Gram-positive (*Staphylococcus aureus, Bacillus subtilis*), four Gram-negative bacteria (*Proteus morganii, Escherichia coli, Shigella flexneri, Salmonella typhi*), and a fungus (*Candida albicans*) species by disc diffusion method. Then, the extracts or fractions with the most potent anti-microbial activities were selected for evaluating their MIC (Minimum Inhibition Concentration). Finally, anti-oxidant potency and total phenol contents of men-

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tioned extracts and fractions were evaluated using DPPH and Folin-Ciocalteu reagents. The results demonstrated significant anti-bacterial activities, especially in methanolic extracts of both species and 40% and 10% methanol/water fractions in *C. involucrata* and *N. menthoides*, respectively. Moreover, anti-oxidant activities and total phenol contents of different extracts confirmed the presence of bioactive ingredients in the methanolic extracts and their relevant fractions. Additional studies are necessary to isolate and characterize bioactive compounds and their *in vivo* anti-microbial properties.

Keywords: antibiotic, anti-oxidant activity, *Campanula*, *Nepeta*

INTRODUCTION

Antibiotic resistance is one of the most important global challenges, which means the ability of microorganisms to thrive and tolerate antibiotics[1]. The spread of various infectious diseases and indiscriminate usage of a wide range of anti-microbial drugs leads to the efforts to encourage people to rational use of antibiotics to decrease the volume of consumption and reduce resistance rates[2]. Antibiotic resistance is one of the main topics with a high priority for WHO (World Health Organization). In 2015, the World Health Assembly adopted the global action plan for the antibiotic resistance problem with five strategic purposes, which include: 1) Improving awareness, 2) Supporting the research, 3) Decreasing the incidence of infection, 4) Optimizing the antibiotic uses, and 5) Development of the economic case for maintainable investment in new drugs, diagnostics, and other interventions for the needs of all countries[3]. Although about 50 years have passed since the discovery of antibiotics in the golden era, antibiotic resistance is still one of the most important issues in developed and developing countries[4,5]. According to previous studies, more than 80% of the prescribed antibiotics for upper respiratory infections are unsuitable and unnecessary, with harmful consequences such as antibiotic resistance, which causes noteworthy morbidity and mortality and imposes a massive global economic burden[1,6]. After the large-scale production of penicillin during World War II, the need to produce and discover herbal medicines increased, and the pharmaceutical companies focused their efforts on finding and producing new antibiotics. Although the use of natural products reduced with the development of chemistry in pharmaceutical industries later, the use of various drugs with natural sources for different health problems is still popular among people. Therefore, for many pharmaceutical companies which continue to natural products discovery research, the overall process for finding and developing novel natural compounds has not changed[7]. Along with the challenges of discovering

antibiotics with natural sources, we selected two Iranian species (*Campanula involucrata* Aucher ex A.DC. and *Nepeta menthoides* Boiss & Buhse) and evaluated possible anti-bacterial and antifungal as well as anti-oxidant activities. Moreover, the total phenol content of active extract of both species was assessed. The genus *Campanula* (Campanulaceae) has 44 species of annual and perennial plants in Iran[8]. The distribution of the plants of this genus is generally in Asia, Europe, and North and Northwest Africa[9,10]. Previous literature reviews have demonstrated different biological and pharmacological activities of the plants of *Campanula* genus, including, anti-oxidant, anti-microbial, anti-inflammatory, antidiabetic, anti- nociceptive, wound healing, and cytotoxic effects[9,11,12]. According to phytochemical studies, these plants are a rich source of flavonoids (especially anthocyanins) and saponin structures[9,11]. Furthermore, the presence of alkaloids, cardiac glycosides, sterols, triterpenoids, and tannins was confirmed in the different species of this genus[13]. The second evaluated species, *Nepeta menthoides*, (Labiatae) is one of 75 identified species of the *Nepeta* genus of Iran[14]. The plants of this genus are distributed in Asia, Europe, and Africa and are used widely in traditional medicine[15]. A wide range of effects, such as anti-asthmatic, anti-spasmodic, anti-septic, antitussive, astringent, blood depurative, diuretic, diaphoretic, emmenagogue, febrifuge, lowering blood pressure, and sedative properties have been reported from the various species of this genus[15,16]. Furthermore, monoterpenoids (nepetalactones and iridoids), sesquiterpenoids, diterpenoids, triterpenoids, and flavonoids were identified as the responsible structural groups of these plants[15].

METHODOLOGY

Plant material

The aerial parts of *C. involucrata* and *N. menthoides* were collected respectively from Goy Zangi Mountain (2800 meters above the sea) and Sahand mountains (3457 meters above the sea) in East Azarbaijan province in Iran. The identity of the studied species has been approved in the herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran. Extraction and fractionation fifty grams of dried and powdered aerial parts of both species were extracted using Soxhlet apparatus with n-Hexane, methylene chloride, and methanol (500mL each, Caledon Company, Canada). The obtained 6 extracts were concentrated separately using a rotary evaporator (Heidolph, Germany) at 45°C. Then 2 grams of methanol extract was weighed and subjected to solid-phase extraction (SPE) using a C18 Sep-Pak cartridge (Waters, USA), with a step gradient of MeOH/water mixture elution (10:90, 20:80, 40:60, 60:40, 80:20 and 100:0). All these fractions were dried using a rotary evaporator at the temperature of 45°C[17].

Anti-microbial assay

Microbial strains

Examined microorganisms included two species of Gram-positive bacteria (*Staphylococcus aureus* PTCC 1112, *Bacillus subtilis* PTCC 1715), four strains of Gram-negative species (*Proteus morganii* PTCC 1078, *Escherichia coli* PTCC 1533, *Shigella flexneri* PTCC 1234, *Salmonella typhi* PTCC1230) and a fungus, *Candida albicans* (PTCC 5027) which were purchased in lyophilized culture from the Iranian bacterial collection center.

Disc diffusion test

The Agar disc diffusion method was used to assess anti-microbial effects as described by Bauer et al.[18]. For the experiments, an overnight culture of microorganisms in Mueller–Hinton broth was used to provide a suspension with turbidity equivalent to the 0.5 McFarland tube (1.5×10^8 bacteria/ml). Briefly, the plates containing Mueller–Hinton agar were inoculated with one of the microorganisms by spreading microbial suspension onto the surface of the medium with a sterile cotton swab. Then, paper discs (6 mm in diameter) were placed on the surface of the inoculated agar. The 50% DMSO and the standard disc of Amikacin were used as negative and positive controls, respectively. The extracts were dissolved in 50% DMSO to a final concentration of 100 mg/mL, then 50 µL of different extracts solutions were added to placed discs on the culture medium, and then they were incubated in a refrigerator for 30 minutes to allow the anti-microbial agents diffuse in the agar. Then, the plates were incubated for 24 hours at 37°C. The anti-microbial effects of extracts were determined by measuring the diameter of inhibition zones (DIZ) around the sterile discs. The experiments were repeated at least three times, and then the mean DIZ was calculated[19,20]. The sensitivity was classified as follows based on the DIZ: not sensitive (DIZ≤8 mm), sensitive (DIZ=9–14 mm), very sensitive (DIZ=15–19 mm), and extremely sensitive (DIZ≥20 mm)[21-23].

Minimum Inhibitory Concentration (MIC)

Extracts or fractions with the most potent anti-bacterial activities were selected to evaluate their MIC value. The MIC was determined by the macro-dilution method with minor modifications to the guidelines of the Clinical and Laboratory Standards Institute[24]. Serial two-fold dilutions of the extracts were prepared in a Mueller–Hinton broth medium in tubes, and then, an equal volume of the bacterial suspension in Mueller–Hinton broth was added to each dilution to result in a final cell density of around 5×10^6 CFU/mL. After incubation at 35°C for 18 hours, the concentration of anti-microbial contained in the first

clear tube is read as the MIC. A tube containing DMSO, culture medium, and the bacterial suspension was utilized as a positive control, and a tube containing extracts, DMSO, and culture medium was used as a negative control. Then, the tubes were incubated at 37°C for 24h.

DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging assay

Anti-oxidant activities of extracts and fractions with different polarities of *C. involucrata* and *N. menthoides* aerial parts were evaluated using the DPPH reagent (Sigma Aldrich, Germany). For preparing the DPPH reagent, 4 mg of DPPH powder was dissolved in 50 mL methanol or chloroform using ultrasonic bath equipment. Methanol and chloroform were used for polar and non-polar extracts, respectively. Then, the stock solution of extracts was prepared at a concentration of 1 mg/mL, and serial dilutions were made in 10 different concentrations in methanol or chloroform. In the next step, 2 mL of diluted solutions of extracts were mixed with 2 mL of DPPH reagent and were allowed to accrue reactions in 30 minutes. Finally, the UV-Vis absorbance of samples was recorded at 517 nm, and the percentage of reduction capacity of DPPH was calculated according to:

$$Reduction\ capacity\ (\%) = (absorbance_{Blank} - absorbance_{sample}) / absorbance_{Blank}$$

The blank solution contained all substances except the extract and standard compound. The final results were reported as RC50 (Reduction Capacity 50%), defined as the extract concentration providing 50% loss of DPPH activity. All tests were repeated three times, and a similar method was performed for quercetin as the positive control[20].

Total phenol content assay (TPC)

The phenolic contents of extracts were measured using the Folin-Ciocalteu reagent (Merck, Germany). The samples were dissolved in the acetone 60% solution to obtain a 5 mg/mL concentration. Then 1 mL of these solutions were mixed with 200 µL Folin-Ciocalteu reagent (1:1 mixed with water) and 1 mL of 2% Na_2CO_3 and incubated at room temperature for 30 minutes. The absorbance of the samples was read at 750 nm using spectrophotometer equipment (Pharmacia Biotech, England). The same procedure was performed for different concentrations of gallic acid as the standard compound, and the sample without any extract was used as blank. The measurements were done in triplicate[25].

RESULTS and DISCUSSION

In the present research, anti-bacterial activities of *C. involucrata* and *N. menthoides* aerial parts extracts and their fractions of the most potent extracts were studied against two Gram-positive, four Gram-negative bacteria, and a fungus. The results are separately represented for both plant species in Table 1 to Table 4.

Table 1. Anti-bacterial activities (DIZs) of *C. involucrata* extracts and methanolic fractions

		DIZ (mm)									
Bacterial strain	**n- Hexane**	**Methylene Chloride**	**Methanolic extract**	**Fr. 10%**	**Fr. 20%**	**Fr. 40%**	**Fr. 60%**	**Fr. 80%**	**Fr. 100%**	**Total extract**	**Amikacin**
Proteus morganii	ND*	ND	ND	ND	ND	ND	ND	ND	ND	ND	25
Staphylococcus aureus	26	29	29	10	21	28	11	ND	ND	29	28
Escherichia coli	ND	ND	27	ND	ND	16	ND	ND	ND	27	29
Bacillus subtilis	10	16	31	ND	ND	18	ND	ND	ND	31	28
Shigella flexneri	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	22
Salmonella typhi	ND	18	31	ND	ND	14	ND	ND	ND	31	26
Candida albicans	ND	ND	ND	ND	ND	-	ND	ND	ND	ND	ND

*Not determined

Table 2. Anti-bacterial activities (DIZs) of *N. menthoides* extracts and methanolic fractions

	DIZ (mm)										
Bacterial strain	**n- Hexane**	**Methylene Chloride**	**Methanolic extract**	**Fr. 10%**	**Fr. 20%**	**Fr. 40%**	**Fr. 60%**	**Fr. 80%**	**Fr. 100%**	**Total extract**	**Amikacin**
Proteus morganii	ND*	ND	ND	ND	ND	ND	ND	ND	ND	ND	21
Staphylococcus aureus	21	40	32	27	21	22	20	19	19	32	23
Escherichia coli	ND	8	18	10	ND	ND	ND	ND	ND	18	26
Bacillus subtilis	19	22	31	25	ND	ND	ND	ND	14	31	22
Shigella flexneri	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	17
Salmonella typhi	20	29	34	18	ND	ND	ND	ND	13	34	17
Candida albicans	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

*Not determined

Table 3. Minimum inhibitory concentration (MIC) values of *C. involucrata* extracts and methanolic fractions

Microorganism	MIC (µg/mL)								
Bacterial strain	**n- Hexane**	**Methylene Chloride**	**Methanolic extract**	**Fr. 10%**	**Fr. 20%**	**Fr. 40%**	**Fr. 60%**	**Fr. 80%**	**Fr. 100%**
Staphylococcus aureus	ND*	125	31.25	250	125	62.5	250	ND	ND
Escherichia coli	ND	500	125	ND	ND	500	ND	ND	ND
Bacillus subtilis	ND	125	62.5	ND	ND	250	ND	ND	ND
Salmonella typhi	ND	500	250	ND	ND	500	ND	ND	ND

*Not determined

Table 4. Minimum inhibitory concentration (MIC) values of *N. menthoides* extracts and methanolic fractions

Microorganism			MIC (µg/mL)						
Bacterial strain	**n- Hexane**	**Methylene Chloride**	**Methanolic extract**	**Fr. 10%**	**Fr. 20%**	**Fr. 40%**	**Fr. 60%**	**Fr. 80%**	**Fr. 100%**
Staphylococcus aureus	ND*	62.5	15.625	31.25	250	250	250	250	250
Escherichia coli	ND	500	62.5	250	ND	ND	ND	ND	ND
Bacillus subtilis	ND	62.5	31.25	62.5	ND	ND	ND	ND	125
Salmonella typhi	ND	125	62.5	250	ND	ND	ND	ND	250

*Not determined

Moreover, the free radical scavenging capacity, total phenol contents of the extracts, and the most potent extract fractions are shown in Tables 5 and 6.

Table 5. Anti-oxidant activities (RC50: mg/mL) of *C. involucrata* and *N. menthoides* extracts and fractions

Plants	n- Hexane	Methylene Chloride	Methanolic extract	Fr. 10%	Fr. 20%	Fr. 40%	Fr. 60%	Fr. 80%	Fr. 100%
C. involucrate	ND*	ND	167 ± 0.0014	ND	149 ± 0.0014	46 ± 0.0007	ND	ND	ND
N. menthoides	ND	ND	178 ± 0.0014	23 ± 0.0007	156 ± 0.0021	256 ± 0.0071	ND	ND	ND
Quercetin	3.9 ± 0.002								

*Not determined

Table 6. Total phenol contents (mg GAE/g) o*f C. involucrata* and *N. menthoides* extracts and fractions

Plants	n- Hexane	Methylene Chloride	Methanolic extract	Fr. 10%	Fr. 20%	Fr. 40%	Fr. 60%	Fr. 80%	Fr. 100%
C. involucrate	-	-	154	137	172	0.461	88	119	11
N. menthoides	-	-	161	180	156	199	58	98	83

The DIZs of three different extracts of *C. involucrata* were studied on the seven microbial species and the results showed that the methanol extract possessed significant anti-bacterial effects against *S. aureus*, *B. subtilis, S. typhi* and, *E. coli* (extremely sensitive (DIZ≥20 mm)). The observed anti-bacterial activities were more potent than the anti-bacterial activity of Amikacin (as a positive standard) in all cases except *E. coli*. Also, among the fractions of methanol extract, the 40% fraction was the most potent but weaker than methanolic extract (Table 1). Moreover, the presented MIC values of extracts and fractions in Table 3 confirmed that the methanol extract was the most active part toward S. aureus (MIC=31.25 µg/ml). The obtained results may be attributed to the synergistic effects of the available compounds in the methanol extract[26]. Several studies have shown notable pharmacological activities of various species of the *Campanula* genus. According to a published study in 2011 in Turkey, whole plant essential oil of *Campanula olympica* demonstrated moderate anti-bacterial and antifungal activities against *Escherichia coli, Yersinia pseudotuberculosis, Pseudomonas aeruginosa, Enterococcus faecalis, Staphylococcus aureus, Bacillus cereus, Mycobacterium smegmatis,* and *Candida albicans*[27]. Based on the other research, the volatile oil of *Campanula portenschlagiana*, an endemic species to Croatia, revealed moderate to potent anti-bacterial activities against tested Gram-positive species (*Enterococcus faecalis, Staphylococcus*

aureus, Clostridium perfringens, Listeria monocytogenes, and *Bacillus cereus*) with MIC values of 62.5 to 125 µg/mL. Moreover, this oil had stronger activities against Gram-negative species, including *Escherichia coli, Klebsiella pneumonia,* and *Pseudomonas aeruginosa*, with MIC values of 7.8 to 62.5 µg/ml[28]. The phytochemical evaluation indicated that diterpene alcohols, the essential oil's major constituents, could be the responsible ingredients in different biological effects[28]. The same study reported that the aqueous extract of *Campanula portenschlagiana* with a total phenol content of 40.6 mg GAE/g showed lower anti-microbial activities than essential oil. The MIC values of the aqueous extract were 125 to 500 µg/ml against Gram-positive and 125 to 250 µg/ml for Gram-negative[28].

In another study, ethanol and methanol extracts *of Campanula glomerata* L. showed potent anti-bacterial activities against *Streptococcus pyogenes* and *Klebsiella pneumonias*, respectively. Moreover, different extracts of *Campanula olympica* Boiss. demonstrated significant anti-bacterial activities toward *Streptococcus pyogenes, Klebsiella pneumonias*, and *Escherichia coli*[29]. In a recent study, the dichloromethane extracts of leaves of *Campanula retrorsa* showed a moderate anti-microbial effect on *Acinetobacter baumanii* and *Candida albicans*[30]. There are limited available data on the *C. involucrata* biological, pharmacological and phytochemical properties. Hashemi and Zarei reported the tyrosinase inhibitory activity of *C. involucrata* species from Kurdistan, Iran. According to their results, 50% inhibition capacity (IC50) of n-hexane extract was 0.575 µg/mL and it indicated a significant inhibition value (75.62%, 75.45% and, 62.26%) at concentration of 1 µg/mL. Therefore, it can be a useful natural source for suppressing unpleasant hyperpigmentation in human skin[31]. Furthermore, the methanol extract of *C. involucrata* exhibited a significant anti-oxidant effect and inhibition (>60%) against the alpha-glucosidase enzyme, where the inhibition capacity (IC50) was 0.02 mg/mL. Consequently, it may be able to prevent the development of diabetic symptoms[32,33]. The phenolic compounds, especially flavonoids, have been isolated in abundance from the methanol extract of *Campanula pyramidalis* and *Campanula alata* species[34,35]. Moreover, the presence of anthocyanin structures from *Campanula medium* petals were reported previously[36]. Therefore, the significant anti-microbial activity of the methanolic extract of the investigated plant could be relate to the presence of flavonoid compounds including anthocyanin compounds. According to Tables 2 and 4, the second tested species, *N. menthoides*, was more potent than *C. involucrata* in most cases. Among the triple extracts of *N. menthoides*, the methanol extract had the most potent anti-bacterial effects. The DIZs of methanol extract on *S. aureus, B.*

subtilis, and *S. typhi* (extremely sensitive [DIZ≥20 mm]) were nearly 1.5 times greater than those of Amikacin. Also, the 10% fraction of methanol extract was the most potent relative to the other fractions. In the case of *N. menthoides*, as in *C. involucrata* species, the anti-bacterial power of the methanolic extract was higher than its isolated fractions. According to previous studies, the essential oil of *Nepeta crispa* showed important anti-bacterial activities against all the tested seven Gram-positive and Gram-negative bacteria and four fungi[16]. The main constituents were found to be 1,8-cineol (47.9%) and 4aα,7α,7a-nepetalactone (20.3%). The other research by Kahkeshani et al. revealed that, unlike the essential oils, 1,8-cineole showed no inhibition on fungi specially *Aspergillus* species, but it was more potent than the essential oil against Gram-negative species[37]. Studies on the different parts of the other species, *Nepeta persica*, indicated that the anti-bacterial effects of the essential oils might be because of their great content of nepetalactone isomers[38]. Further studies showed that the 50% methanolic extract of *N. menthoides* had significant inhibitory effects against the Gram-positive bacterial strains, and there was a direct relationship between total flavonoid contents of *N. menthoides* extracts and anti-bacterial activities. Additionally, *N. menthoides* was introduced as a good source of natural bioactive structures[14].

There are several studies about the anti-bacterial and antifungal effects of different species of *Nepeta*. In a recently published review article, traditional uses and pharmacological effects, as well as phytochemical properties of the plants of *Nepeta* genus were described. The authors have frequently mentioned the anti-microbial activities of various species of the *Nepeta* genus[39]. Moreover, new details about these effects of various species in different areas are being updated. For example, methanol extract of *Nepeta juncea* leaves showed high biological effects such as anti-microbial activity[40]. Among different extracts of *Nepeta cataria*, the maximum inhibition percentage toward examined bacteria species was 250-1000 µg/mL; and methanol and ethanol-based extracts were the potent parts[41]. Primary phytochemical analysis of this species confirmed the presence of phenolic compounds, tannins, flavonoids, cardiac glycosides, terpenoids, anthraquinones, and alkaloids in the extracts[41]. Generally, based on the literature, most of these compounds have demonstrated significant anti-microbial activities[42-47]. The other biological properties studied in this research were the measurement of anti-oxidant activities of different extracts and fractions of two mentioned plant species. Based on anti-oxidant results in Table 5, the methanolic extract of *C. involucrata* and its 40% fraction and the methanolic extract of *N. menthoides* and its 10% fraction were the most potent parts. Moreover, the evaluation of the results of phenolic contents

(Table 6) established that only methanolic extracts possessed acceptable contents of active natural phenolic compounds with TPC values of 154 mg GAE/g (*C. involucrata*) and 161 mg GAE/g (*N. menthoides*). Similar outcomes were reported previously in different species of *Campanula* and *Nepeta* species[13,30,48,49]. This evidence indicates the presence of numerous active natural compounds in various species of both plant genera that can be important for future pharmacological, biological, and clinical studies.

STATEMENT OF ETHICS

There are no ethical issues with human or animal subjects.

CONFLICT OF INTEREST STATEMENT

Nothing to declare.

AUTHOR CONTRIBUTIONS

Design: Fariba Heshmati-Afshar and Somayeh Hallaj-Nezhadi

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Analysis of data: Solmaz Asnaashari and Somayeh Hallaj-Nezhadi

Drafting of the manuscript: Solmaz Asnaashari and Somayeh Hallaj-Nezhadi.

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