

Original Article

Ecological Requirements, Antioxidant Activity and New Chemotype Essential Oil from *Achillea millefolium* L. and *Achillea micrantha* Wild. in North of Iran (Golestan Province)

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Abstract

In many field observation, ecological equipment, phenology and ethnopharmacological data of *Achillea millefolium* L. and *A. micrantha* Wild. were studied. The inflorescences of plants were collected in different locations of Golestan province: Chaharbagh (2000 m) and Dozan (2200 m) respectively. Essential oils were obtained by steam distillation (Clevenger) and analyzed by gas chromatography-mass spectrometry and (GC-MS). Total phenol (TP) and total flavonoid (TF) were determined with spectro photo meteri. Antioxidant properties were obtained by three radical scavenging activity methods: reducing power (RP), total antioxidant capacity (TAC) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH). Field observation showed that both species of *Achillea* are perennial aromatic plants which can grow 30 to 70 cm tall, flowers appeared in mid to late of May where the annual average rainfall were 399-345 mm, in sandy loam to silty clay loam soil. These plants has been used in traditional medicine as antibacterial, anti infection ,anti spasm, sedative, astringent, anti nociceptive and wound healing. Flowers essential oils of *A. millefoilium* and *A. micrantha* were characterized by higher amounts of similar constituents: binapacryle (63.82%, 83.63%), 1-8 cineol (14.97%, 3.76%) and α -selinene (4.81%, 4.49%) respectively. TP content (12.34 \pm 0.264 to 18.44 \pm 0.085 mgGAEg⁻¹) and TF contents (61.003 \pm 2.38 to 80.30 \pm 5.793 mgQUE g⁻¹) were measured in *A. micrantha* and *A. millefolium*, respectively. *A. micrantha* had more antioxidant activity with IC₅₀ (0.184 \pm 0.0475 μ g/ml in dry weight in DPPH method) than *A. millefolium* (IC₅₀ 0.178 \pm 0.178 μ g/ml in dry weight in RP method). According to the results, there is a positive correlation between antioxidant activity and important secondary metabolites (TP, TF), this could help to study more about the application of these plants in traditional medicine as an antiseptic, anti spasm and antibacterial agent.

Key words: *A. millefolium* L., *A. micrantha* Wild., Autecology, Essential oil, TF, TP, Antioxidant capacity, Golestan province, Iran

Introduction

Secondary metabolites in plants act as a defense mechanism against ecological stresses, that predation by herbivores, microorganisms and pathogens, they can also produce similar substances as a part of their normal growth and development or in response to stresses. It is believed that these metabolites may have biological effect in preventing disease due to their antioxidant effects

[1,2]. There are many different *Achillea* species growing wild in Europe and Asia (Iran and Turkey) which have been known to have ethno pharmacological effects. They are used in traditional remedies possesses as a strong ant oxidative, antimicrobial, anticancer, anti inflammatory or liver protective activities [3], for wound healing and abdominal pain, to treat of diarrhea, flatulence, dysmenorrheal and hemorrhoid [4-6]. The essential oil yield of *Achillea* and their

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components is related to genetic structure, type of species, climatic factors, soil condition and phase of growth of plant [7,8]. In similar researches, the main active secondary metabolites in *Achillea* species included flavonoids: apeginenin, rutin, luteolin and campherol [1], monoterpenes which are considered as important major constituents such as alpha-pinene, 1,8-cineole and camphor and their amounts are varied within different species based on ecological factors and climatic condition in different geographical regions [1,9-14]. Antibacterial screening against a wide spectrum of pathogens as well as antioxidant properties of essential oils and flavonoids from several *Achillea* species and their compositions were also investigated [12,13,15]. Antioxidant activity of phenolic and flavonoid compounds is attributed to their redox properties [16,17]. They could protect body against free radicals that are involved in diseases like Cancer, AIDS and Neurodegenerative [18] that's the reason why studies on antioxidant scavenging has greatly increased recently [19]. *Achillea millefolium* L. and *A. micrantha* Wild. are aromatic plants growing wild in different regions in the North of Iran. For centuries, they have been used in traditional medicine to treat many kind of disease including inflammation, pains, dysmenorrhea, diarrhea, stomach cramps, flatulence, gastritis and gastrointestinal disturbances. In according to these effects, the aim of the present study was to evaluate the ecological equipments, ethno pharmacology, essential oil investment and antioxidant activities of *A. millefolium* L. and *A. micrantha* Wild.

Materials and Methods

Field Observation

The inflorescences of *A. millefolium* L. and *A. micrantha* Wild. were collected from Chaharbagh (2000m) and Dozin (2200m) Mountain areas in the North of Iran (Golestan Province) during August to September 2011. Ecological factors, phenological characteristics and ethno pharmacological data of plants were also obtained in different field observations such as using local people and healers experiences in these regions (45-67 years old).

Plant Materials

The voucher specimen of plants (*Achillea millefolium* L. with herbarium No. 4001 and *A. micrantha* Wild. with herbarium No. 4002) were identified in Mazandaran University and have been deposited in the Herbarium Museum of the Islamic

Azad University of Gorgan branch. The influences were separated, dried in shade and grinded into fine powder and maintained at room temperature (21–23 °C). The prepared powder was kept in tight containers protected completely from light to perform the extraction of the secondary metabolites.

Essential Oils

Essential oils of plants were obtained by hydro distillation method (Clavenger apparatus). The yields for *A. millefolium* and *A. micrantha* were found to be 0.98% and 1.2% (v/w), respectively and were analyzed by GC/MS.

Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS) Analyses

The GC analysis was carried out with a Varian CP3800 system, while the Detector were MS Model Varian Saturn 2200 (Capillary Column VF5 (30 m×0.25 mm, 0.25 µm film thickness) was used with helium as carrier gas. GC oven temperature was kept at 60 °C for 10 min and programmed to increase to 240 °C at a rate of 3 °C/min. The injector temperature was at 260 °C with transformation temperature at 270 °C. Column flow were 1.7 ml/min. FID detector temperature was set to 300 °C. In order to obtain the same elution order with GC-MS, simultaneous injection was carried out using the same column and appropriate operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

Identification of the Essential Oil Components

Identification of essential oil components was carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to a series of nalkanes. Computer matching against commercial (Wiley GC-MS, MassFinder 3, Adams Library [20]).

Chemicals

2,2'-diphenyl-1-picrylhydrazyl (DPPH) and quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) were purchased from Sigma Chemical Co. (St., Louis, USA). Gallic acid, Folin-Ciocalteu reagent and methanol were purchased from Merck Co. (Germany).

Extract Preparation for Phytochemical and Antioxidant Tests

Three gram of the flower samples with 100 ml (methanol 80%) were extracted by maceration method. Extracts were filtered with Whatman No. 1 filter paper. The filtrates obtained from extracts were evaporated into dry rotary evaporator at 40 °C and were stored at 4 °C [18-21].

Determination of Total Phenolic Content

The total phenolic content of the *Achillea millefolium* and *A. micrantha* extracts was determined using the Folin-Ciocalteu Reagent. Total phenolic content was estimated by the Folin Ciocalteu method, based on the procedure suggested by Pourmorad et al. [18]. Then 0.5ml of plant extracts or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu Reagent (5ml) and aqueous Na₂CO₃ (4ml, 1M). The mixtures were allowed to stand for 15 min and the total phenols were determined by colorimetry at 765 nm. Gallic acid was used as a standard for calibration curve. Total phenol values were expressed in terms of mg equal gallic acid in 1 gr powder dry plant.

Determination of Total Flavonoid Content

Total flavonoids content were determined by aluminum chloride method [18]. Extract plants (0.5 ml) were separately mixed with 1.5 ml of solvent, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. They were kept at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with a spectrophotometer. Total flavonoid values were expressed in terms of mg equal quercetin in one gram powder dry plant [18].

Antioxidant Activity Tests

Reducing Power Assay

The reducing power assay was determined according to Arabshahi-Delouee and Urooj method [22]. At first, the dried extract (12.5 to 1000 µg) in 1 ml of the corresponding solvent was combined with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (K₃Fe(CN)₆; 10 g/l), after the mixture was incubated at 50 °C for 30 min. Then, 2.5 ml of trichloroacetic acid (100 g/l) were added and the mixture centrifuged at 1650 g for 10 min. Then, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml of FeCl₃ (1 g/l), and the samples absorbance was measured at 700 nm.

Total antioxidant capacity

This experimental procedure was adapted from Arabshahi-Delouee and Urooj method [22], which is based on the reduction of Mo (VI) to Mo (V) by the sample and observation of a green phosphate/Mo (V) complex at acidic pH. An aliquot of 0.1 ml of sample solution, containing 12.5 to 1000 µg of dried extract in corresponding solvent, was combined in a tube with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). They were incubated in a thermal block at 95 °C for 90 min. Then we got cold the samples and measured their absorbance at 695 nm. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent was used for the sample, and was incubated under the same conditions as the rest of the samples.

2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging Capacity Assay

The ability of the extract for free radical scavenging was assessed by the method of Kirca and Arslan [2]. The aliquots of plant extract (20 to 40 to 60 to 80 to 100 µl) were mixed with a methanolic solution of DPPH- (1 mm, 600 µl) and brought to 6 ml with solvent. After incubating in dark and room temperature that absorbance was measured at 517 nm. A DPPH- blank sample (containing 5.4 ml of methanol and 600 µl of DPPH-solution) was prepared. The percent decrease in absorbance was recorded for each concentration and percentage inhibition was calculated according to the following formula: %inhibition= [(ADPPH_ AExtract) /ADPPH]×100 ADPPH is the absorbance value of the DPPH-blank sample and extract is the absorbance value of the test solution. The plots of the 'percentage inhibitions amounts of dried plants (mg) in the extract' were used to find the concentration at which 50% radical scavenging occurred (IC₅₀) [2].

Results

Aut ecology, Phenology and Ethno pharmacology

In these natural regions, natural population of *Achillea* species were found in cold mountain area (Chaharbagh 2000 m, 70 km distance from south east of Gorgan city and Dozin (2200 m, 40-50 km South east of Minudasht city in Golestan province, results showed that this perennial plants could grow about 30-70 cm tall, roots could reach a depth of up to 30cm. Dormant plants commenced growth in the middle of April, grew through May and June, but

their shoots development was formed through April to September. White and yellow inflorescences appeared in mid to late of May and fruits normally one month after the first flowers were formed (June to July). These natural habitats have annual average rainfall is 399 mm in Dozin region and 345 mm in Charbagh mountain in which temprate average 18.3-18.1 0C respectively. *A. millefolium* L. and *A. micrantha* Wild. were found on the soil with $\text{PH}_{\text{KCl } \frac{1}{4}} = 8.1-7.9$, EC in 0.49-0.34 in Sandy loam to silty clay loam soil, respectively. Chaharbagh and Dozin's soils were poor in nitrogen (0.07-0.06%) and the amounts of phosphorus varied from 15 to 18 ppm and potassium from 143 to 135 mg ppm (Table 1).

Achillea millefolium and *A. micrantha* (Asteraceae) are locally known as "Marambu" have been used in traditional medicine of these regions for grazing, and in combination with other herbs as an anti-pathogen, anti-inflammatory, antispasmodic, pain killer, disinfectants, also a powerful healer for the treatment of wounds, skin infections, gastrointestinal and ulcer. Additionally, for the treatment of abdominal pain, dysmenorrheal, stop internal and external bleeding and menstrual pain have been considered. Additionally, local farmers bestrew dried flowers on their farms to avoid pathogens, pest and insects in their land. Despite, the uses of 7-10 *peganum* seeds with flowers of *A. micrantha* and *Cuminum cyminum* to treat of dysmenorrheal, but in combination of powder of *Provsikia abrotanoides* to treat leishmaniasis infection.

Table 1 Characterisation of natural habitats

Species	<i>A. millefolium</i>	<i>A. micrantha</i>
Location	Chaharbagh	Dozin
Sand (%)	45	29
Silt (%)	40	46
Clay (%)	15	25
Soil type	S.Si.L	Si.L
Organic carbon	0.624	0.643
Organic matter	1.075	1.109
pH	7.8	8.1
EC	0.34	0.49
N%	0.06	0.07
P (ppm)	18	15
K (ppm)	135	143
Fe (ppm)	4.14	3.22
Mn (ppm)	7.3	4.16
Zn (ppm)	0.36	0.56
Cu (ppm)	2.28	1.5

Essential Oil

According to obtained results in Table2, the yields for *A. millefolium* and *A. micrantha* were found to be 0.98% and 1.2% (v/w), respectively. The oils were characterized by higher amount of binapacryle (63.82-83.63%), 1-8 cineol (14.79-3.76%) and α -selinene (4.81%-4.49%) for *A. millefolium* and *A. micrantha* respectively, other main constituents of the oil for *A. millefolium* were camphor (3.34%), thujone (3.12%), santolina trien(1.97%) and for *A. micrantha* Wild. were santolina trien(2.60%).

Total Phenol and Flavonoid

The highest content of flavonoids and total phenols accumulated in the *A. micrantha* wild. during blooming (Table 3, Fig. 1, Fig. 2). The difference between the lowest and the highest levels of flavonoids in 2 species of yarrow was 20.6%.

Antioxidant Activity

Table 3 indicated the total phenolic and flavonoids contents and their antioxidant activities of methanolic extracts of *A. micrantha* and *A. millefolium*. Furthermore, the analysis of the extracts showed more scavenging effect against DPPH radical. Despite the antioxidant capacity with respect to their IC_{50} values was showed that methanolic extract of *A. micrantha* have more effects than *A. millefolium* extract Specially in DDPH method with $\text{IC}_{50} 0.184 \pm 0.0475 (\mu\text{g/ml})$.

Correlation coefficient showed that total phenolic content was responsible for antiradical efficiency in the extract of *A. micrantha* because the antioxidant and total phenolic content levels are also positively and significantly correlated. It has been recognized that flavonoids show antioxidant activity. Whereas, this findings has been showed that these methanol extracts with the highest content of secondary metabolites could be used as an important medicinal plants with high potency in scavenging of free radicals Specially in *A. micrantha* and DPPH method.

Discussion

The essential oil components are important portions within the phytochemistry of *Achillea* species [13, 7]. Monoterpenes, such as pinene, 1,8-cineole, camphor, artemisia ketone and sesquiterpenes, such as caryophyllene, germacrene, azulene and derivatives as well as sesquiterpene lactones, alkamides, flavonoids, lignans, and triterpenes are considered as important major constituents and their amounts vary within different species in many different regions [7,12,15,23-26].

About different chemotypes variation in *Achillea* species, Rahimmalek et al. [14] reported that there are high level of chemical polymorphism in *Achillea* species due to genetic, environment, climatic factors, growth phase, development of organs and plant part [27,28]. Toncer et al. [29] also

reported that *Achillea* species adapts to new environments and morphologic and chemical composition can be affected by environmental conditions.

Table 2 The most important essential oil composition of *A. millefolium* and *A. micrantha*

No	Compounds	Retention time	Total%	
			<i>A. micrantha</i>	<i>A. millefolium</i>
1	α -pinene	5.11	-	0.59
2	camphor	5.55	-	0.73
3	β -terpine	6.12	-	0.2
4	β -pinene	6.29	-	0.43
5	Santolina triene	6.72	2.60	1.97
6	Terpineol	7.43	-	0.16
7	p-cymene	7.69	0.27	1.65
8	1,8-cineole	7.99	3.76	14.97
9	Binapacryle	8.79	83.63	63.82
10	1,5-heptadien-4-ol, 3,3,6-trimethyl	9.62	0.76	0.55
11	Thujene	10.70	-	3.12
12	α -thujene	11.14	-	0.45
13	d-(+)-camphor	12.34	-	3.34
14	2-naphthalenamine,1,2,4a,5,6,7,8,8a-octahydro-4a-methyl	12.94	-	0.31
15	Borneol	13.33	-	0.31
16	Terpine-4-ol	13.65	-	0.46
17	α -terpineol	14.29	-	0.47
18	β -caryophyllene	23.45	0.38	0.27
19	α -eudesmol	32.65	0.83	0.82
20	α -seline	32.78	4.49	4.81
Total oil			96.664	99.51
Essential oil (%)			1.2%	0.98%

Table 3 Comparison of secondary metabolites (TF, TP) in both plants extract

Species	Metabolites	Flavonoid (mg QUE g ⁻¹)	Phenol (mg GAE g ⁻¹)
<i>A. millefolium</i> L.		80.30±5.793	18.44±0.085
<i>A. micrantha</i> Willd.		61.003±2.38	12.34±0.264

Table 4 Comparison of secondary metabolites (TF, TP) and antioxidant activity in both *Achillea* species extract (μ g/ml)

Antioxidant activity	<i>A. millefolium</i>	<i>A. micrantha</i>	BHA	BHT
IC ₅₀ DDPH	0.178 ± 0.178	0.184 ± 0.047	-	-
IC ₅₀ TAC	1.549 ± 0.008	1.888 ± 0.005	3.25 ± 0.351	2.93 ± 0.404
IC ₅₀ RP	1.0403 ± 0.019	0.814 ± 0.002	2.66 ± 3.74	2.463 ± 0.173

There have been studied that identified main compounds within *Achillea* species such as 1,8-cineol, α -selinene, santolina triene, α -pinene, camphor, borneol, p-cymen, camphene, terpinene 4-ol and thujen [23,29-37], but, we couldn't find binopacryl as a essential oil compounds of *Achillea* species in any similar records.

Binopacryl is the main constituents of these essential oils from both plant species in this research, In some

reports, it is one of nitrophenol component which could have pesticide and fungicide effects [38].

This finding was confirmed traditional uses of these plants as antipathogen in farms in addition of other study [39], so we have reported the new chemotypes of *A. millefolium* and *A. micrantha* based on binopacryle in North of Iran, that has not reported in any *Achillea* species in previous studies.

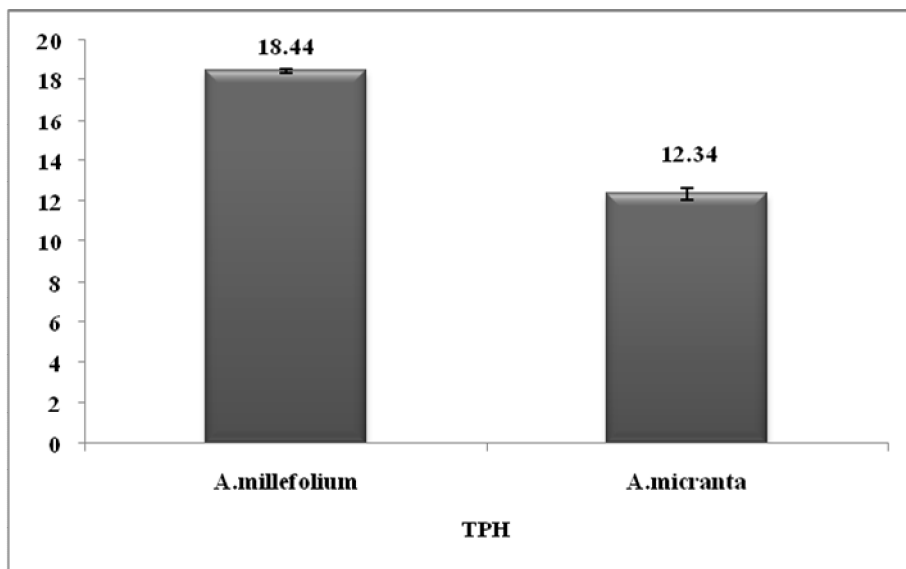


Fig. 1 Total phenol contents of *A. millefolium* & *A. micrantha* (mg GAE g⁻¹)

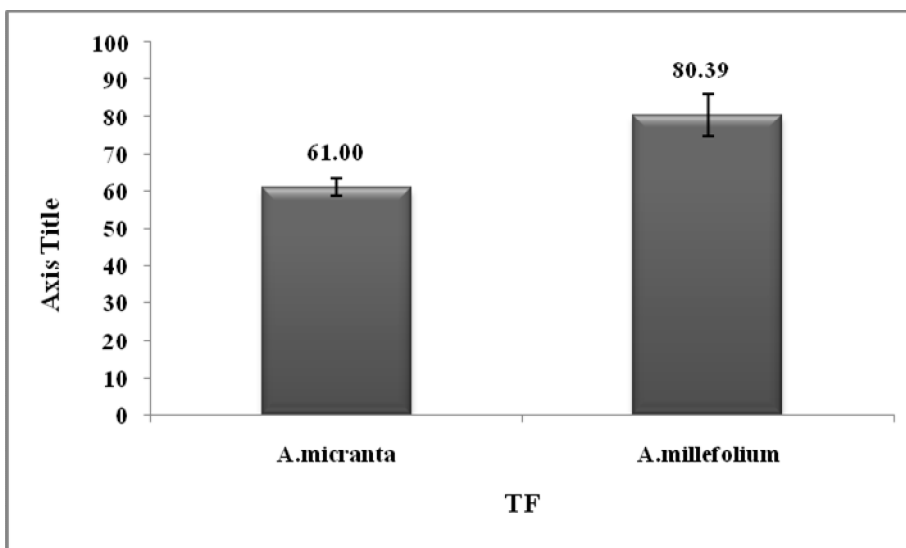


Fig. 2 Total flavenoids of *A. micrantha* & *A. millefolium* (mg QUE g⁻¹)

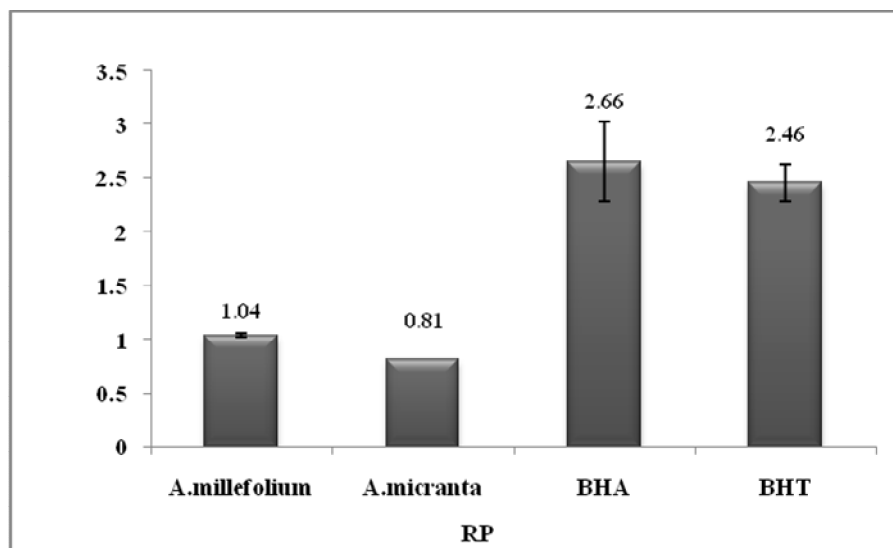


Fig. 3 Antioxidant activity of both extracts (IC₅₀) in RP method

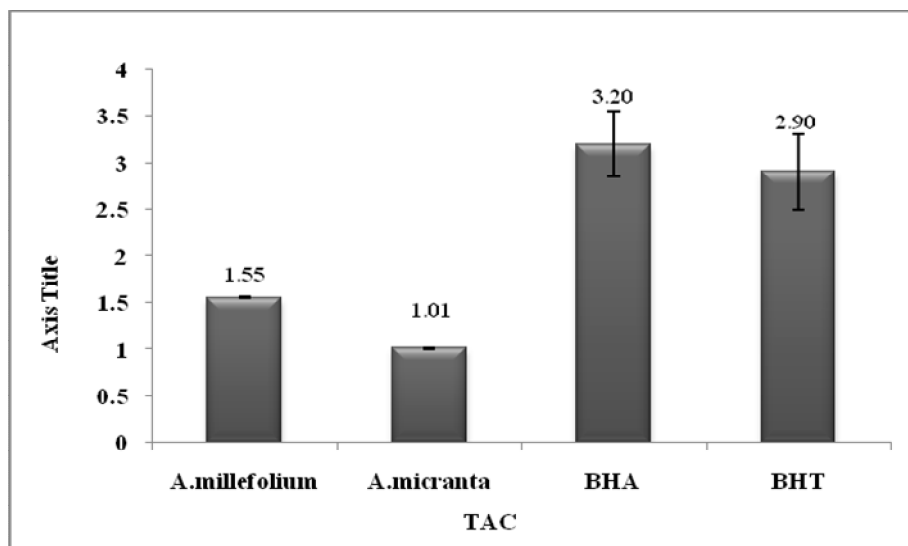


Fig. 4 Antioxidant activity of both extracts (IC₅₀) in TAC method

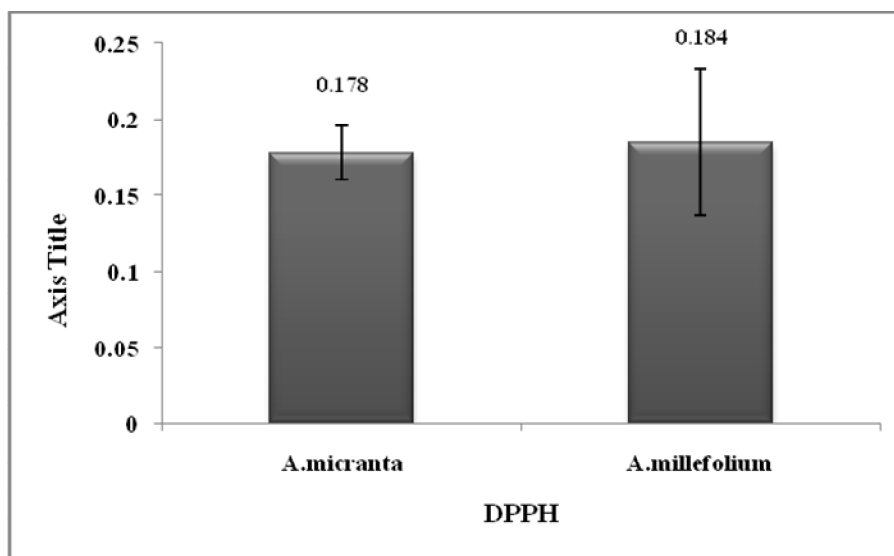


Fig. 5 Antioxidant activity of both extracts (IC₅₀) in DPPH method

Some researches uncovered the presence of new chemotypes based on major chemical components of essential oil from *Achillea* samples were collected from different parts of their country [8,29,40].

Total Phenol and Flavonoid

Polyphenols are the most secondary antioxidant compounds in medicinal plants, which have important role in blocked activity of free radicals. Results in many similar reports, showed that phenols and flavonoids are the most important secondary metabolites of *Achillea* species, which have many pharmacological effects could be attributed to the presence of these valuable constituents [10].

The highest content of flavonoids and total phenols accumulated in the *A. micrantha* during blooming (80.6%) (Table 3, Fig. 1 and 2). The difference between the lowest and the highest levels of flavonoids in 2 species of yarrow was 20.6%.

Table 4 indicated that the TP and TF contents in *A. millefolium* L. were more than *A. micrantha* Wild., so antioxidant capacity with respect to their IC₅₀ values was showed that methanolic extract of *A. millefolium* have more antioxidant capacity than *A. micrantha* extract Specially in RP and TAC methods. According to our results in Table 3 and Fig. 1 and 2, TP content (18.44±0.085 to 12.34±0.264 mgGAE/g⁻¹) and total flavonoids (80.30±5.793 to 61.003±2.38 mgQUE/g⁻¹) in *A. millefolium* and *A. micrantha*. Polyphenols have been reported to have multiple biological effects including antioxidant activity [41]. Due to their redox properties Specially in DPPH method with IC₅₀ 0.178±0.178 in *A. millefolium* lower than 0.184±0.0475 µg/ml in *A. micrantha* The high content of secondary metabolites (TP, TF) in both *Achillea* species could explain its high radical scavenging activity. Previous studies demonstrated that there is a direct relationship between amount of TP and TF contents and antioxidant activity in many medicinal plants [18, 42-46]. It has been reported that the high antioxidant activity of extracts in some medicinal plants (*Onosma dichloroanthum*, *Artemisia annua*, *Sylibum marianum* and *Heracleum gorganicum*) were directly depended on amount of TP and TF contents [47,48].

In various studies belongs to *Achillea* was observed the high correlation between quantity of TP and antioxidant activity [49,50]. Some flavonoids agents have been found in this family which, through scavenging or chelating process, showed antioxidant activity [18,19].

Conclusion

It can be concluded that high level of the binopacryle in two endemic species of *A. millefolium* L. and *A. micrantha* Wild. and their chemical polymorphism among of them is due to genetic and environmental factors as well as their interaction. The data presented in this study demonstrated that *A. millefolium* and *A. micrantha* possess also antibacterial and antioxidant activities against free radical produced in different condition. They have been used by the rural healers in the traditional medicine, in single or combination with other herbs as antiseptic, sedative, anti-inflammatory and anti pathogene to treat of wounds, skin infections, gastrointestinal and ulcer, abdominal pain, leishmaniasis infection, dysmenorrheal, stop internal and external bleeding and menstrual pain have been considered.

These results suggested that the phenolic compounds contributed significantly to the antioxidant capacity of the investigated plant species which results of many research shows such positive correlation between total phenolic content and antioxidant activity. Considering its many uses in traditional medicine in this region, this study can be a fond of future research of production of natural medicines with low risks and antioxidants capacity. As well, *A. millefolium* can be used as a natural pesticides for commercial and agricultural purposes.

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