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Original Article

Essential Oil Composition, Total Phenol, Flavonoid, Anthocyanin and Antioxidant Activities in Different Parts of *Artemisia annua* L. in Two Localities (North of Iran)

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Abstract

In this study, we evaluate the different biological activities of *Artemisia annua* L., locally known as "Moureh", in various altitudes in North of Iran, which has been used as sedative, fever few, anti inflammation, insecticide and anti infection to treat many current diseases. Parts of plants were collected from two different localities (23-1000 m) in Mazandaran province, North of Iran. The most important of secondary metabolites of total phenolics (TP), total flavonoids (TF) and total anthocyanin (TA) content of extracts were investigated by spectrophotometry method and their antioxidant activity were obtained by Total Antioxidant Capacity (TAC), Reducing Power (RP) and 1,1-diphenyl-2-picryl hydrazyl radical scavenging (DPPH). The essential oils were obtained by hydro distillated in a Clevenger-type apparatus for 5h and analyzed by GC/MS. Results indicate that the main components in essential oils were identified as Artemisia ketone (25.54- 13.6%), followed by 1,8-cineole (11.98-13.26%), camphor (11.89-13.68%), α-pinene (10.11-9.29%) in AFRACHAL (1000m) and DOLAT ABAD (23m) regions , respectively. TP content had significant variation in different plant parts and regions, ranging from (11.22 to 16.94) mgGAEg^{-1}, TF content (11.62 to 63.74) mgQUE $g^{-1}$and quantity of TA (0.03 to 3.59) mgCGEg^{-1}.The highest contents of secondary metabolites were found in aerial parts when compared with the other parts. Amount of antioxidant activity (IC50) in various parts of *A. annua* L. was measured (1.98 to 4.2) in DPPH, (7.07 to 7.46) in TAC and (5.26 to 8.04) in RP methods. In general, the highest contents of activities were found in aerial parts when compared with the other parts, whereas this part with the highest amount of IC_{50} had the weakest antioxidant activity.

Key words*:* *Artemisia annua* L., Essential oil, Phenolic content, Antioxidant capacity, Iran

Introduction

Reactive oxygen species including free radicals and non free radicals along with different forms of active oxygen are involved in diverse physicochemical processes and causing more than one hundred disorders in humans such as neurodegenerative disorders, cancer, ischemia, cardiovascular diseases, arthrosclerosis, arthritis, AIDS, Alzheimer's, Parkinson's, cataracts and inflammation. Antioxidants prevent diseases by mechanisms such as scavenging free radicals, against oxidative stress and inhibiting lipid peroxidation [31].

Therefore supplementing a food product with antioxidant compounds of plant source may provide a health benefit as well. Secondary metabolites, especially phenol compounds and terpenes in medicine plants have been known to therapeutic activities like antioxidant, antimicrobial, anticancer, anti-inflammatory and etc. in many researches, high correlation was reported between the antioxidant capacity and total phenol and flavonoid contents of medicinal plants [39, 43]. The antioxidant activity of secondary metabolites is due to their redox properties, ability to chelate metals and quenching of singlet oxygen [41]. Flavonoids with chelate metals

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such as iron chelating have been recommended for malaria patients [19]. Different studies reported that phenolic compounds have protection against a wide range of diseases such as coronary heart disease, stroke, and certain types of cancers [21, 3] and exhibit biological activities such as: anti-inflammatory, immune-stimulating agents antiallergenic, anti-artherogenic, anti-microbial, antithrombotic, anti stress, anti hyperglycemia and vasodilatory effects [10].

Artemisia annua L. belong to Asteraceae family, comprising with up to 500 species in world and about approximately 34 native *Artemisia* spp. in Iran [44, 46]. Approximately 200 species of *Artemisia* grow in China, where more than 50 of them have been used in traditional medicine of this country [42], which the tea and decoction of *Artemisia annua* L. is famous to an anti-malarial drug plant that has been used for many centuries for treat human fever and malaria [2], Also in ancient Greece and Roman the essential oil of *Artemisia* species were used in infusion as poison antidote, activates the blood circulation, anti-helminthic and for its abortive qualities indicated in gastric insufficiency [44]. *A. annua* L. is a rich source of biologically active natural products which has been used to treatment of malaria, gastrointestinal helminthosis, hemorrhoid, skin rashes, diarrhea and some other diseases [7-19, 46].

Many secondary metabolites of terpene peroxides such as artemisia ketone, artemisinic alcohol, arteannuin B and myrcene hydroperoxide, over 50 different phenolic compounds belonging to five major groups (flavones, flavonols, coumarins, phenolic acids, and a miscellaneous group) which the main coumarins are coumarin, aesculetin, iso-fraxidin, scopoletin, scopolin, tomentin, and also other phenolic compounds such as 2,4-dihydroxy-6-methoxy-acetophenone, 5-nonadecyl-3-O-methyletherresorcinol, 2,2,6-trihydroxychromene, and 2,2-dihydroxy-6-methoxychromene have been isolated of this plant [11-14-19,46], the high antioxidant activity of *A. annua* extract is due to its high phenolic content [19]. Artemisinin (sesquiterpene lactone) is a quite expensive compound with very low concentration which the only commercial source available is *A. annua* [2]. From approximately 400 species of artemisias, *A. annua* L. is the main source of artemisinin [20]. Different studies show that Artemisinin-derived drugs are effective against bacteria, fungi, insects and parasites such as *Plasmodium* spp., *Coccidia* spp., *Babesia* spp., *Leishmania* spp. *Neospora caninum* and *Schistosoma* spp. [4-26-28-29-45-49,50] that effective in the health of humans and animals. The aerial parts of A. *annua* L. are highly aromatic and known to give an essential oil of some economic importance, which is rich in monoterpenes, sesquiterpenes, diterpenes and phenyl propanoids [23].

The aim of the present study is to find out how the geographic location influences on the qualitative and quantitative composition of secondary metabolites and essential oil of *Artemisia annua* L. and evaluation of antioxidant activities of different parts of plant, which has been used by the rural healers in North of Iran to prevent and treat of malarial, parasitic, antibacterial, anti inflammation, sedative, dysmenorrhea, diarrhea and infectious disease.

Materials and Methods

Plant materials

The different parts (stem, root and aerial parts) of *Artemisia annua* L. were collected in AFRACHAL (1000 m) and DOLAT ABAD (23 m) regions of Mazandaran province in North of Iran during Sep to Oct 2010. This voucher of specimen was identified and has been deposited at the Herbarium Museum of the Islamic Azad University of Gorgan branch (Golestan province). The plant materials were dried and ground to a fine powder using a laboratory mill, were maintained at room temperature (21–23 °C), and protected from light.

Phytochemical tests

2, 2-Diphenyl-1-picryl hydrazyl (DPPH), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), methanol and other compounds were purchased from international companies.

Extract preparation

Extract preparation for phytochemical tests

The dried stem, root and aerial parts of plant (5g) were extracted overnight with 100 ml of methanol, in a mechanical shaker at room temperature. Each extract of plant was filtered with Whatman No. 1 filter paper and stored at 4 °C.

Extract preparation for antioxidant activity

A 45 gr of different parts (root and aerial parts) of *Artemisia annua* L. were extracted with 300 ml of methanol solvent in a mechanical shaker at room temperature. Extracts were filtered with Whatman No. 1 filter paper. The filtrates obtained from extract were evaporated into dry at 40°C in a rotary from evaporator and stored at 4 °C [8].

Phytochemical tests

Total phenols determination

Total phenolic content were determined by Folin Ciocalteu method [35]. A 0.5 ml of samples or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml) and aqueous Na_2CO_3 (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined by colorimeters at 765 nm. Gallic acid was used as a standard for calibration curve. Total phenol values

are expressed in terms of mg equal gallic acid in 1 gr powder dry plant.

Total flavonoids determination
Total flavonoids content was estimated by the Aluminum chloride method, based on the procedure of [35]. Plant extracts (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. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with a spectrophotometer, and quercetin was used as a standard for calibration curve. Total flavonoids values are expressed in terms of mg equal quercetin in 1gr powder dry plant.

Total anthocyanin determination
Total anthocyanin content was determined by the pH-differential method described by Giusti [22], using 2 buffer systems: potassium chloride buffer, pH 1 (1.86 g KCl in 1 L of distilled water, pH value adjusted to 1.0 with concentrated HCl), and sodium acetate buffer, pH 4.5 (54.43 g CH3CO2Na·3H2O in 1 L of distilled water, pH value adjusted to 4.5 with concentrated HCl). The sample diluted with corresponding buffer and the absorbance was measured at 510 and 700 nm. Total anthocyanins were calculated as cyanidin-3-glucoside according to the following equation:

$$TAC = \left(\frac{A \times MW \times DF \times 100}{MA}\right)$$

$A = (A510 - A700)_{pH1} - (A510 - A700)_{pH4.5}$
MW: 449.2 g/mol for cyanidin-3-glucoside
DF = dilution factor; MA: 26900

Antioxidant Activity Tests

Reducing Power assay
This assay is based on Arabshahi-Delouee method. First, The dried extract (12.5–1000 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^{-1}), after the mixture was incubated at 50°C for 30 min. Then, 2.5 ml of trichloroacetic acid (100 g l^{-1}) were added and the mixture centrifuged at 1650g 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 $FeCl_3$ (1 g l^{-1}), and the samples absorbance was measured at 700 nm [8].

1,1-diphenyl-2-picryl hydrazyl radical scavenging capacity Assay
The ability of the extracts for free radical scavenging was assessed by the method suggested by Arabshahi-Delouee and Urooj [8]. Briefly, 1ml of a 1mM methanolic solution of DPPH was mixed with 3ml of extract solution in methanol (containing 12.5–1000 µg of dried extract). The mixture was then vortexes vigorously and left for 30 min at room temperature in the dark. The absorbance was measured at 517 nm and activity was expressed as percentage DPPH scavenging relative to control using the following equation:
DPPH scavenging activity (%) $=[(A_{control} - A_{sample}) / A_{control}] \times 100$

Total Antioxidant Capacity
This experimental procedure was adapted from Arabshahi-Delouee method, 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 12.5-1000µg 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 $^{\circ C}$ 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 [8].

Isolation of essential oil
The essential oils of aerial parts of plant in blooming were obtained by hydro distillated in a Clevenger-type apparatus for 5h according to the method recommended in the British Pharmacopoeia and was kept refrigerated and protected from direct light until the analysis time.

Gas Chromatography - Mass Spectrometry (GC-MS)
The essential oil was analyzed by GC/MS (6890 N Network GC system and 5975 B Intet MSD). The capillary conditions were as follows; carrier gas, helium with a flow rate of 1.7mL/min; injected 1µLof the essential oil and ionization potential 70ev. The initial temperature of column was 50 ˚C (held 5min) then heated to 250 ˚C, then heated to 250 ˚C and kept constant for 20min. The same condition of temperature programming used for n-alkenes mixture (C7-C31) to calculate the retention indices (RI). The identification of each component was studied by mass spectral data, literature (Adams R, 1995) and NIST computer library. The relative percentage of the oil constituent was calculated.

Statistical analysis
For all assays, data were expressed as means ± S.E. and differences at $P<0.05$ were considered statistically significant.

Results and Discussion

Total phenolics, flavonoids and anthocyanin
Secondary metabolites contents of different parts of *Artemisia annua* L. in various regions show in Table

1, comparison of the results indicated that the TP content of various parts of *A. annua* L. had significant variation, ranging from 11.22±0.74 to 16.94±0.52 mgGAEg^{-1}, TF content 11.62±0.59 to 63.74 ±1.01 mgQUE $g^{-1}$and quantity of TA 0.03±0.002 to 3.59±0.4 mgCGEg^{-1}. The highest contents of secondary metabolites of TP (15.58 and 16.94 mgEGAgr^{-1}), TF (57.28 and 63.74 mgEQUgr^{-1}) and TA (3.59 and 3.34 mgECGgr^{-1}) were found in aerial parts when compared with the other parts, whereas the lowest contents TP (11.22mgGAEg^{-1}) and TF (11.62 mgQUE g^{-1}) were in stem extract of AFRACHAL region (1000m), and the lowest TA detected in the root extract of DOLAT ABAD region (23m) (Table 1 and Fig 1-2,3).

Amount of secondary metabolites (phenolics, flavonoids and anthocyanin**)** has been associated with increased levels of reactive oxygen species, which are by products of aerobic metabolism or with biotic, abiotic and stresses [6, 17]. Results of Seddik [38] show that high flavonoids and polyphenol contents of *A. herba alba* Asso. were present in the ethyl acetate phase, while the aqueous phase contains smaller amounts of these compounds.

Also in another studies by Brown [13] indicate that the artemisinin content of *A. annua* has ranging between 0.01% and 1%, depending on variety, and can even be as high as 1.4% in some cultivated strains. Flavonoids are well known for their antioxidant activity due to their redox properties, which can delay or inhibit oxidation of lipids and other molecules by inhibiting the beginning of oxidizing chain reactions [19]. Survey of results showed that was a negative correlation between total phenol and flavonoid contents and antioxidant activity for aerial parts extract of plant. These results were in opposite with the findings of many research groups who reported direct relationships between total phenolic content and antioxidant activity [15-18-27-35-41, 43]. Also in other studies about variety of species (*Sylibum marrianum*, *Lithospermum erythrorhizon* , *Cordia multispicata* , *C. multispicata* and *Tournefortia bicolor*, *Ehretia laevis*, *Cordia myxa* and *Borago officinalis*), showed direct relationships between of them [16-24, 33].

Table 1 Comparison of secondary metabolites of different parts of *Artemisia annua* L. in various regions

Metabolite	(AFRACHAL region) (1000 m)			(DOLAT ABAD region) (23 m)		
	Aerial part	Stem	Root	Aerial part	Stem	Root
Flavonoid (mg QUE g^{-1})	63.74 ± 1.01	11.62 ± 0.59	21.76 ± 0.33	57.28 ± 0.25	21.48 ± 0.66	11.88 ± 0.89
Phenol (mg GAE g^{-1})	16.94 ± 0.52	11.22 ± 0.74	14.9 ± 0.6	15.58 ± 0.89	13.96 ± 0.86	13.24 ± 0.66
Anthocyanin (mg ECG g^{-1})	3.59 ± 0.4	0.26 ± 0.11	0.56 ± 0.09	3.34 ± 0.36	0.31 ± 0.05	0.03 ± 0.002

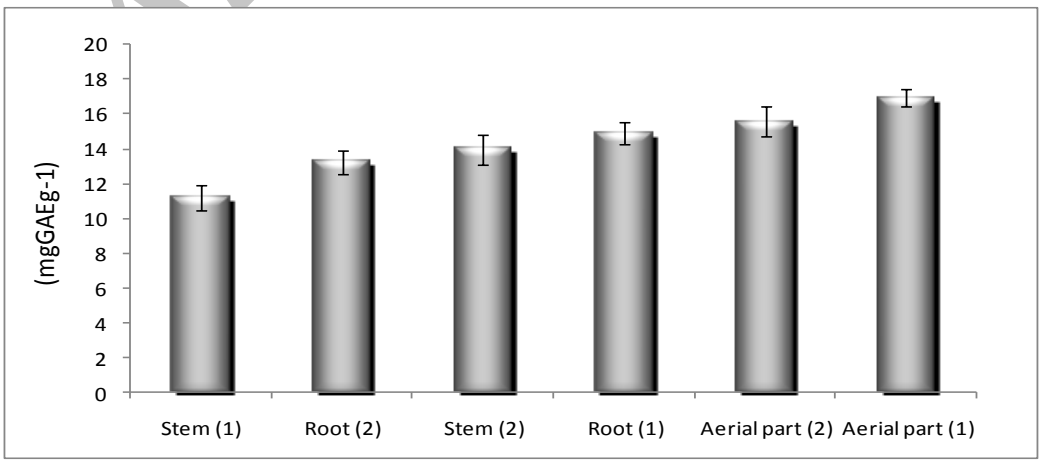

Fig 1. Total phenol contents of different parts of *Artemisia annua* L.
Sample 1. AFRACHAL region (1000 m), Sample 2. DOLAT ABAD region (23 m)

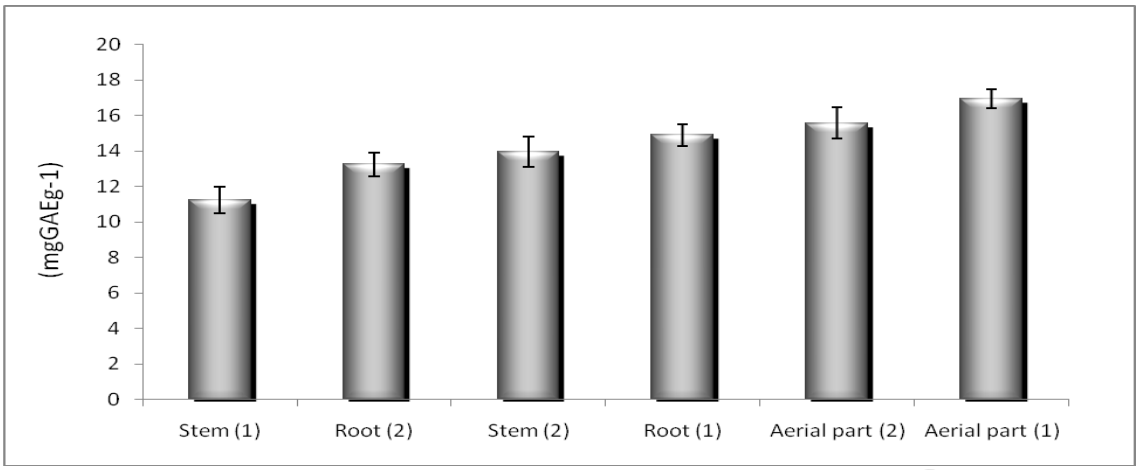

Fig 2. Total flavonoid contents of various parts of *Artemisia annua* L.
Sample 1. AFRACHAL region (1000 m), Sample 2. DOLAT ABAD region (23 m)

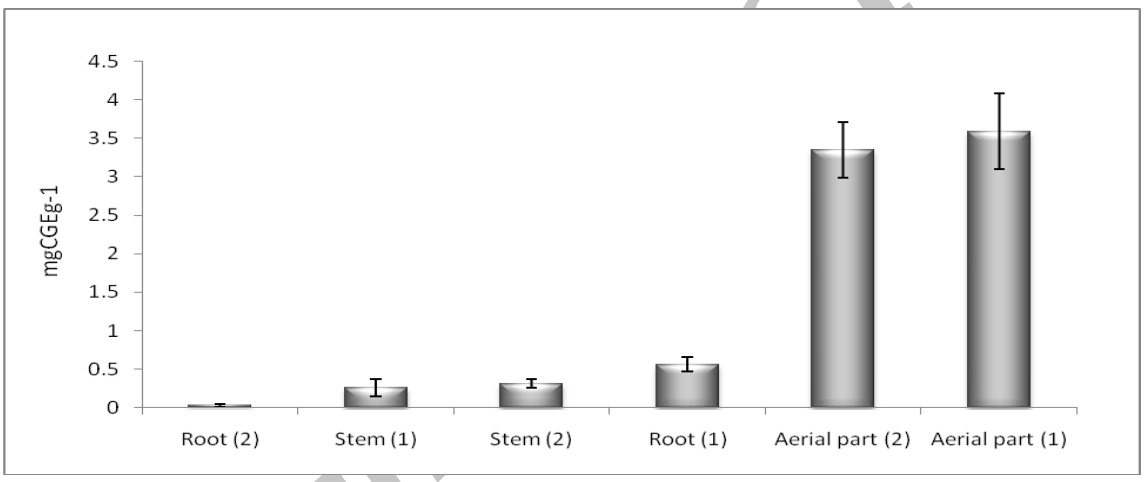

Fig 3. Total anthocyanin contents of different parts of *Artemisia annua* L.
Sample 1. AFRACHAL region (1000m), Sample 2: DOLAT ABAD region (23m)

Antioxidant activity

Fig 4-6 and Table 2 shows antioxidant activity by inhibition of the free radical. Amount of IC50 in various parts of *A. annua* L. was (1.98±0.21 to 4.2±0.63) in DPPH method, (7.07±0.86 to 7.46±0.86) in TAC and (5.26±0.87 to 8.04±0.84) in RP methods. The highest antioxidant activity and radical scavenging effect were observed in root extract with IC_{50} (1.98 to 2.2) in Dpph, (5.26 to 5.83) in PR and (6.89 to 7.01) in TAC methods, whereas aerial parts with the highest amount of IC_{50} had the weakest antioxidant activity.

Reactive oxygen species are involved in diverse physicochemical processes in the human body [36] which have main role in the pathogenesis of different diseases [16].

In research by Brisibe [12], total antioxidant capacity reported for the leaves 1,125 µmol TE/g dry matter and 1,234 µmol TE/g dry matter for the inflorescences and the antioxidant activity values for the stems and roots were similar with 292 and 287 µmol TE/g dry matter, respectively.

Total Antioxidants in Hot-water extracts of *Artemisia monosperma* reported 675.33 µmol Trolox per 100 ml [5].

The traditional tea of *A. annua* is a main source of both antioxidant phenolics (mostly flavonoids) and artemisinin [37, 48].

Another report indicates that the high antioxidant activity of *A. annua* L. extract is most likely due to its high phenolic content and this capacity is stable to boiling [19]. The results of Laciar [30] show that *A. echegarayi* essential oil presented moderate antioxidant activity and its antioxidant activity was lower than that of quercetin, a powerful natural antioxidant.

Table 2 Comparison of IC_{50} of different parts of *Artemisia annua* L. in various regions

Antioxidant activity	Roots		Aerial parts		BHA	BHT
	(DOLAT ABAD region)	(AFRACHAL region)	(DOLAT ABAD region)	(AFRACHAL region)		
IC_{50} TAC	7.01 ± 0.88	6.89 ± 0.88	7.46 ± 0.86	7.07 ± 0.86	3.46 ± 0.88	3.09 ± 0.84
IC_{50} RP	5.26 ± 0.87	5.83 ± 0.9	8.04 ± 0.84	7.56 ± 0.88	2.79 ± 0.95	2.6 ± 0.91
IC_{50} DPPH	2.2 ± 0.43	1.98 ± 0.21	4.2 ± 0.51	2.4 ± 0.32	-	-

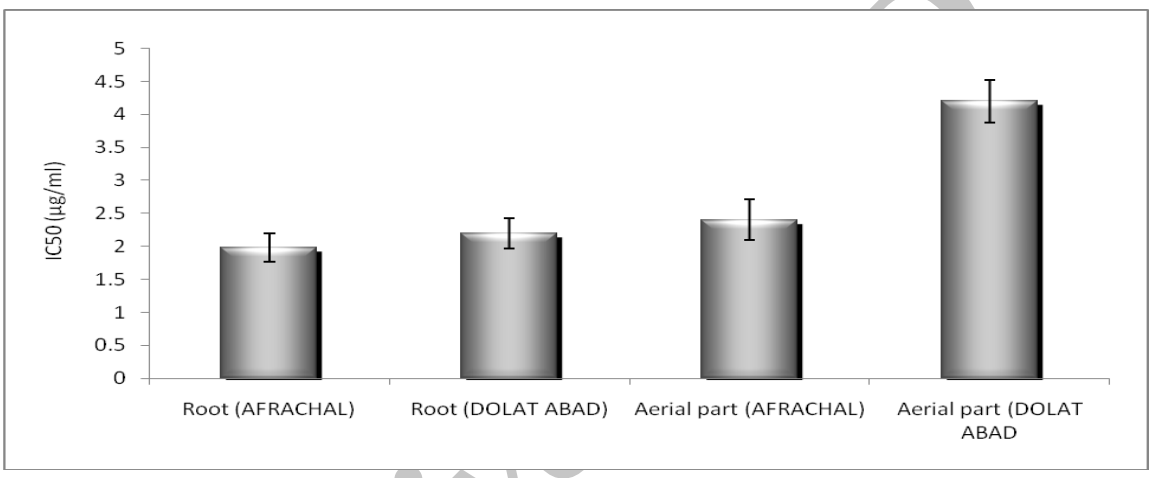

Fig 4. Amounts of IC_{50} in various parts of *Artemisia annua* L. in DPPH method

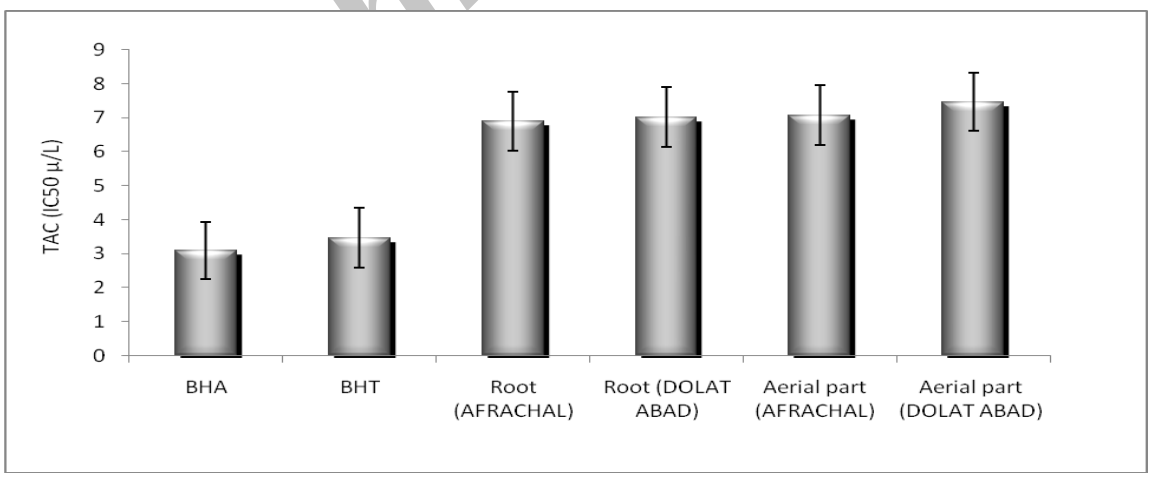

Fig 5. Amounts of IC_{50} in various parts of *Artemisia annua* L. in TAC method

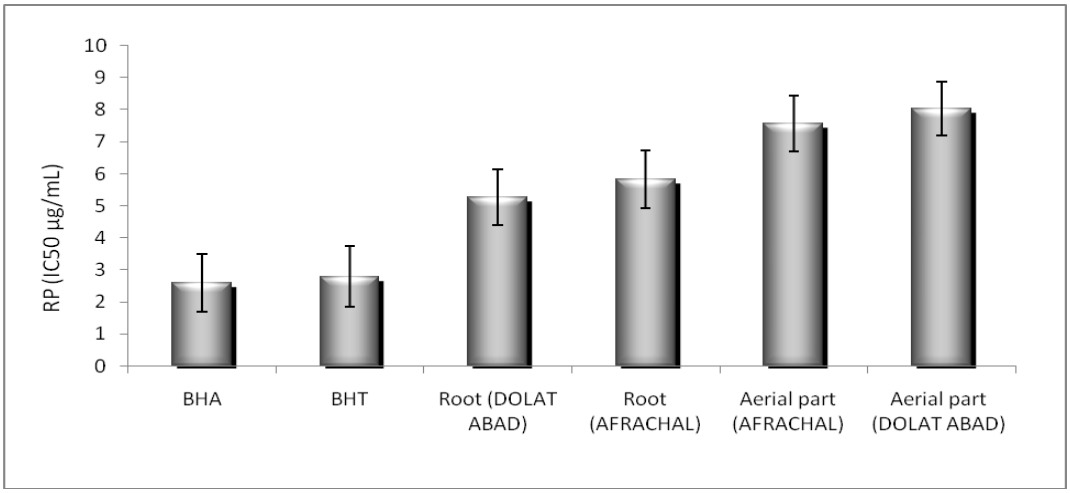

Fig 6. Amounts of IC_{50} in various parts of *Artemisia annua* L. in RP method

Table 3 The main chemical constituents of the flowering aerial parts of *Artemisia annua* L. essential oil

Compound	AFRACHAL region (1000 m)	DOLAT ABAD region (23 m)
α-pinene	10.11	9.29
Camphene	2.99	2.77
Thujene	1.46	1.89
β-pinene	1.24	0.94
Myrcene	2.57	1.93
1,8-cineole	13.26	11.98
γ-terpinene	0.72	1.12
Artemisia ketons	25.54	13.6
Artemisia alcohol	2.08	2.09
Camphore	11.89	13.68
Pinocarvone	1.03	2.44
Borneol L	0.69	0.84
Naphthalene	5.09	7.01

Essential oil

The essential oil analysis of *Artemisia annua* L. led to the identification of approximately 43 main compounds accounting for the 99.99% of the total essential oil in AFRACHAL (1000m) and 47 compounds accounting for the 93.71% in DOLAT ABAD (23m) regions (Table 3). The main essential oil components were identified as *Artemisia* ketenes (25.54- 13.6%), followed by 1,8-cineole (11.98-13.26%), camphor (11.89-13.68%), α-pinene (10.11-9.29%) in AFRACHAL (1000 m) and DOLAT ABAD (23 m) regions respectively. The chemical composition of the essential oil had been demonstrated in Table 3.

In previous study by Verdian-rizi [47] in Iran, the major constituents (48%), 1,8-cineole (9.39%), camphene (6.98%) and spathulenol (4.69%) were identified in the pre-flowering stage, in the volatile of flowering stage camphor (43.50%), 1,8-cineole (13.90%), spathulenol (3.73%) and Artemisia ketone (3.37%) and in the oil obtained from post-flowering stage, camphor (36.75%), 1,8-cineole (12.00%), spathulenol (4.50%) and pinene were the principal components of this oil.

The majority of the compounds identified in the essential oil of *Artemisia annua* growing in Ethiopia reported monoterpenes (57.89%), Sesquiterpenes (36.84%) and phenols comprised (5.55%). Among

the identified monoterpenes 52.17% were monoterpene alcohols, 30.43% - monoterpene hydrocarbons, 13.04% - monoterpene aldehydes and 4.35 % were monotrpene oxides, and camphor was identified as the major component (43.84%) [34].

In other study on the chemistry of *A. annua* L. by Juteau [25] show that the essential oil of *A. annua* L. aerial parts of species growing in France consisted of camphor (44%), germacrene D (16%), *trans*-pinocarveol (11%), β-selinene (9%), β-caryophyllene (8.9%) and Artemisia ketone (3%). In fact essential oil contents show variations in plants of different geographical origin.

The essential oil of *A. annua* aerial parts, that were obtained from plants growing at India consisted of main constituents artemisia ketone (58.8%), 1,8-cineole (10.2%) and camphor (15.8%) while the chief components of the essential oil from aerial parts of plants grown in Kashmir contained artemisia ketone (52.3%), 1,8-cineole (13.1%) and camphor (15.5%) [9].

Accordingly, it seems differences in among of chemical compositions of the *A. annua* L. essential oils from the viewpoint of qualitative and quantitative might depended on different geographical and environmental conditions such as different temperature, soils and altitudes which play an important role on the biological activity of *A. annua* L. essential oil.

Conclusion

The present study indicate the aerial part of *Artemisia annua* L. with highest amount of TP, TF and TA compounds as an important part of this plant, which could provide potential natural sources compounds to prevent and treatment of diseases.

These findings demonstrate the aerial part as the best organ with the most secondary metabolites of plant for future research and also confirmed the interesting of this plant uses by the rural healers to prevent and treat of current infectious disease.

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