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Survey of artemisinin production of *Artemisia annua* (anti-malarial medicinal plant) bioecotypes available in Iran by HPLC method

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ABSTRACT

In this study, 120 bioecotype seeds of Artemisia annua were collected from different sites in Iran. In order to characterize elite Artemisia annua bioecotypes with high artemisinin producibility, the bioecotypes were analyzed via the HPLC method. Among the 120 bioecotypes collected, 5 bioecotypes (S4, M9, Gu17, M33 and M47) did not have any artemisinin content, while 12 bioecotypes (Gu3, Gu5, Gu7, Gu19, M26, M29, M35, M36, M41, M43, M51 and M53) had higher artemisinin than other bioecotypes. From 53 bioecotypes available in the province of Mazandaran, 35 bioecotypes had more than 0.4% artemisinin and thus were high artemisinin. Also, bioecotypes collected from moderate and rainfall provinces of Iran (Mazandaran and Guilan) had highest mean of the artemisinin content (9.56±8.02 mg/1 g of plant and 7.65±8.06 mg/1 g of plant, respectively) among 7 provinces. Thus, climate conditions affect on the growth and development of the Artemisia annua plants and the expression level of artemisinin in Artemisia plants. Also, bioecotype Gu5 of Artemisia annua can be an ideal choice for industrial production of this medicine because it contained the highest artemisinin level (about 30 mg/1 g of plant) among all bioecotypes studied.

Key words: *Artemisia annua*, Malaria, Artemisinin, Bioecotypes, HPLC

Abbreviations: *A: Ardabil province, EA: East Azerbaijan province, Gu: Guilan province, Go: Golestan province, M: Mazandaran province, S: Semnan province, T: Tehran province, S.D:Standard Deviation. HPLC: High Pressure Liquid Chromatography. ATC: Artemisinin Combination Therapy. DAD: Diode Array Detector.*

INTRODUCTION

In spite of all advances in medical sciences, malaria is still a global health problem causing death to about one million people annually [1]. At the end of 2004, 107 countries and colonies with approximately 3.2 billion people lived in areas where transmission of the parasite is a danger [2]. Two hundred and forty three million cases of malaria were identified in the world in 2008, causing 863,000 deaths, mostly in African children [3]. Just like other moist places in the world, this disease is a problem in southeast and south of Iran.

As artemisinin cannot be synthesized chemically in any economically feasible way, *Artemisia annua* is the only practical source of this multifunctional drug [4]. Also, artemisinin is a sesquiterpene lactone, containing an unusual endoperoxide group. However, artemisinin combination therapy (ACT) is currently the most effective means to treat and reduce the transmission rate of malaria [5-9]. Artemisinin has also been characterized in being effective against

other Parasites including *Leishmania* [10-12], *Schistosoma* [11], *Toxoplasma* [13] and *Trypanomosa* [14-15]. It can be applied in the treatment of hepatitis [16] some viruses [17-18] and a range of cancer cell lines, including breast cancer, prostate, human leukemia, colon, melanoma, ovarian, renal and small-cell lung carcinomas [19-20]. In addition, Hirt *et al* (2008) suggested that artemisinin could protect HIV positive people from Kaposi's sarcoma [21]. Artemisinin is a relatively safe drug with no obvious serious side effects [22]. In 2005, the world health Organization declared that the request level grew from two million therapeutic periods in 2003 to 30 million in 2004, 70 million in 2005 and 130 million in 2006. This development was resulted in a corresponding increase in artemisinin requests and the need for rapid and considerable increase in the amount of artemisinin in order to supply the growing demand [23]. The cost of the artemisinin plants for ACT is $4.20 for any therapeutic period of adult (4 tablets, two times per day during 3 days) [24]. Unfortunately, Artemisinin supply available in the world can't meet future requirements [25-26] and these results in an inadequate supply of artemisinin in international markets [27]. Artemisinin is extracted from a medicinal herb *Artemisia annua* L., a member of the *Asteraceae* family [28].

The relatively low yield (0.01 to 0.5%, DW) of artemisinin in native grown *A. annua* greatly limits the commercialization of this medicine [27]. In Iran, there are various species of *Artemisia* and also, different bioecotypes of *A. annua.* The goal of the current study was to explore high artemisinin yield bioecotypes from new sources and to compare artemisinin concentrations in different bioecotypes of *Artemisia annua* found in different areas of Iran.

MATERIALS AND METHODS

Extraction, purification and analysis of artemisinin from bioecotypes of *Artemisia annua* was carried out using method described by Mannan *et al.* (2010) with some modifications [29]. HPLC of samples were performed in triplicate. One hundred twenty bioecotype seeds of *Artemisia annua* were collected from a variety of sites in Iran (fig 1). In order to characterize elite *Artemisia annua* bioecotypes with high artemisinin yield, the bioecotypes were analyzed via HPLC method. Bioecotypes were given herbarium numbers and were deposited in the herbarium collection of Genetic and Biologic Resources National Center of Iran. These *Artemisia* bioecotypes were screened to measure the artemisinin concentration in their leaves. For analysis and quantification of artemisinin, five plants per *Artemisia* bioecotypes were harvested at any given location. Before HPLC, seeds of bioecotypes were cultured separately in any distinct pertri dish. *A. annua* seed was germinated in a photoperiod of 16 h and a temperature regiment of 25^{o}C/20^{o}C. After 48 h, germinated seeds were transferred to distinct pots containing special soil. Then, the pots were put in a growth chamber with enough light and humidity and temperature regiment of 25^{o}C/20^{o}C [28]. Plantlets were kept up to seedling stage. Sampling from all 120 bioecotypes was done.

Artemisinin available in the leaves of each of these plants was analyzed by HPLC. One gram each of the fresh leaf parts of every *Artemisia* bioecotype was taken and placed in an oven at 60 °C for three days. Leaves included whole leaves with short stalks inclusive and plucked from the upper surface of the seedlings, which were maximally exposed to light. Their dry weights were measured; the tissues were ground with mortar and pestle and put in 5 ml of HPLC grade toluene (Sigma) to make a homogenous mixture. These mixtures were placed in a sonicator (Elma™, Germany) for 30 min.

During sonication, artemisinin was released into toluene, which was separated from cellular debris by centrifugation at 2000×g and -8ºC for 20 minutes (Eppendorf centrifuge, model 5810R). Supernatant was decanted and saved in dram vials. Pellets were re-suspended in 5ml toluene, vortexed, sonicated again for 30 minutes, centrifuged and decanted as before. The pellets were discarded and both supernatants were pooled; extracts for each replicate were air dried before storing at -20ºC for further analysis. The dried extracts and dilutions of standard artemisinin were converted to a Q260 derivative of artemisinin as follows: each extract was solubilized in 400 µl of methanol (Sigma) and 1600 µl of 0.2% (w/v) NaOH; then hydrolyzed for 45 min at 50 ºC. The reaction was stopped by adding 1600 µl of 0.2 M acetic acid (Merck) and placing the test tube on ice. To make a final volume of 4 ml, 400 µl of methanol was added. Samples at this stage contained a Q260 derivative of artemisinin, and were filtered through a 0.45 m filter before injecting into HPLC for analysis. All samples were hydrolyzed just before use. For HPLC analysis, the mobile phase was prepared by combining 45% (v/v) methanol (Sigma) and 55% 0.01M sodium phosphate buffer (pH7.0). All samples were analyzed with a Zorbax SB C18 column (150 × 4.6 mm) 5µm; Agilent Technologies USA). Flow of the mobile phase through the column (stationary phase) was optimized to 1 ml/min. Artemisinin was detected by using a Diode Array Detector (G1315B-DAD) at 258 nm absorbance and its retention time was 12 min. Artemisinin concentration was calculated as a percentage of dry weight. As 120 bioecotypes of A*rtemisia annua*

available in Iran were diverse in artemisinin content in different ecosystems, it was necessary to consider the significance of differences between bioecotypes. On this basis, statistical analysis was done by SPSS software version 16.0. In this analysis, Mean, Mean of square and standard deviations of the artemisinin amount in bioecotypes of different ecosystems was calculated.

RESULTAS AND DISCUSSION

Studies of other researchers in the world indicated significant differences in artemisinin content of different *Artemisia* species [28, 30]. Heidarzadeh *et al.* (2012) showed that species of *Artemisia vulgaris* had the highest content of artemisinin, followed by *Artemisia dracunculus* and *Artemisia absinthium*, while *Artemisia biennis* artemisinin showed the lowest content [30]. Also, our results showed that there are considerable differences, even between bioecotypes of *Artemisia annua*. However, artemisinin found in this species was very low [31]. A*rtemisia annua* samples from China were differed in artemisinin content and were reported to contain only 0.01% to 0.50% artemisinin dry weight [32] whereas the samples obtained from other European origins indicated artemisinin content in range from 0.03% to 0.22% dry weight [33].

The Artemisinin amount in studied bioecotypes of this research, was very variable and in a range between 0-30 mg (Table1 and Figure2). *Artemisia* plants that produce more than 0.4% artemisinin were identified as plants with high artemisinin yield [34]. Over half of the bioecotypes had more than 0.4% artemisinin in their leaves. Among the 120 bioecotypes collected, 12 bioecotypes (Gu3, Gu5, Gu7, Gu19, M26, M29, M35, M36, M41, M43, M51 and M53) had much higher artemisinin pharmaceutical level than other bioecotypes. They had over 20 mg artemisinin/1 g of plant material. Conversely, 5 bioecotypes (S4, M9, Gu17, M33 and M47) did not have any artemisinin in their leaves. The variation in the content of artemisinin might be having various reasons. In addition, the use of diverse methods for extraction and analysis depends on so much to the variation among different samples, the time of collection and the preparation of samples [35]. Also, environmental factors, such as light, temperature and availability of nutrients (salt, water and etc), rainfall, and types of soil, harvesting time, different seasons, altitude and geographic locations were reported to alter the artemisinin yield [31, 36-39]. However, previous observation indicates that the growth of the *Artemisia annua* plants and the variations in artemisinin content were attributed more strongly to environmental factors than to genetic variations [40]. As shown, bioecotype Gu5 had the highest artemisinin level (about 30 mg artemisinin/1 g of plant) (3%) in all of bioecotypes and this was found in Guilan province. The highest standard deviation of the Mean of the artemisinin content in bioecotypes of different ecosystems was found in the province of Mazandaran. Separately, the highest of mean of square and standard deviation within an ecosystem was found in the province of Guilan. Also, in the Mazandaran, Golestan, Guilan, Ardabil, East Azerbaijan and Ardabil provinces, there were 27, 12, 11, 4 and 2 bioecotypes with 4-20 mg artemisinin/1 g of plant (0.4-2%), respectively (Table 2). From the above 12 bioecotypes, there were 8 bioecotypes in the province of Mazandaran and 4 bioecotypes in the province of Guilan. Our results indicated significant differences between Mazandaran and Tehran, Semnan provinces in artemisinin content of bioecotypes. Also, statistical analysis revealed no significant differences in artemisinin content of bioecotypes available in Mazandaran, Guilan, Golestan, East Azerbaijan and Ardabil provinces ($p< 0.05$). Highest and lowest mean of the artemisinin content were observed in Mazandaran and Tehran provinces, respectively (Table 3). Also, bioecotypes collected from moderate and rainfall provinces of Iran (Mazandaran and Guilan) had highest mean of the artemisinin content (9.56±8.02 mg/1 g of plant and 7.65±8.06 mg/1 g of plant, respectively) among 7 provinces (Table 2, 3). In province studies, statistical analysis indicated no significant differences ($p<0.05$) between bioecotypes A2 and A3 in Ardabil province. However, there were significant differences between bioecotypes A1, A4 and A5. Analysis of variance represented significant differences between all bioecotypes in East Azerbaijan. In the Guilan province, lowest mean of the artemisinin content was observed in bioecotype Gu17. Also, bioecotypes Gu6 and Gu8 did not differ significantly ($p<0.05$) with bioecotype Gu16. Statistical analysis revealed significant differences between bioecotypes Gu2, Gu3, Gu4, Gu5, Gu6, Gu7, Gu8, Gu9, Gu10, Gu11, Gu12, Gu14, Gu15, Gu16, Gu17, Gu18, Gu19, Gu20, Gu21, Gu22, Gu23, Gu24, Gu25, Gu26, Gu27, Gu28 and Gu29 with bioecotype Gu1 (2.96±0.06 mg/1 g of plant). Bioecotype Gu1 had no significant difference only with bioecotype Gu2. In the Golestan province, bioecotypes Go9, Go14, Go18 and Go21 had significantly lower artemisinin content than the bioecotypes Go5, Go6 and Go7. There were 6.85±0.3 mg/1 g of plant and 6.75±0.25 mg artemisinin/1 g of plant in bioecotypes Go2 and Go10, respectively. In the Mazandaran province, highest mean of the artemisinin content was observed in bioecotypes M35 and M44. Statistical analysis indicated no significant differences between bioecotypes M48, M47, M46, M38, M36, M33, M32, M25, M23, M20, M17, M12, M11, M9, M8, M5 and M3. In the Semnan province, highest and lowest mean of the artemisinin content were observed in bioecotypes S5 and S4, respectively. In this

province, statistical analysis revealed that there is no significant difference ($p< 0.05$) between bioecotypes S3 and S5 and also, S1 and S2 (Table 2). Overall, Mazandaran Province is a favorable environment for the cultivation of this medicine plant. Among the 53 bioecotypes available in this province, 35 bioecotypes had more than 0.4% artemisinin and were high artemisinin (Table 2). Also, bioecotype Gu5 of *Artemisia annua* had the highest artemisinin level (about 30 mg artemisinin/1 g of plant) (3%) of all the bioecotypes. On obtained results, it can be found that weather and climate have considerable effect on the expression level of artemisinin in *Artemisia* plants. These provinces have moderate weather with high rainfall. Klayman (1989) demonstrated that extra humidity of the tropics was not suitable for the cultivation of *Artemisia annua* [32].

Table1- Artemisinin amount in *Artemisia annua* bioecotypes available in different sites of Iran

Bioecotype No.	Sampling site	Mean of artemisinin amount in 1g of plant (mg)±S.D	Bioecotype No.	Sampling site	Mean of artemisinin amount in 1g of plant (mg)±S.D	Bioecotype No.	Sampling site	Mean of artemisinin amount in 1g of plant (mg)±S.D
A**1	Ardebil- first of osalom road toward Khalkhal	14.1±0.21	Go4	Golestan- Gorgan-Aliabad road, near Fazelabad	8.69±0.01	M21	Mazandaran: Chamestan, Kiala	10.50±0.32
A2	Ardebil- 5km after Esalm to Khalkhal	2.21±0.01	Go5	Golestan- Namteloo to Azadshahr, near to Emamiye vilage	15.34±0.19	M22	Mazandaran: Chamestan toward Amol, Abdollahdeh	6.16±0.16
A3	Ardebil- 20km after Heyran gardane, toward Astara	4.51±0.09	Go6	Golestan- 8 Km. before Azadshar from Aliabad	13.85±0.15	M23	.: Mazandaran- Chamestan toward Amol, 10km to Amol	3.52±0.02
A4	Ardebil- 10 km to Astara of Ardebil.	13.37±0.11	Go7	Golestan- 22 km from Azadshahr towards Shahrood	14.77±0.13	M24	Mazandaran: Amol toward Babolsar, 25km to Babol	2.093±0.004
A5	Ardebil- entrance of Germi from Ardebil	5.54±0.08	Go8	Golestan- 5 km. before Gonbad from Azadshahr	9.51±1.2	M25	Mazandaran: 15 km before Amol from Babol	0.92±0.07
EA**1	East Azerbaijan- 22 km after Oskanlu towards Khodaafarn	10.23±0.25	Go9	Golestan- after Gonbad towards Kalaleh Opposite of Asbdivan square	0.61±0.02	M26	Mazandaran- Babol, Babolsar, exit of Babol	**21.31±0.3**
EA2	East Azerbaijan- Oskolo toward Aynalo, Kolasofla village	0.21±0.04	Go10	Golestan- 25 Km before Kolaleh from Gonbad	6.75±0.25	M27	Mazandaran- Babolsar, Behmiz	1.89±0.17
EA3	East Azerbaijan- Khoda-Afarin (Asheghlou)	11.21±0.35	Go11	Golestan- 10 Km before Kalaleh from Gonbad, before Kalaleh-Mashhad junction	9.01±0.09	M28	Mazandaran- Behmiz toward Sari, Kiakola	15.92±0.6
Gu**1	Guilan- 35km to Deilaman.	2.96±0.06	Go12	Golestan- Kalaleh, before Sofian	2.93±0.07	M29	Mazandaran: Ghaemshahr toward Sari, first of Pashakola	**24.27±0.2**
Gu2	Guilan- Siahkol toward Lahijan, 5km to Lahijan	3.98±0.14	Go13	Golestan- Kalaleh, before Haji-Beig village	2.28±0.18	M30	Mazandaran- Sari toward Kiasar, 68km to Kiasar	17.64±0.31
Gu3	Guilan- midlle of Lahijan & Amlash, 10km to Amlash	**22.81*±0.09**	Go14	Golestan- first of Goli-dagh and Moraveh-Tapeh junction	0.79±0.13	M31	Mazandaran- Sari toward Kiasar, 50km to Kiasar	14.67±0.6
Gu4	Guilan- Otaghvar	11.09±0.09	Go15	Golestan- after Goledagh 2way & Maravetape, 20km after Gholaf village	7.32±0.06	M32	Mazandaran- Sari towards Kiasar	0.45±0.03
Gu5	Guilan- Khorma village	**30.06±0.05**	Go16	Golestan- Kalaleh towards Maraveh-tapeh, After Chatal	5.59±0.24	M33	Mazandaran- Kiasar toward Sari, exit of Sari	0
Gu6	Guilan- Siahrostagh, middle of Roodsar & Rahimabad.	0.48±0.07	Go17	Golestan- Kalaleh towards Maravehtapeh, 20 km	4.69±0.14	M34	Mazandaran- Takam towards Farim	**21.41±0.24**
Gu7	Guilan- middle of Rahimabad & Garmabad dasht, 10km to Garmabad dasht.	**21.15±0.15**	Go18	Golestan- 3km after hesarche toward Raz	0.91±0.04	M35	Mazandaran- Sari towards Farim	**27.52±0.3**
Gu8	Guilan- Roodsar, before Sarom, Bargadasht village.	0.54±0.04	Go19	Golestan- 2 Km after ashkhane towards Kalaleh	2.78±0.09	M36	Mazandaran- Sari toward Farahabad	1.16±0.08
Gu9	Guilan- after Kolachay toward Chaboksar.	13.67±0.02	Go20	Golestan- Ashkhane towards Kalaleh, middle TangehGol and Tangehrah, near Madarsoo River	0.94±0.06	M37	Mazandaran- Formabad, Soote village	12.98±0.02

Gu10	Guilan- Loshan toward jirand, Loshan bala	3.91±0.01	Go21	Golestan- Kalaleh junction towards Tangeh-Rah, before Darabad	0.90±0.08	M38	Mazandaran- Neka, near the Niroogah	4.65±0.25
Gu11	Guilan- in jirand, rand of line of water.	0.84±0.06	Go22	Golestan- Galikesh road toward Tangrah, near Tangrah	3.49±0.01	M39	Mazandarn- Neka, Zaghmarz	11.00±0.65
Gu12	Guilan- after Roodbar toward Rostamabad	7.33±0.33	Go23	Golestan- Galikesh road toward Tangrah, first of Tangrah	1.52±0.31	M40	Mazandaran- Neka toward Behshahr, exit of Neka	9.03±0.06
Gu13	Guilan- after jibabad	3.47±0.07	M**1	Mazandaran- Orym towards Pole-sefid, between Orim and Sorkhab	14.90±0.4	M41	Mazandaran- Behshahr toward Galoogah, 7km to Galoogah	**20.33±0.3**
Gu14	Guilan- Rasht toward Qazvin, 10km after Rasht.	7.59±0.08	M2	Mazandaran- 5 km after Pole-sefid, before Zirab	15.84±0.14	M42	Mazandaran- Galoogah toward Bandar gaz, 5km to Bandar gaz	11.11±0.1
Gu15	Guilan- after Aghaseyed sharif 3 way	7.18±0.09	M3	Mazandaran- the first road of Amol Towards Tehran	0.75±0.13	M43	Mazandaran- Galoogah toward Dibaj, 7km to Galoogah	16.38±0.02
Gu16	Guilan- 20km to Masoole of Fooman	0.64±0.09	M4	Mazandaran- Ramsar, 7km to Javaherdeh.	15.76±0.27	M44	Mazandarn- Galoogah toward Dibaj, 20km after Galoogah	**27.65±0.5**
Gu17	Guilan- 10km to Masoole of Fooman	0	M5	Mazandaran- Ramsar, 5km to Javaherdeh.	4.97±0.05	M45	Mazandaran- Ghaemshahr toward Firoozkoh, 25km after Ghaemshahr	12.52±0.18
Gu18	Guilan: entrance of Masuleh village from Fooman	9.32±0.21	M6	Mazandaran- Ramsar, Katalm toward Galesh mahale.	6.20±0.2	M46	Mazandaran- Ghaemshahr toward Firoozkoh, 5km before Zirab	0.56±0.05
Gu19	Guilan- in Some'esara	7.07±0.17	M7	Mazandaran- Ramsar, Katalm toward Jannatrodbar.	9.24±0.22	M47	Mazandaran- Ghaemshahr towards Firoozkooh 5 Km after Zirab	0
Gu20	Guilan- Some'esara toward Talesh, Ziabar, Nopashan	**25.09±0.07**	M8	Mazandaran- Ramsar, katalm toward Jannatrodbar.	3.40±0.3	M48	Mazandaran- 30km before Alasht from Pole-sefid	0.8±0.37
Gu21	Guilan- some'esara, rezvanshahr, changarian village	7.35±0.34	M9	Mazandaran- Rramsar, 5km to Jannatrodbar	0	M49	Mazandaran- Alasht	1.37±0.21
Gu22	Guilan- middle of Rezvanshahr & Talesh	10.31±0.25	M10	Mazandaran- Ramsar toward Tonekabon, Shirood	10.99±0.01	M50	Mazandaran- Polsefid toward Alasht, 10km to Alasht	**24.03±0.24**
Gu23	Guilan- Talesh towards Astara, Lisar	0.26±0.01	M11	Mazandaran: Tonekabon, Formabad toward Dohezar	0.65±0.04	M51	Mazandaran- Ghaemshahrtoward babol, before Dorikonde	7.46±0.37
Gu24	Guilan- Talesh toward Astara, Chober	2.08±0.06	M12	Mazandaran- Tonekabon, Dohezar village	0.80±0.07	M52	Mazandaran- Aamol toward Mahmoodabad, before Kohlarkaj	**22±0.62**
Gu25	Guilan- shopping center toward Astara beach	1.89±0.09	M13	Mazandaran- Tonekabon	12.16±0.1	M53	Mazandaran- befor Balade, Taker village	15.51±0.06
Gu26	Guilan- hadigh, keshabil, in village	8.27±0.09	M14	Mazandaran- Abasabad, Siahmahale	6.78±0.13	S**1	Semnan- Azadshahr road toward shahrood, 42 km to khosh yilagh	1.40±0.6
Gu27	Guilan- Hadigh, village	10.5±0.54	M15	Mazandaran- Kelardasht toward Marzanabad	8.05±0.2	S2	Semnan- 115 Km after Azad-Shar towards	1.72±0.1

							Shahrod	
Gu28	Guilan- Anzali toward Rasht, befor Khamam, Zirdeh.	0.89±0.15	M16	Mazandaran- Marzanabad toward Gachsar, 5-10km to Gachsar	12.20±0.1	S3	Semnan- after Minoodasht toward Azadshahr	5.23±0.11
Gu29	Guilan- Sangar	0.92±0.08	M17	Mazandarn- Noshahr toward Noor, exit of Noshahr	4.32±0.07	S4	Semnan- before Azadshahr, Minoodasht road to Azadshahr	0
Go**1	Golestan- 37 Km from Gorgan towards Bandare-gaz	10.21±0.21	M18	Mazandaran- Noshahr toward noor, Malekar	6.55±0.4	S5	Semnan- after Varsak, Khatirkoh toward Mazandaran	5.26±0.06
Go2	Golestan- after jalin toward Aliabad	6.85±0.3	M19	Mazandaran: middle of Sisangan & Noor, Sibon towny, Vazivar	2.87±0.09	T**1	Tehran: Qazvin- Rasht road, Gonbad	0.60±0.07
Go3	Golestan- Gorgan towards Aliabad, before Ganareh, opposite of gas station	1.58±0.06	M20	Mazandaran: Chamestan toward Raeeskola	3.56±0.15	T2	Tehran: Qazvin- Rasht road, after police way, first of Fooman	0.35±0.05

**Bold numbers indicate bioecotypes with over 2% artemisinin. ** A, EA, Gu, Go, M, S and T are abbreviation to Ardabil, East Azerbaijan, Guilan, Golestan, Mazandaran, Semnan and Tehran provinces, respectively. S.D is abbreviation of Standard Deviation.*

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Table2- Mean and standard deviation of artemisinin amount in *Artemisia annua* bioecotypes of different provinces of Iran

province name	Bioecotype No.	Mean of artemisinin amount in 1 g plant (mg)	Number of bioecotypes in Ecosystem	Mean of population	Mean of square	Standard Deviation inter province	Standard Deviation between provinces
Ardabil	A*1	14.10^{a}	5	7.95	30.22	5.03	52.01
	A2	2.21^{d}					
	A3	4.51^{d}					
	A4	13.37^{b}					
	A5	5.54^{c}					
East Azerbaijan	EA*1	10.23^{b}	3	7.22	36.93	5.27	51.87
	EA2	0.21^{c}					
	EA3	11.21^{a}					
Guilan	Gu*1	2.96^{n}	29	7.64	64.95	7.97	58.46
	Gu2	3.98^{l}					
	Gu3	22.81^{c}					
	Gu4	11.09^{f}					
	Gu5	30.06^{a}					
	Gu6	0.48^{qr}					
	Gu7	21.15^{d}					
	Gu8	0.54^{qr}					
	Gu9	13.67^{e}					
	Gu10	3.91^{m}					
	Gu11	0.84^{p}					
	Gu12	7.33^{jk}					
	Gu13	3.47^{n}					
	Gu14	7.59^{j}					
	Gu15	7.18^{l}					
	Gu16	0.64^{pq}					
	Gu17	0.00^{s}					
	Gu18	9.32^{h}					
	Gu19	7.07^{l}					
	Gu20	25.09^{b}					
	Gu21	7.35^{jk}					
	Gu22	10.31^{g}					
	Gu23	0.26^{rs}					
	Gu24	2.08^{o}					
	Gu25	1.89^{o}					
	Gu26	8.27^{i}					
	Gu27	10.50^{g}					
	Gu28	0.89^{p}					
	Gu29	0.92^{p}					
Golestan	Go*1	10.21^{d}	23	5.72	22.20	4.64	32.61
	Go2	6.85^{gh}					
	Go3	1.58^{n}					
	Go4	8.69^{f}					
	Go5	15.34^{a}					
	Go6	13.85^{c}					
	Go7	14.77^{b}					
	Go8	9.51^{e}					
	Go9	0.61^{o}					
	Go10	6.75^{h}					
	Go11	9.01^{f}					
	Go12	2.93^{l}					
	Go13	2.39^{m}					
	Go14	0.79^{o}					
	Go15	7.32^{g}					
	Go16	5.72^{i}					
	Go17	4.69^{j}					
	Go18	0.91^{o}					
	Go19	2.78^{lm}					
	Go20	0.94^{o}					
	Go21	0.88^{o}					
	Go22	3.49^{k}					
	Go23	1.52^{n}					
	M*1	14.90^{defgh}	53	9.56	64.38	7.97	91.47

	M2	15.84^{defgh}					
	M3	0.75^{o}					
	M4	15.76^{defgh}					
	M5	4.97^{lmno}					
	M6	6.20^{klmn}					
	M7	9.24^{jkl}					
	M8	3.40^{mno}					
	M9	0.00^{o}					
	M10	10.99^{hijk}					
	M11	0.65^{o}					
	M12	0.80^{o}					
	M13	12.16^{ghij}					
	M14	6.81^{klmn}					
	M15	8.05^{jklm}					
	M16	12.20^{ghij}					
Mazandaran	M17	4.32^{lmno}					
	M18	6.55^{klmn}					
	M19	2.87^{no}					
	M20	3.56^{mno}					
	M21	10.50^{ijk}					
	M22	6.16^{klmn}					
	M23	3.52^{mno}					
	M24	2.09^{no}					
	M25	0.92^{o}					
	M26	21.31^{bc}					
	M27	1.89^{no}					
	M28	15.92^{defgh}					
	M29	24.27^{ab}					
	M30	17.64^{cdef}					
	M31	14.67^{fghi}					
	M32	0.45^{o}					
	M33	0.00^{o}					
	M34	21.55^{bc}					
	M35	27.52^{a}					
	M36	1.14^{o}					
	M37	12.98^{ghij}					
	M38	4.65^{lmno}					
	M39	11.00^{hijk}					
	M40	9.03^{jkl}					
	M41	20.33^{bcd}					
	M42	11.11^{hijk}					
	M43	16.38^{defg}					
	M44	27.62^{a}					
	M45	12.52^{ghij}					
	M46	0.56^{o}					
	M47	0.00^{o}					
	M48	0.62^{o}					
	M49	1.37^{jkl}					
	M50	24.03^{cde}					
	M51	7.46^{ghij}					
	M52	22^{bc}					
	M53	15.51^{efghi}					
	S*1	1.40^{b}					
	S2	1.72^{b}					
Semnan	S3	5.23^{a}	5	2.72	5.71	2.23	7.39
	S4	0.00^{c}					
	S5	5.26^{a}					
	T*1	0.60					
Tehran	T2	0.35	2	0.475	0.03	0.1473	0.22

**A, EA, Gu, Go, M, S and T are abbreviation to Ardabil, East Azerbaijan, Guilan, Golestan, Mazandaran, Semnan and Tehran provinces, respectively. Different letters in the column (Mean of artemisinin amount in 1 g plant (mg)) represent to be significant (p> 0.05) differences between means.*

Table 3- Artemisinin content comparison of Iranian *Artemisia annua* bioecotypes

Province name	Mean of artemisinin amount in 1g of plant (mg)±S.D	Number of bioecotypes in any province
Ardabil	7.95±5.03^{ab}	5
East Azerbaijan	7.22±5.27^{ab}	3
Guilan	7.64±7.97^{ab}	29
Golestan	5.72±4.64^{ab}	23
Mazandaran	9.57±7.98^{a}	53
Semnan	2.72±2.23^{bc}	5
Tehran	0.48±0.15^{c}	2

Different letters in the column (Mean of artemisinin amount in 1 g plant (mg)) represent to be significant (p> 0.05) differences between means. S.D is abbreviation of Standard Deviation.

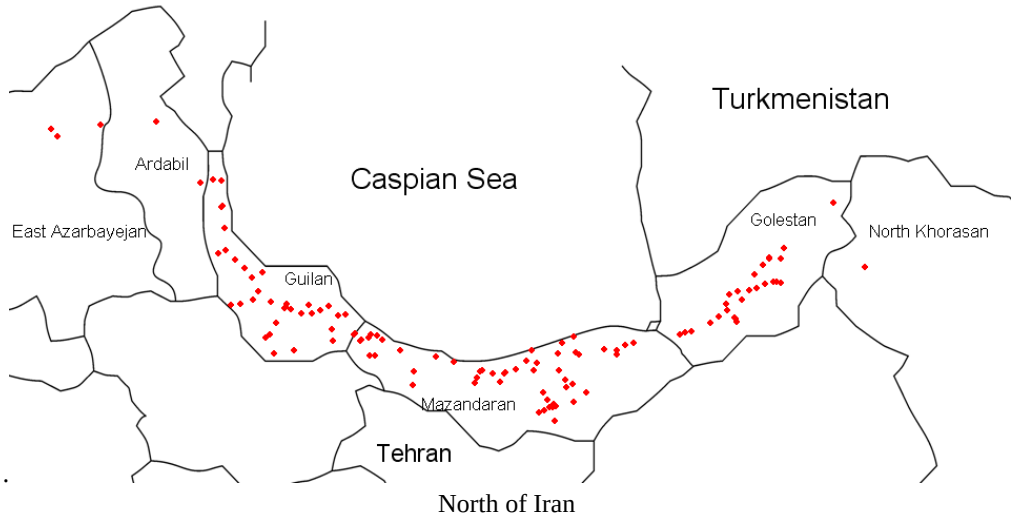

Figure1- Geographical distribution map of *Artemisia annua* bioecotypes available in Iran. Red dots represent sampling sites of *Artemisia annua* bioecotypes in northern provinces of Iran

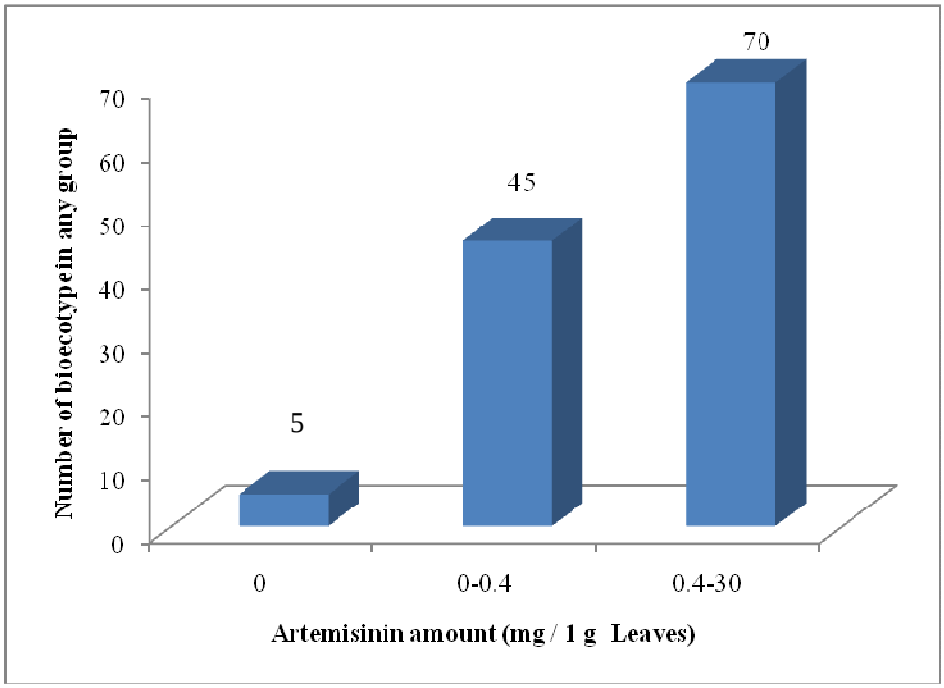

Figure2. Classification of *Artemisia annua* bioecotypes on artemisinin amounts. Bioecotypes with artemisinin amount (0.4-30, 0-0.4 and 0 mg/1g leaves) indicate High artemisinin bioecotypes, low artemisinin bioecotypes and bioecotypes without artemisinin, respectively. 70, 45 and 5 represent abundance of bioecotypes in any group

CONCLUSION

Statistical analysis indicated significant differences in mean of artemisinin content of bioecotypes available in Mazandaran and two provinces Tehran and Semnan ($p< 0.05$). HPLC report represented the bioecotype (Gu5) with highest artemisinin produciblity about 30 mg / 1 g of plant medicine among all of studied bioecotypes. In other words, this bioecotype produces 3% artemisinin in 1 g of plant leaves. Moreover, 12 bioecotypes produce over 2% artemisinin (20 mg artemisinin /1 g of plant leaves). Our results indicated that climate conditions considerable effect on the growth and development of the *Artemisia annua* plants and the expression level of artemisinin in *Artemisia* plants. On obtained results, the bioecotype Gu5 of *Artemisia annua* can be an ideal choice for the industrial production of this Medicine.

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