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Isolation and identification of the phenolic compounds from *Pulicaria inuloides* and their antibacterial and antioxidant activities

NABIL QAID M. AL-HAJJ
State Key Laboratory of Food Science and Technology
School of Food Science and Technology, Jiangnan University
Wuxi, P.R. China
Marine Science and Biological Research Authority
Aden, Yemen
ALGABR METHAQ
Department of Chemistry, Faculty of Applied Science
Hajjah University, Yemen
HUSNAIN RAZA
KHAMIS ALI OMAR
A. AL-FARGA
HONGXIN WANG
RIYADH THABIT
State Key Laboratory of Food Science and Technology
School of Food Science and Technology, Jiangnan University
Wuxi, P.R. China

Abstract:

The phenolic compounds of P. inuloides were extracted by ultrasonic device followed by loading on column of macroporous resin chromatography for isolation and then identification was done using high performance liquid chramoatography. The antioxidant activities were determined by 2,2-diphenyl-1,1-picrylhydrazyl (DPPH) and beta-carotene assays. P. inuloides phenolic constituents inhibited the all tested microorganisms except E. coli, Salmonella typhimurium and Shigella dysenteriae with a minimum inhibitory concentration (MIC) of 30.0 µg/mL against Bacillus subtilis in 50% ethanol. Totally 13 fatty acids were determined including 6 unsaturated fatty

acids as linoleic acid was found to be in the highest amount (5.74%) followed by Oleic acid acid (2.37%) and 6,9,12-Octadecatrienoic acid (1.61%). γ-tocopherol was the highest value of about 58.00% among the total tocopherols, In addition, the highest level of K, Mg, Na, Fe and Ca of 159.5, 29.5, 14.2, 13.875 and 5.225 mg/100 g was found in P. inuloides. The Scanning electron microscopy (SEM) revealed or showed the interior structures of P. inuloides.

Key words: Aromatic plant; phenolic compounds, vitamins, fatty acid, antioxidant, antimicrobial activity, scanning electron microscopy

1. INTRODUCTION

The genus Pulicaria belongs to the family Asteraceae (tribe Inuleae), and consists of more than 67 species found throughout the world. Members of this genus contain various bioactive compounds such as monoterpenes, flavonoids, acetylenes, isocomene along with sesquiterpene acetylenes, isocomene and sesquiterpene lactones [1]**.** The traditional medicinal plants still play a vital role to cover the basic health needs in developing countries. Herbal remedies used in the traditional folk medicine provide an interesting and still largely an unexplored source for the creation and the development of new potential drugs. Medicinal plants have been used in folk medicine for thousands of years. In the codex of Ebers, an Egyptian medical papyrus dated about 1550 B.C., of more than 800 therapeutic formulae, 22 mentioned garlic for a variety of ailments including heart problems, headache, bites, worms and tumors [2]. Plant-derived polyphenols receive considerable interest because of their potential antioxidant and antimicrobial properties. Consumers conscious about the possible adverse health effect of certain chemical preservatives, have increased the demand for foods with longer shelf-life, pressurizing the food industry to withdraw chemically synthesized additives and

to use "natural" alternatives [3]. Many plants are considered to be the excellent source of phenolic compounds that could be used, not only to preserve foods, but also to contribute to a healthy diet [4]. The plant (poly) phenols are the diverse group of higher secondary metabolites, possessing an aromatic ring bearing one or more hydroxy substituents, derived from the shikimate pathway and phenylpropanoid metabolism [5]. Phenolic compounds exhibit a considerable free radical scavenging (antioxidant) activity; which is determined by their reactivity as hydrogen or electron donating agents, the stability of the resulting antioxidant-derived radical, their reactivity with other antioxidants and finally, their metal chelation properties [6]. Plant polyphenols are considered to be antimicrobial agents and are proposed as potential natural food preservatives [7]. Although, the antimicrobial activity of phenolic compounds has recently been discovered, however the mechanism of their action on microorganisms has not yet been presented. Furthermore, contradictory data have been reported by different authors for the same antimicrobial compound [7]. Fatty Acids (FAs) are released usually by the actions of lipases (from different sources) during lipolysis [8]. Short-chain fatty acids have a direct impact on the flavour, free fatty acids (FFAs) also act as precursor molecules in a series of catabolic reactions leading to the production of other flavouring compounds such as methyl ketones, esters and thioesters [9]. In addation, minerals content of *P. inuloides* leaves and plant structure were also examined.

No previous study on the physiochemical properties, chemical composition or antioxidant activity of the phenolic compounds extracted from the Pulicaria (*Pulicaria inuloides*) has been reported so far. Thus, in the current research, the physiochemical properties, chemical composition and antioxidant and antibacterial activity of the phenolic isolated from *Pulicaria inuloides* were evaluated. This may lead to the

investigation of *Pulicaria inuloides* as an option for the food industrial and medical applications.

2. MATERIALS AND METHODS

2.1. Plant collection, identification and documentation

Dried *Pulicaria inuloides* leaves were collected on August, 2014 from Bany-Mater in Sana'a city, Republic of Yemen and transported to the Food Nutrition laboratory in Jiangnan University, People's Republic of China. The plant was taxonomically identified by Prof. Abdellah Amine (College of Agriculture, Sana'a University). A voucher specimen of the plant material was deposited at Department of Biology, College of Agriculture, Sana'a University. The samples were kept dry at room temperature in desiccators and then milled using a laboratory scale hammer mill (Debarker Co. Ltd., Beijing, China). Analytical reagents and solvents were obtained from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China.

2. 2. Chemicals and Equipments:

Folin-Ciocalteu reagent, gallic acid and quercetin reagent, methylimidazole, butyl bromide were purchased from Sigma (USA) and D-101 macroporous resin from Tianjin Dajun Co., Ltd. Spectrophotometer (Shanghai-Techcomp, UV 2300), balance (Shanghai-Mettle Toledo, AB 204–N), rotary evaporator (Shanghai-Biochemical Equipment), water bath (Shanghai-Hengzi) and Microwave (Beijing- Xianghu Science & Technology.XH-200A) were used.

2.3. Determination of tocopherol composition

To determine tocopherol, the NY/T 1598-2008 method was used [10]. A solution of 250 mg *P. inuloides* oil, 25 mL of ethanol, 5 mL of 10% ascorbic acid and 10 mL of a 50% KOH solution was saponified in a capped flask in a water bath at 90 °C for 1 h.

After cooling, 100 mL of distilled water was added and mixed. The solution was extracted with 50 mL of diethyl ether. The upper layer was collected and washed with distilled water until a neutral pH was reached. The organic layer was then separated and dried over Na_2SO_4. After filtration of this solution, the solvent was evaporated until fully dried under a vacuum at 40 °C. The dry matter was then dissolved in 2 mL of ethanol and centrifuged at 5,000 rpm for 5 min. The upper layer (10 μL) was injected for the HPLC analysis. The HPLC system (Waters, USA) included a water 2996 PAD detector and a spherisorb silica column (250 mm × 4.6 mm, 5 μm), operating at 30 °C. The separation of tocopherols was based on a mobile phase of methanol/ water (98:2, v/v) at a flow rate of 1.0 mL/min with detection at 290 nm. Tocopherols were calculated as mg/g oil.

2.4. Preparation of fatty acid compounds

The ether-soluble parts of the petroleum ether extracts of each of the three Clematis species were subjected to saponification to obtain the fatty acids. About 3 g of the residue in each case was saponified by refluxing with 100 mL of 10% alcoholic KOH for 8 hrs. The solvents were separately distilled off nearly to dryness. Dilution was done with 100 mL water and the mixtures were extracted with ether till complete utilization of the unsaponifiable matters. The aqueous alkaline solutions left were acidified with 10% diluted HCl and the liberated fatty acids were extracted with ether yielding 1.73, 1.29 and 1.18% respectively. The fatty acids were subjected to methylation using methanol and dry H_2SO_4 [11,12].

2.5. Determination of Fatty acid Constituents

Fatty acids were converted to their methyl esters (FAME) following the method of He and Xia [13, 14] with a slight modification. In brief, 1μL of FAME sample was injected into

the gas chromatograph (Series PEG30 M) equipped with a flame ionization detector. GC separation was conducted on a capillary column (PEG30 M; 30m × 0.32mm × 0.50 μm). The carrier gas was nitrogen and the column flow rate rate was 0.8 mL/min(Use one style of units representation.). Initially, the oven temperature was calibrated at 190 °C for 1 min, which was increased from 190 to 230 °C at a rate of 3 °C/min and then maintained at 230 °C for 10 min. The temperatures of the injection port and detector were 240 and 250 °C, respectively. The peaks were identified on the chromatogram according to retention data from analyzed standard samples. Finally, the fatty acid contents were calculated as percentages (%).

2.6. Determination of phenolic compounds by macroporous resin

The sample of *Pulicaria inuloides* leaves (10 g.) was crushed into the powder in a grinder (ZSJD, Kaichuang mechanism Co., Ltd., China). Furthermore, 50% ethanol (v/v), 95% ethanol (v/v), 70% acetone (v/v) and 100% methanol were used for extracting phenolic compounds from the Pulicaria leaves. The extracts were placed at 40 ^{0}C, 250 rpm for 3 hours in an ultrasonic device. The extracts were then filtered and concentrated to dryness at 30°C under reduced pressure using a rotary evaporator (SWB-1, Shenbo Instrument Co., Shanghai, China). The crude extracts were dissolved in ethanol, acetone and methanol, respectively, then loaded on a glass column (6 cm×100 cm) of macroporous resin (HPD-100, Cangzhou Bon Adsorber Technology Co., Ltd., China). In the macroporous resin column chromatography, water, with solvents was used to desorbs target components at a successively flow of 1.6 BV/h. Each elution was collected, dried at 30 ^{0}C under reduced pressure and re-dissolved in ethanol (1 mL) for HPLC analysis.

2.7. Identification and quantification of phenolic compounds using high-performance liquid chromatography (HPLC)

The phenolic compound profiles were isolated according to the procedure proposed [18]. The analytical HPLC system employed consisted of a JASCO high-performance liquid chromatograph coupled with a UV-Vis multiwavelength detector (MD-910 JASCO). The analytical data were evaluated using a JASCO data processing system (DP-L910/ V). The separation was achieved on a Waters Spherisorb 5µm ODS2 4.6×250 mm column at ambient temperature. The mobile phase consisted of water with 1% glacial acetic acid (solvent A), water with 6% glacial acetic acid (solvent B), and water/acetonitrile (65:30 v/v) with 5% glacial acetic acid (solvent C). The gradient used was similar to that used for the determination of phenolics in wine [11] with some modifications: 100% A, 0-10 min; 100% B, 10-30 min; 90% B/10% C, 30-50 min; 80% B/20% C, 50-60 min; 70% B/30% C, 60-70 min; 100% C, 70-105 min; 100% A, 105-110 min; post-time, 10 min before the next injection. The flow rate was 0.5 mL/min with injection volume of 20µL and 280nm monitoring wavelength.. The identification of each compound was based on a combination of retention time and spectral matching.

2.7.1. Standards preparation

Standard samples, were dissolved with methanol (HPLC grade), the concentration of each standard was: Ferulic acid 1mg, cinnamic acid 1mg, gallic acid 1 mg, p-coumaric acid 1 mg, ascorbic acid 1 mg, vanillic acid 1 mg, Caffeic acid 1mg, benzoic acid 1 mg and chlorogenic acid 1 mg stored at 4 ^{o}C.

3. ANTIOXIDANT ASSAY

3.1. Determination of DPPH radical scavenging activity

The antioxidant activities of the phenolic compounds of *P. inuloides* were determined by 2, 2-diphenyl-1,1-picrylhydrazyl (DPPH) free radical scavenging assay, as previously described [19], [20]. Each concentration of phenolics was mixed individually into 1 mL methanol solution containing 0.1 mM DPPH radicals. The reaction mixture was shaken thoroughly, incubated in the dark for 30 min at room temperature, and measured at an absorbance of 517 nm (2100, Unico, Shanghai, China). The free radical scavenging activity was calculated using the following equation 1:

$$\text{DPPH (\%)} = \{(An - Am)/Am\}100 \quad \text{..... (1)}$$

where An is absorbance of the control (without phenolic acids), and Am is absorbance of the sample.

3.2. Antioxidant assay using the β-carotene bleaching method

Antioxidant activities of the phenolic compounds were determined as previously described [21], with some modifications. The ß-carotene (0.1 mg) was added to a boiling flask together with linoleic acid (20 mg) and Tween 40 dissolved in chloroform. After evaporating the chloroform under vacuum at 50 °C using rotary evaporator, 50 mL oxygenated distilled water was added, and the mixture emulsified for 1 min. Thereafter, 5 mg of each essential oil was added separately to 4.8 mL of the emulsion. Absorbance at 470 nm was measured using a spectrophotometer before (t = 0 h) and after a 2-h incubation at 50 °C (t = 2 h). All measurements were performed in duplicate, and antioxidant activity, assessed as

percent inhibition of ß-carotene bleaching, was calculated using equation 2:

$$\% \text{ inhibition} = \{AA\ (2h) - AC\ (2h)/(AC\ (0h) - AC\ (2h)\}100 \ldots\ldots (2)$$

Where AA (2h) is absorbance of the sample at t = 2 h, AC (2h) is absorbance of the control at t = 2 h, and AC (0h) is absorbance of the control at t = 0 h, and AC (2h) is absorbance of the control at t = 2 h.

4. ANTIMICROBIAL ASSAY

4.1. Tested compounds

Phenolic acids of *Pulicaria inuloides* were tested for antimicrobial activity. They were sterilized by filtration using 0.45µm membrane filter before testing. Amoxicillin (Smithkline Beecham) served as positive controls for the tested bacteria, whereas Nystatin (Bristol-Myers Squibb) served as positive control for *Candida albicans* and dimethylsulfoxide (DMSO) (10%) solution was tested as solvent control.

4.2. Microorganisms

Seven strains of bacteria were tested*: Staphylococcus aureus* 6538, *Streptococcus penumoniae* ATCC25922, *Bacillus subtilis* ATCC-6633, *Escherichia coli* ATCC 25922, *Shigella dysenteriae* 51302, *Salmonella Typhimurium* 50013 and one yeast: *Candida albicans* (ATCC 10231); were purchased from China General Microbiological Culture Collection Center (Beijing, China).

4.3. Preparation of inoculate

A 18 h-old culture of the selected bacteria/yeast was mixed with sterile physiological saline and the turbidity was corrected by adding sterile physiological saline until a Mac Farland

turbidity standard of 0.5 (106 colony forming units (CFU) per ml).

4.4. Disc diffusion assay

Antimicrobial activity of the essential oils was determined by the disc diffusion assay [22] with some modifications. Whatman no. sterile filter paper discs (6 mm) were impregnated with the phenolic compounds (10µl). Commercial antibacterial test discs (Oxoid) containing 30µg/disc of Amoxicillin were used. The solution of nystatin (6 mg/ml) was prepared in DMSO (10%) and deposited on the filter paper disc to give 30 µg/disc. Disc impregnated with10% of DMSO served as a solvent control. Tryptic soy agar was inoculated with the micro-organism (104 colony forming units/mL).

4.5. Determination of the minimal inhibitory concentration (MIC)

To determine the MIC of each spice extract, a broth micro-dilution experiment was performed according to the method of Jorgensen and Turnidge [23], ninety-six-well culture plates were prepared, and serial two-fold dilutions of the extracts were dispensed into the plate wells. The volume of dispensed extract was 0.1 mL per well in the concentration range of 50 mg/mL to 5 mg/mL. The concentration values were expressed on the basis of lyophilized materials dissolved in water. The same volume (0.1 mL) of overnight bacterial culture at a density of 105CFU/mL was added to the wells, and the culture plates were placed in an incubator set at 37 °C for 24 h. The lowest concentration of the plant extract required to inhibit the visible growth of the tested microorganism was designated as the MIC.

4.6. Minerals content

Minerals were determined in *P. inuloides* from the ash which was prepared and dissolved in 6 M hydrochloric acid and

made up to 10 mL.(This sentence is little confusing as you didn't mention any initial volume of the HCl and making up 10mL of the final volume. Kinldy reconfirm it.) Calcium content was estimated by the titrimetric method of [24] and iron content was estimated by UV-Visible spectrophotometer (Shimadzu, UV-160A model) at 480 nm [24]. Phosphorus was analyzed by Ranganna method [25]. The blue colour developed was read at 650 nm in UV-Visible spectrophotometer and expressed as phosphorus (mg/100 g meal). Other minerals were estimated by atomic absorption spectroscopy (Shimadzu AA 6701F, Atomic absorption flame emission spectrophotometer) equipped with hollow cathode lamp.

4.7. Scanning electron microscopy (SEM)

Structural analysis of *P. inuloides* was carried out using a scanning electron microscope (Quanta-200 FEI, Netherland). The samples were coated before loading to the scanning electron microscope. The coated samples were loaded into the system and the image was viewed under 1.0 KV potential using secondary electron image. The image was captured using 11.20 mm Ricoh Camera of 600x Mag.

5. STATISTICAL ANALYSIS

Two-way analysis of variance (ANOVA) of the data was carried out using Statistical Packages for the Social Sciences (SPSS 19.0) software. Mean differences were established by the Duncan's multiple range tests. The significant differences ($p<0.05$) between the means were performed to determine the effect of solvent polarity on the phenolic contents and antioxidant activity of Pulicaria leaf extracts.

6. RESULTS

6.1. Chemical Composition of the fatty acids and vitamins

The fatty acid compositions of *Pulicaria inuloides* oil was shown in **Table 1.** Of the thirteen fatty acids determined, six were unsaturated**.** Linoleic acid was found in the highest content (5.74%), followed by Oleic acid (2.37%) and 6,9,12-Octadecatrienoic acid (1.61%). (These sentnences are already been written in the abstracts, I think you need to use different structure of sentences in this paragraph.)

6.2. Tocopherols content

The tocopherol contents (α, δ and γ) of the *Pulicaria inuloides* oil were shown in **Table 1.** γ-tocopherol was the highest content and was about 58% of the total tocopherols, followed by α-tocopherol and δ-tocopherol (17.30% and 10.10%, respectively).

Table 1. Fatty acid (%) and tocopherols (mg/100 g oil) composition of *Pulicaria inuloides*

Fatty acid	Composition (%)
ΣSFA	34.76
Myristic acid	0.32± 0.01
Heptadecanoic acid	1.02± 0.02
Palmitic acid	0.10±0.02
Docosanoic acid	5.31±0.03
Stearic acid	16.97± 0.04
Propanoic acid	10.37±0.02
Tetracosanoic acid	0.67± 0.01
ΣUFA	11.00
Linoleic acid	5.74± 0.02
Heneicosanoic acid	1.15± 0.1
Oleic acid	2.37± 0.17
Octadecanoic acid	1.40± 0.03
6,9,12-Octadecatrienoic acid	1.61± 0.50
Eicosenoic acid	1.33± 0.20
Ratio SFA/UFA	1.18
Tocopherols (mg·100 g-1 oil)	
Total	58

α-Tocopherol	17.3±0.00
ß-Tocopherol	ND
δ-Tocopherol	10.10±0.41
γ-Tocopherol	30.6±0.01

All determinations were carried out in triplicate and mean value ± standard deviation was reported. SFA, saturated fatty acids; UFA, unsaturated fatty acids; ND, not detected.

6.3. Chemical composition of the phenolic compounds

Phenolic compounds determination of the *Pulicaria inuloides* leaves were performed by HPLC analysis. As it is shown in Table 2, nine phenolic components were isolated and identified, including Ferulic acid, Cinnamic acid, Ascorbic acid, P-coumaric acid, Gallic acid, Vanillic acid, Caffeic acid, Benzoic acid and chorogenic acid using column chromatography and macroporous resin chromatography as shown in Fig. 1.

Fig 1. Macroporous resin column chromatography used in isolation of phenolic compounds.

Table 5.2. Main phenolic constituents identified in *Pulicaria inuloides* leaves extracts

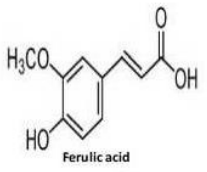
Constituents (mg/g DM)	Ethanol (%50)	Ethanol (%95)	Methanol (%50)	Methanol (%95)
Ferulic acid	0.10 ± 0.00^{Ca}	0.41 ± 0.01^{Ab}	$0.48\pm0.00A^{Bc}$	$0.42\pm0.00A^{b}$
Cinnamic acid	0.29 ± 0.01^{Da}	9.50 ± 0.02^{Eb}	$0.34\pm0.00A^{Ba}$	$0.29\pm0.00A^{a}$
Gallic acid	11.88 ± 0.00^{Ib}	7.76 ± 0.01^{Da}	6.86 ± 0.00^{Ea}	$7.82\pm0.01C^{a}$

P-coumaric acid	0.08±0.02^{Aa}	3.63±0.01^{Cc}	0.44±0.01^{Ab}	0.07 ±0.00A^{a}
Ascorbic acid	2.05±0.01^{Ha}	2.16±0.00^{Ba}	2.44±0.01^{Cb}	3.15±0.00^{Bb}
Vanillic acid	0. 86±0.01^{Fb}	0.611±0.00^{Aa}	3.66±0.00^{Dd}	1.15±0.02A^{Bc}
Caffeic acid	1.66±0.02^{Gb}	ND	0.96±0.00^{Ba}	ND
Benzoic acid	0.00^{Eb}0.41±	ND	ND	0.18±0.02^{Aa}
Chlorogenic acid	0.10±0.00^{Ba}	ND	ND	ND

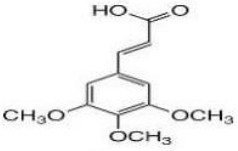
Each value is expressed as an average ±standard error (n =3).

Means in the same column with same capital letters are not significantly different (P < 0.05).

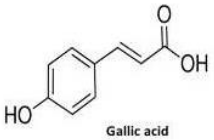
Means in the same row with same lowercase letters are not significantly different (P < 0.05); ND means not detected.



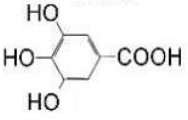
Ferulic acid



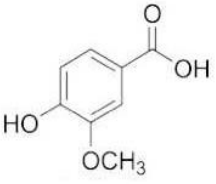
Cinnamic acid



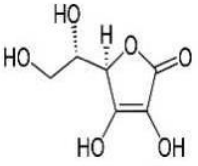
Gallic acid



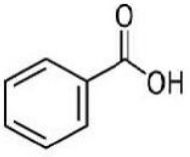
P-coumaric acid



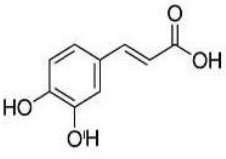
Ascorbic acid



Vanillic acid



Benzoic acid



Caffeic acid

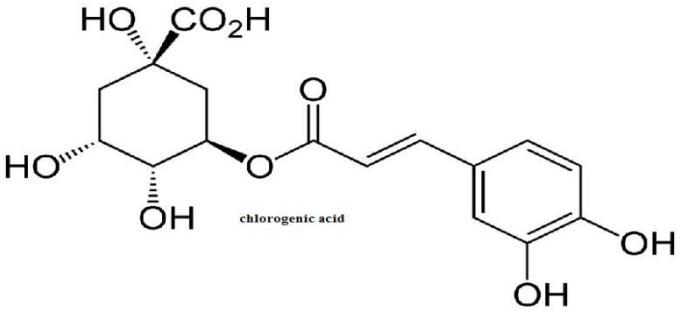


Figure 2. Phenolic compounds isolated by HPLC

6.4. Antioxidant activity

Table 3. Antioxidant activity by DPPH scavenging of phenolic compounds extracts from *P. inuloides* and reference antioxidants (V.C and TBHQ) (means ± S.D.).

Solvent				
	Ethanol (50%)	**Ethanol (95%)**	**Methanol (50%)**	**Methanol (95%)**
Extract	43.97±0. 18^{c}	80.60±2.25^{b}	31.18±0.17^{d}	85.47 ±3.15^{a}
V.C	80.33±1.69^{b}	94.57±0. 16^{a}	77.77±2.84^{c}	96.17±0.06^{a}
TBHQ	90.77± 0.21^{a}	95.01 ± 0.12^{a}	84.60±2.25^{b}	98.08±0.10^{a}

Each value is expressed as average ± standard error (SE) (n =3).
Mean followed by different superscript letters in the same row represents significant difference P<0.05).

Table 4. % inhibition of linoleic acid oxidation of extracts from *P. inuloides*

Solvent				
	Ethanol (50%)	**Ethanol (95%)**	**Methanol (50%)**	**Methanol (95%)**
Extract	5.31 ± 0.35^{d}	17.6 ± 2.2^{c}	30.16 ± 0.40^{b}	55.23 ±0.12^{a}
V.C	74.6 ± 2.2^{b}	80.57±0. 16^{c}	75.64±1.32^{b}	90.17±1.06^{a}
TBHQ	82.7 ± 1.8^{b}	91.11 ± 1.10^{a}	82.44±0.32^{b}	93.06±1.17^{a}

Each value is expressed as average ± standard error (SE) (n =3).
Mean followed by different superscript letters in the same row represents significant difference P<0.05).

7. ANTIMICROBIAL ACTIVITY

The phenolic compound extracts were screened for their antimicrobial activities against gram-positive bacteria (*S. aureus*, *S. penumoniae*, *B. subtilis*), gram-negative bacteria (*E. coli*, *S. dysenteria* and *S. Typhimurium*) and fungi.
The minimum inhibitory concentration MIC was calculated against each bacterial strain using broth macro dilution method and results were shown in Table 5.

Table 5. Antimicrobial activities of *Pulicaria inuloides* leave extracts

Microorganism	Indices	Antimicrobial activity			
		Ethanol (50%)	Ethanol (95%)	Methanol (50%)	Methanol (95%)
Staphylococcus aureus		++	+	+	+
	MIC (mg/mL)	10	20	7.0	25
Streptococcus penumoniae		+	++	+	++
	MIC (mg/mL)	10	6.0	10	0.3
Bacillus subtilis		+	+	+	++
	MIC (mg/mL)	11	8.0	30	.09
Escherichia coli		-	-	-	-
	MIC (mg/mL)	ND	ND	ND	ND
Shigella dysenteriae		-	-	-	-
	MIC (mg/mL)	ND	ND	ND	ND
Salmonella typhimurium		-	-	-	-
	MIC (mg/mL)	ND	ND	ND	ND
Candida albicans		+	++	+	++
	MIC (mg/mL)	16	.02	10	.04

Inhibition zone <6 mm: no antimicrobial activity (–); inhibition zone 6-7.5 mm: antimicrobial activity (+); inhibition zone >7.5 mm: high antimicrobial (++) Activity.
MIC: minimal inhibitory concentration (mg/mL)

8. MINERAL DETERMINATION

Mineral contents of *P. inuloides* were determined by atomic absorption spectroscopy**.** The concentrations were calculated on a dry weight basis. Table 5.6 showed difference between the content of plant from minerals. This may be due to various fractions of dissolved organic matter.

Table 6. The mineral elements in whole plant of *P. inulodies*

Elements	Mg/100g
Pb	0.01**0.081**±
Cd	0.09±0.22
Zn	0.3±0.72
Fe	13.875±152
Cu	0.144±0.31
Mn	0.645±0.71
K	159.5±1.51
Na	14.2±0.62
Mg	29.5±0.64
Ca	5.225±0.12
P	0.0024±0.01

Values are means ± standard deviation of three determinations.

9. SCANNING ELECTRON MICROSCOPY (SEM)

Plant structure of *P. inuloides* leaves was examined by Scaning electron microscopy (I think this structure needs to be explained.)

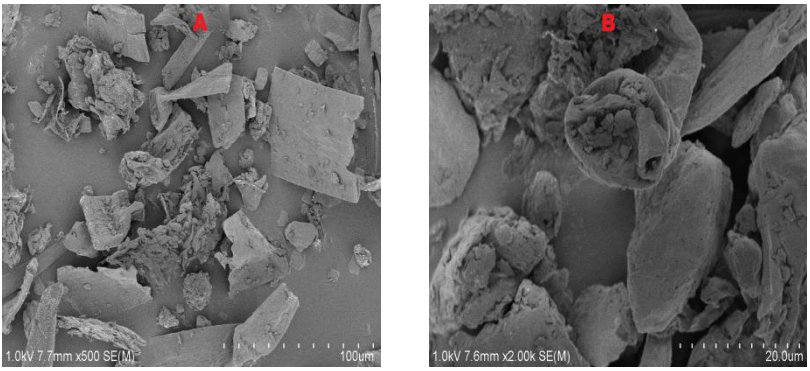


Figure 3. Scanning electron microscopic (SEM) pictures of *P. inuloides*

10. DISCUSSION

10.1. Chemical Composition of the fatty acid and vitamins

Of the thirteen fatty acids determined, six were unsaturated. Linoleic acid was found in the highest content (5.74%), followed by Oleic acid (2.37%). This is the first report for the fatty acids profile of *Pulicaria inuloides*.(Repeated these sentences for the 3rd time.) The results of fatty acid profiling of *Pulicaria inuloides* could be important from chemotaxonomic point of view. Octadecadienoic acid was previously reported to have a powerful antiviral activity against H. Simplex (HSV) and Parainfluenza viruses (PIV) [26]. This fatty acid may has favorable nutritional implications and beneficial physiological effects in the prevention of diseases such as cancer and coronary heart disease [27]. Linolenic acid and its derivatives eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have received attention for their effects on brain and retina function [28], suppressive effects on coronary heart disease [29], anti-inflammatory properties [30] and involvement in infant development [31], [32].

Oils and derived compounds are a main source of tocopherols. α-tocopherol is benefitial to human nutrition due to its higher biological activity than other tocopherols [33]. The total tocopherol content in this crude oil was 58.00 mg/100 g oil, higher than that of *Cucumis melovar. Flexuosus*. Tocopherols have been reported to inhibit lipid oxidation in oxygen radical absorbance capacity ORAC assays [34] and in radical trapping antioxidant parameter assays [35].

10.2. Identification of phenolic Constituents

Polyphenols are a broad family of naturally-occurring physiologically active compounds in nutrition and medicine because of their potent antioxidant capacity and possible protective effects on human health [36]. The results demonstrated that the *Pulicaria inuloides* leaves were rich in phenolic compounds, which were widely known as antioxidants and antimicrobial agents [37], [36]. Those phenolic constituents varied with different extract solvents. The major constituents were Gallic acid 11.88 %), Ascorbic acid (2.05%) and Caffic acid (1.66%) in 50% ethanol extracts, as well as Cinnamic acid (9.50%), Gallic acid (7.76%), P-coumaric acid (3.63%) and Ascorbic acid (2.16%) in 95% ethanol extracts. Gallic acid (6.68% and 7.82), Vanillic acid (3.66% and 1.15%) and Ascorbic acid (2.44% and 5.15) were main constituents in 50% and 95% methanol extracts, respectively. Furthermore, Caffic acids were not detected in 95% ethanol and 50% methanol extracts. Chlorogenic acid (0.10 mg/g) only was found in 50% ethanol. The highest level of Gallic acid was found in 50% ethanol extract, while there was no significant difference in 95% ethanol and 95 methanol extracts ($P>0.05$). Cinnamic acid in 95% ethanol extracts was higher than in other extracts, and there were no obvious variations among the other three extracts. The concentration of Vanillic acid was greatest in 50% methanol extract followed by methanol 95% extracts, while the

smallest amount was in 95% ethanol extracts. [39] reported the level of caffic acid and quercetin in the 95% ethanol of blueberry leaves, was 0.36 and 1.24 mg/g dry matter, respectively. These different results might be dependent on species, extraction method and growth location.

10.3. DPPH radical scavenging activity

DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule [40]. The reduction capability of DPPH was determined by examining the decrease in its absorbance at 517 nm, which was induced by antioxidants. The scavenging ability of the plant extracts on DPPH was likely due to the hydrogen donating ability of the polyphenolic compounds in the extracts. In the present studies, the values of the plant extracts were from ethanol (43.97 and 80.60 µg/mL) and methanol (31.18 and 85.47 µg/mL) Table 3. These results were lower than what had previously been reported [21]. These differences referred to many factors such as type of solvent, composition of solvent and apparatuses used in the extraction. Overall, the results indicated that the solvent extracting power was exerted.

10.4. β-Carotene bleaching

In the ß-carotene bleaching assays, all sample extracts had lower antioxidant activities than BHT. The antioxidant activities of the extracts were: methanol (95 and 50%) > ethanol (95 and 50%) (Table 4). There were significant differences ($p < 0.05$) among the antioxidant activities of all extracts. ß-carotene has biological activity and is an important physiological compound. The presence of different antioxidants can hinder the extent of ß-carotene bleaching by neutralizing the linoleate free radical formed during the system [41,42]. In ß-carotene bleaching assays, the linoleic acid free radical formed after abstraction of a hydrogen atom from one of its diallylic

methylene groups attacks the highly unsaturated ß-carotene molecules.

10.5. Antimicrobial activity

The results indicated that the extracts of *P. inuloides* had antibacterial potential and could be used in the treatment of the infectious diseases caused by the resistant microorganisms. The extracts of *P. inuloides* inhibited the growth of micro-organisms with various degrees. Table 5 displayed that 50% ethanol extracts of *P. inuloides* leaves were active against three bacterias and a fungi, and the highest inhibitory effects were found on *S. aureus*. Both 95% ethanolic and 95% methanolic extracts had higher antimicrobial activities for fungi than for other four bacteria. These variations might be associated with different structures of cell surface between gram negative and gram-positive bacteria [38]. The higher antifungal activity exhibited the methanol portion and 95% ethanol may be due to the presence of substantial amounts of polar constitutes from the leaves. This was in agreement with the previous research reported on the antifungal properties of the leaf extract of Aloe Vera extracted with pure methanol and ethanol [43].

The minimum inhibitory concentration (MIC) of all active extracts of *Pulicaria inuloides* was calculated using macro dilution method. The MIC values for specie like *B. subtilis* were higher in 50% methanol extracts than 95% methanol. For *S. aureus*, the smallest MIC value was found in 50% ethanol extracts, and the greatest one was in 95% methanol extracts. The MIC values for fungi were higher in both 50% ethanol and 50% methanol than those in other solvents. The results also confirmed that the gram-positive bacterial strains were more susceptible to the plant extracts as compared to gram negative bacteria [21]. Our results were in agreement with the fact that gram positive bacteria had only an outer peptidoglycan layer which was not an effective

permeability barrier [44]. The solvents used in the extraction procedure were found to have pronounced effect on the solubility of the antibacterial compounds [45]. The high bioactivity of phenolic acid is possibly related to its chemical structure as a phenol derivative. In fact, according to [46,47], phenols are more active than alcohols than aldehydes than ketones than ethers and esters than hydrocarbons (phenols > alcohols > aldehydes > ketones > ethers > hydrocarbons). This may also explain the inactivity of *E. coli* and *s. typhimurium*. Many researches showed phenolic compounds in the plant had contributed to the antimicrobial properties, and the antimicrobial capacity was dependent on the mode of action of polyphenols, source and concentration of phenolic compounds, extraction methods, varieties and microorganism species [48]. Moreover, there are the synergistic effect of all kinds of phenolic compounds on bacterial stains and fungi species [49].

10.6. Mineral content

Potassium and sodium were the highest whereas magnesium was the most highly concentrated trace element (Table 6). The Fe content of the sample was 13.875, while Ca was 5.225 mg/100 g respectively. The Pb content in the sample was 0.81 mg/100g. These values were higher than that for the *P. undulata* from Iran [50]. However, toxic mineral concentrations of the studied plant were lower. This work attempts to contribute to the knowledge of nutritional properties of these plants.

10.7. Scanning electron microscopy (SEM)

A scanning electron microscope (SEM) is a type of electron microscope that produces images of a sample by scanning it with a focused beam of electrons Figure 3.

Figures (A) and (B) were from the same internodal cell of P. inuloides.), which showed, at a higher magnification, an area

where a number of completed coated vesicles on the plasma membrane were visible.

The TEM has been particularly important for basic studies of the structure and function of plant cell organelles such as microtubules, coated vesicles and monitoring internal changes during the development of cultured explants [51].

11. CONCLUSION

The current study indicated that *Pulicaria inuloides* leaves had favorable antioxidant and antimicrobial potential and could be a good source of natural antioxidants and antimicrobials. The results of present study showed that ethanolic extracts from *P. inuloides* leaves had the highest levels of phenolic compounds. Among 9 phenolic compounds which were isolated and identified by HPLC, caffeic acids, benzoic and chorgenic were not detected in 95% ethanol extracts. Caffeic and chorgenic acids were not found in methanol extracts. All extracts except *E. coli*, *S. dysenteriae* and *S. typhimurium* were active against gram-positive bacteria and fungi. Both 95% ethanol and methanol extracts showed the highest inhibitory effects against *S. penumoniae* and MIC was lowest. Both 95% ethanol and methanol extracts demonstrated the greatest inhibition zone and the smallest MIC for fungi. More research in the future is required to investigate the effects of plant extracted parameters (such as solvent concentration, temperature, time) on the functionalities of the extracts and the stability of extraction methods.

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