کارگاه‌های آموزشی مرکز اطلاعات علمی

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اصول تنظیم قراردادها

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Antibacterial, antifungal and antioxidant activity of four species from *Arnebia* genus growing wild in Iran

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Abstract

Antibacterial, antifungal and antioxidant activities of methanolic crude extracts from aerial parts of four *Arnebia* species namely *Arnebia fimbriopetala*, *Arnebia linearifolia*, *Arnebia garandiflora*, *Arnebia tubata* were screened. Total phenolic contents were determined by Folin-Ciocalteu procedure and ranged from 2.2 ± 0.13 (*A. fimbriopetala*) to 1.6 ± 1.0 mg (*Arnebia tubata*) gallic acid µmol equivalent /g extract. The antioxidant activities were evaluated using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assays. All of the four *Arnebia* polar extracts showed good radical scavenging activity with an averaged IC_{50} value of 3.3 ± 0.15 µg/ml. DPPH assay results showed good correlations with the total phenolic contents of the plants, measured by the Folin-Ciocalteau assay. The antimicrobial activity was tested by using the disc-diffusion (DD) method and determining the minimal inhibitory concentration (MIC) using the macro dilution broth technique. The tested plants showed a mild antimicrobial activity against all nine tested strains. The best result belonged to the methanol extract of *A. linearifolia* against *Bacillus pumilus* with MIC value of 85µg/ml. Ampicillin, gentamicin and ketoconazole were used as references. The results obtained indicate that tested plant species from *Arnebia* genus may become important in the obtainment of noticeable sources of compounds with health protective potential, antioxidant and antimicrobial activity.

Keywords: *Arnebia*; Antimicrobial; Antioxidant; Total Phenol; free radical

1. Introduction

Since ancient times the crude herbal extracts of aromatic plants have been in use for different purposes, such as food, drugs and perfumery (Farjam, 2012). Crude extracts of fruits, herbs, vegetables, cereals, and other plant materials rich in phenolics are of increasing interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. The preservative effect of many plant spices and herbs suggests the presence of antioxidative and antimicrobial constituents in their tissues (Hirasa and Takemasa, 1998). Many medicinal plants contain large amounts of antioxidants other than vitamin C, vitamin E, and carotenoids. Antioxidants play important roles in the scavenging of free radicals and/or chain breaking of the oxidation reactions both in vivo and in vitro. The inhibition of oxidative reactions in food, pharmaceutical and cosmetic products and the prevention of oxidative stress related diseases in the human body are some of the useful potential functions of antioxidants (Velioglu et al., 1998). Application of synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxytanisole (BHA), Trolox and Quercetin in food processing has led to the appearance of remarkable side effects. For example, these substances can exhibit carcinogenic effects in living organisms and enlarge the liver size and increase microsomal enzyme activity (Moure et al., 2001; Ames, 1983). Due to these limitations, there is an increasing interest in finding naturally and biologically produced antioxidants capable of inhibiting free radical reactions, retarding oxidative rancidity of lipids, protecting the human body from diseases, and preserving foods from spoiling (Pryor, 1991; Ito et al., 1983). Natural antioxidants present in the diet prevent oxidative damages and they may have a substantial impact on human health. The current interest is toward natural antioxidants, especially plant polyphenolics. Tea and herbal infusions are an important source of antioxidant phenolic compounds in our diet (Albayrak et al.,

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2013). Microbial contamination is another important issue in the field of food, beverage, cosmetic, and pharmaceutical industries. Increased consumer demand on organic natural food stuff from one side and the observation of growing cases of microbial resistance to existing preservatives from the other has encouraged the world food research community toward seeking new natural antimicrobial substances. According to these facts, the plant kingdom with a remarkable diversity in producing natural compounds has attained a special interest and, today, accessing to plant materials with dual antioxidant and antimicrobial capabilities is an ideal goal in the field of research on food additives (Ebrahimabadi et al., 2010).

Arnebia is a genus of the family *Boraginaceae*. The Generic name *Arnebia* originates from the Arabic name Sagaret-el-Arneb. In Iran, a survey of the ethnobotanical studies indicated the use of several of this plant species by the inhabitants of the area, especially by those habiting the rural areas for wounds healing purpose (Ghasemi-Pirbalouti, 2009; Ghorbani, 2005; Zargari, 1990). For example, in nomadic tribes (Bakhtyari) in the southwest of Iran, the roots of *Arnebia euchroma* with goat lipid is used as a remedy for burn wounds. *Arnebia euchroma* Rolye. (Johnst.) (*Boraginaceae*), a well-known traditional herb used in tribal medicine of Iran, is locally known as "Sorkh Giyah or Heveh Choaeh". A literature review shows that there are some reports on the biological investigation of *Arnebia* genus. Ethanol extracts of the roots of *Arnebia nobilis* showed bactericidal and fungicidal activity when put through a wide range of biological tests (Sukila et al., 1968). Acetylshikonin isolated from *Arnebia euchroma (Royle) Johnst* cell suspension cultures exhibits specific *in vivo* and *in vitro* antitumor effects (Xiong at al., 2009). Naphthaquinones from *Arnebia hispidissima* (Lehm.) DC. exhibits *In vivo* and *In vitro* anti-inflammation activity (Singh et al., 2004). Shikonin derivatives isolated from the roots of *Arnebia euchroma* have been reported to have antimicrobial, anti-inflammatory and anti-tumour activities and thus are considered as important compounds for potential medicinal use (Ghorbani, 2005).

In this study the methanolic crude extracts of four species from *Arnebia* genus namely *Arnebia fimbriopetala*, *Arnebia linearifolia*, *Arnebia garandiflora*, *Arnebia tubata* were comparatively screened for their antimicrobial and antioxidant properties. To the best of our knowledge, there is no report on these properties in methanolic extracts of the aforementioned species in the literature. Thus, the present research reports (i) in vitro antioxidant activity profiles of these plant extracts using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, (ii) total phenolic compounds content of the plant extracts as gallic acid equivalents, and (iii) antimicrobial potentials of the plant extracts against six bacteria and three fungi using two complementary methods, namely disc-diffusion method and determining the minimal inhibitory concentration (MIC).

2. Material and Methods

2.1. Reagents and microbial strains

Trolox (water soluble equivalent of vitamin E) and Quercetin were obtained from Acros Organics. Acetic acid glacial, dimethyl sulphoxide, Folin-Ciocalteau reagent, hexane, methanol, sodium acetate and sodium carbonate were purchased from Merck. Galic acid, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and hydrochloric acid 32% were obtained from Sigma–Aldrich. The bacteria that were used in this study were *Bacillus pumilus* (PTCC 1319), *Escherichia coli* (PTCC 1533), *Kocuria varians* (PTCC 1484), *Pseudomonas aeruginosa* (PTCC 1310), *Salmonella typhi* (PTCC 1609), and *Listeria monocytogenes* (PTCC 1298). The fungal strains that were used in this study were *Aspergillus niger* (PTCC 5154), *Aspergillus flavus* (PTCC 5006) and *Candida glabrata* (PTCC 5297). All microorganisms were obtained from the Persian type culture collection (PTCC), Tehran, Iran.

2.2. Plant samples

The plant materials were collected in June 2012 from the south of Iran. The plants were identified by Dr. Khosravi, Department of Biology, University of Shiraz, Iran and voucher specimens were deposited at the herbarium of the Medicinal and Natural Products Chemistry Research Centre, Shiraz, Iran. Aerial parts of plants were air-dried at room temperature (25°C) in the shade. They were cleaned and cut into small pieces before extractions.

2.3. Extraction

Methanolic extracts of the plants were prepared as follows: 7.5 g dry plant, after being defatted with light petroleum, was macerated in 200 mL methanol for 2 days with one change of solvent after 1 day. The extract was filtered and then concentrated in a rotary vapor for less than 10 min. Powders were weighed to calculate the yield, and kept at -20°C until used. Shortly before each experiment, the powder was dissolved in methanol at the desired concentration and tested for antioxidant activity and total phenolic content.

2.4. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial compound that will inhibit the visible growth of a microorganism after overnight incubation. MIC of the four sample extracts against bacterial strains was determined through a micro dilution tube method as recommended by Bauer and his co-workers with slight modifications (Bauer at al., 1966). In this method, various test concentrations of extracts were made in the wells of microtiter plate (96 wells) from 512 to 8 mg/mL by serial dilutions in sterile Brain Heart Infusion (BHI) broth (Fig 1a). Turbidity of the test inoculums of the sex bacteria and three fungal strains in BHI broth was adjusted to McFarland's standard No. 0.517. (Matthew, et al., 1997). Turbidity tube and 10 mL of these standard inoculums were added to each well. The microtiter plate was then incubated at 37ºC for 24h. The end result of the test was the minimum concentration of test compound that was able to restrict bacterial growth to a level lower than 0.05 at 650 nm. Positive controls include; gentamicin, ampicillin and ketoconazole for Gram-negative bacteria, Gram-positive bacteria and fungi, respectively.

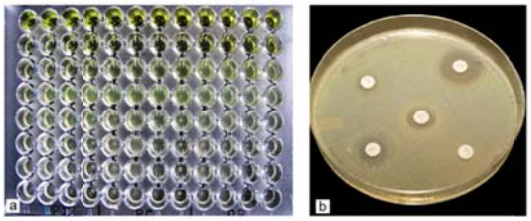

Fig. 1. Assessment of antimicrobial activity: a) 96-well microplate in MIC method. b) The disc immersed in inoculated agar in disk diffusion assay

2.5. Disk diffusion assay

Determinations of antimicrobial activities of four dried *Arnebia* extracts were also accomplished by agar disk diffusion method (Murray, et al., 1999) using 400 µL of suspension containing 10^8 CFU/mL of bacteria and 10^6 CFU/mL of fungi, spread on the nutrient agar (NA) and potato dextrose agar (PDA) mediums, respectively. The disc (6 mm diameter) impregnated with 10 µL of the extracts (dissolved in DMSO to a final concentration of 4 mg/ml) and DMSO (negative control) was placed on the inoculated agar, which was incubated for either 24 h at 37°C (bacteria) or 48 h at 30ºC (fungi) (Fig 1b). Gentamicin (10 µg/mL) and nystatin (100 IU) were used as the positive controls for bacteria and fungi, respectively. The diameters of clear inhibition zones around the discs indicated the presence of antimicrobial activity. All tests and analyses were carried out in triplicate.

2.6. DPPH radical scavenging activity

Radical scavenging activity of plant extracts against the stable free radical DPPH was measured as described previously by Blois (Yoo et al. 2005), Briefly, 0.1 mM solution of DPPH was prepared in methanol and 4 mL of this solution was added to 1 mL of sample solution in DMSO at different concentrations (1.25, 2.5, 5 µM). Thirty min later, the absorbance was measured at 517 nm. Lowered absorbance of the reaction mixture indicated higher free radical scavenging activity and was calculated as per the following equation:

$$\%I = [(A_{DPPH} - A_P)/A_{DPPH}] \times 100$$

Where A_{DPPH} and A_P were the absorbance of the DPPH solutions containing methanol and plant extract, respectively. The dose-response curve was plotted by using the software SigmaPlot for Windows version 8.0 and IC_{50} values for extracts were calculated. Quercetin was tested at the final concentration of 10 µM and used as the reference compound.

2.7. Total phenolic content (TPC)

Total phenolic content in sample extracts was determined by the Folin-Ciocalteau colorimetric method, as described by Slinkard and Singleton (1977). A sample of stock solution (1.0 mg/mL) was diluted in methanol to final concentrations of 1000, 800, 600, 400, and 200 µg/mL. A 0.1 mL aliquot of sample was pipetted into a test tube containing 0.9 mL of methanol, then 0.05 mL Folin-Ciocalteu's reagent was added, and the flask thoroughly shaken. After 3 min, 0.5 mL of 5% Na_2CO_3 solution was added and the mixture was allowed to stand for 2 h with intermittent shaking. Then, 2.5 mL of methanol was added and the mixture was left to stand in the dark for 1 h. The absorbance measurements were recorded at765 nm. The same procedure was repeated for the standard gallic acid solutions and the concentration of total phenolic compounds in the extracts was expressed as mg of gallic acid equivalent per g of sample. Tests were carried out in triplicate and the gallic acid equivalent value was reported as mean ± SD of triplicate.

2.8. Statistical analysis

Regression analyses were performed by SigmaPlot 2002 for Windows version 8.0.

3. Results

3.1. Antimicrobial activity

The antimicrobial activity of methanol extract fractions of four *Arnebia* species extracts were evaluated against a set of 9 microorganisms and their potency was assessed qualitatively and quantitatively by minimum inhibitory concentration (MIC) values and the disk diffusion (DD) method. The results are given in Table 1 and indicate that, at tested concentrations, the plant has mild antimicrobial activity against tested microorganisms. Our findings showed that in the MIC method, the best inhibition belonged to the methanol extract from aerial part of *A. linearifolia* against *Bacillus pumilus* one of the tested gram-positive bacteria with MIC value of 85 µg/ml. Moreover, this plant extract showed mild inhabitation against all gram-negative bacteria strains and *Kocuria varians* with MIC value of 128 µg/ml. The *A.fimbriopetala* extract proved to be active against four out of the six bacterial strains used and was particularly active against *Escherichia coli*, *Salmonella typhi*, *Bacillus pumilus* and *Listeria monocytogenes* (MIC values of 128µg/ml). Also, Methanolic extract of *A.garandiflora* showed similar amount in MIC value against *Kocuria varians*, *Listeria monocytogenes*, *Escherichia coli*. Ultimately *A.tubata* was found active against *Salmonella typhi*, *Bacillus pumilus* with the same amount of MIC (128 µg/ml). In disk diffusion method, the diameters of inhibition zones were used as a measure of antimicrobial activity. In this method, the best result was recorded for *A.garandiflora* against *Listeria monocytogenes* with inhibition zone: 14.5 ± 0.86 mm.

Table 1. Antimicrobial activity of the of the crude extract of four tested *Arnebia* extracts

Test microorganisms	*A. fimbriopetala*		*A. linearifolia*		*A. garandiflora*		*A. tubata*	
	MIC	DD	MIC	DD	MIC	DD	MIC	DD
Gram-negative bacteria								
Escherichia coli	128	9.3 ±0.57	128	6.9± 0.55	128	14.3± 1.15	512	6.4± 0
Pseudomonas aeroginosa	170.6	10.3±1.15	128	9.3 ± 1.52	256	15 ± 1	512	6.4 ± 0
Salmonella typhi	128	12.6±1.15	128	11.6± 1.52	512	14 ± 0.86	128	7.5 ± 0.5
Gram-positive bacteria								
Bacillus pumilus	128	11.8±1.25	85.3	8.3 ± 0.57	256	12.1 ± 0.76	128	9.6 ± 0.57
Kocuria varians	256	12.5±1.32	128	11 ± 1	128	10±1	256	10.1 ± 1
Listeria monocytogenes	128	7.6±0.28	256	10.6 ± 1.52	128	14.5 ± 0.86	256	8.5 ± 1.32
Fungi								
Aspergillus flavus	256	6.4±0	256	7.16±0.28	256	9±1	512	6.4±0
Candida glabrata	426.6	6.6±0.34	512	6.4±0	512	7.6±0.28	512	6.4±0
Aspergillus niger	512	6.4±0	512	6.6±0.34	512	10.3±0.57	512	6.4±0

[a]Minimum Inhibitory Concentration (range of concentration: 8–512 µg/ml)
[b]DD (Disk diffusion method), Inhibition zones in diameter (mm) around the impregnated disks (Mean ± SD).
[c]In MIC assessment, Ampicillin, gentamicin and ketoconazole were used as references for Gram-positive, Gram-negative bacteria and fungus, respectively. In disc diffusion method, Gentamicin (10 µg/mL) and nystatin (100 IU) were used as the positive controls for bacteria and fungi, respectively.

3.2. Antioxidant activity

Methanol extract of four *Arnebia* species were subjected to screening for their possible antioxidant activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay method. DPPH is a stable free radical which can readily experience reduction in the presence of an antioxidant. It shows a maximum ultraviolet and visible (UV–Vis) absorbance at 517 nm. The reduction in the intensity of absorption at 517 nm of methanol solutions of DPPH radical in the presence of antioxidants is usually taken as a measure of their antioxidant activity. In this study,

the ability of samples to scavenge DPPH radical was determined on the basis of their concentrations providing 50% inhibition (IC_{50}). Methanol extract of four *Arnebia* species and positive control (Quercetin) IC_{50} values are given in Table 2, Fig. 2. All four *Arnebia* polar extracts showed good radical scavenging activity with an averaged IC_{50} value of 3.3 ± 0.15 µg/ml, about 22% of the potency of synthetic standard Quercetin.

Table 2. Antioxidant activity and total phenolic contents of four tested *Arnebia* extracts

Sample	DPPH IC_{50} (µg/ml)	Total phenolic content (µmol eq galic acid/g extract)
Arnebia fimbriopetala	3.7 ± 0.26	2.2± 0.13
Arnebia linearifolia	3.4 ± 0.30	2.0± 0.12
Arnebia garandiflora	3.3 ± 0.15	2.1±0.13
Arnebia tubata	3.1 ± 0.25	1.6±0.10
Quercetin	0.72±0.47	-

Values represent the mean of three experiments ± SD. Quercetin was tested as a reference compound in the DPPH

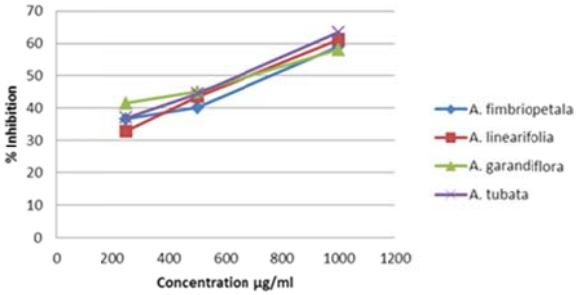

Fig 2. Graphical representation of % antioxidant activity of tested *Arnebia* extracts

3.3. Amount of total phenolic constituents

Total phenolic content of the plant extracts were determined using a colorimetric assay method based on Folin–Ciocalteu reagent reduction. Results, expressed as gallic acid equivalents, are given in Table 2. DPPH assay results showed good correlations with the total phenolic contents of the plants, measured by the Folin- Ciocalteau assay (r^2 = 0.926, 0.913, 0.931, 0.912 for *A. fimbriopetala* , *A. linearifolia*, *A. garandiflora*, *A. tubata* respectively, $p < 0.0001$).

4. Discussion

Almost all plants of *Arnebia spp.* used in the traditional medicine exhibit moderate antimicrobial activities when tested individually as reported earlier (Abdulameer and Al-Mussawi, 2010; Koca at al., 2010). Four evaluated extracts showed mildly significant activity against Gram-positive and Gram-negative bacteria, and fungi. This means that they can show an inhibiting activity on disease causing Gram-negative and Gram-positive bacteria. This is particularly interesting from a medical point of view because this microbial agent is responsible for severe opportunistic infections. Most of the crude extracts exhibit bactericidal activity rather than bacteriostatic (Gnanamani et al., 2003; Kharea et al., 2005), which simply highlights the effectiveness of traditional medicine. The antifungal activity of the plant extracts was also screened. The extracts showed mildly significant activity against fungal. However, it is worthy to note that MIC values of assayed plant extracts are still comparatively higher as compared to those reported for other species.

Many substances may be antimicrobial, but only a few of them will be potential therapeutic agents for the simple reason that mammalian cells are more sensitive to chemical inhibition than microbial cells. As per the studies by Cowan (1999), the useful antimicrobial phytochemicals generally function through various mechanisms. Although we did not investigate chemical constituent of the extracts in this study, good radical scavenging activity and total phenolic contents results of the plants extracts could be responsible for phenolic toxicity to microorganisms. It can include enzyme inhibition by the oxidized compounds possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins. Similarly the quinones, the probable targets in microbial cells are surfaced exposed adhesions, cell wall polypeptides and membrane bound enzymes. The activity of flavonoids found in plants as their glycosides is due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. The more lipophilic flavonoids may also disrupt microbial membrane and the alkaloids have their ability to interact with DNA of microbial cells.

The results of antioxidant activity show that phenolic compounds provide the major contribution to the antioxidant activity of the plant extracts measured by these assays. This is in line with the observation of other authors who found similar correlations between total phenolic content and anti-oxidant activity of various plants.

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