Pergularia tomentosa, from Traditional Uses to Ecology and Phytochemistry

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Abstract

Background: ***Pergularia tomentosa*** **L. has several traditional uses and biological activities which need to be more investigated.**

Objective: In this study, some ecological and phytochemical properties of *Pergularia tomentosa* were assessed. The flavonoids and cardenolides in the aqueous extract of leaves were identified with LCMS techniques.

Methods: Some quantity characteristics of *P. tomentosa* were measured. Extraction was performed according to the traditional method (aqueous extract at room temperature). Metabolite profiling was conducted using untargeted liquid chromatography coupled to high resolution mass spectrometry (LCeESI-OrbitrapeMS). Data were analyzed by Xcalibur version 2.1.

Results: Results showed that length of roots, leaves and fruits and the height of aerial parts were 23-30 cm, 2.5-3.2 cm, 4.9- 5.4 cm and 40-58.00 cm, respectively. Physiography and canopy cover were two important variables on these properties. We also identified 19 natural products in aqueous extract, 15 phenolics and flavonoids and 4 cardenolides. Three flavonoids (8, 11 and 14) and one cardenolide (17) had been previously isolated in *P. tomentosa* and 3 cardenolides (5, 18 and 19) has been reported for the first time in this plant.

Conclusion: Results indicated that the leaves of *P. tomentosa* are a rich source of flavonoids and cardenolides. These findings suggest that leaves of this plant can be a good source of natural antioxidant and antitumor compounds. However, this plant needs special conservational efforts. Therefore, planting this plant under the canopy or along the borders of waterways can help in an appropriate habitat for its conservation and reclamation.

Keywords: ***P. tomentosa,*** **Cardenolide, Flavonoid, LC-ESI-Orbitrap-MS/MS, Quantity characteristics**

Introduction

Pergularia tomentosa L. is an evergreen perennial shrub belonging to the Apocynaceae family (subfamily: Asclepiadeae) and has a peculiar odor [1]. The height of this shrubby plant species is less than one meter, but in suitable condition reaches to higher sizes. Etymologically, tomentosa means as hairy, because the plant covered by a lot of small hairs which causes a greenish color [2]. This plant is widely distributed in horn of Africa (north Sudan, Egypt, Ethiopia, Algeria, Jordan, Niger and Kenya) and Middle East (Iran, Oman, Pakistan, Afghanistan, Saudi Arabia) [3, 4].

This plant is used in folk remedies as an antirheumatic, laxative, abortive and treatment of some skin diseases, asthma and bronchitis [5]. This plant also used for treatment of chills, helminthiases, allergies and constipation. In some cases, it has been reported that *P. tomentosa* has been used for the wool remover from the skin of some animals especially sheep [6, 7]. According to the previous reports, this plant has different biological effects (such as cytotoxic, antioxidant and antibacterial) and these activities are due to its cardenolide and flavonoid compounds [8, 9, 10, 5, 3, 11]. The presence of these valuable phytochimcals can be investigated more by the very high analytical methods like LC-ESI-Orbitrap-MS/MS. It is a promising method for identifying the known compounds and compared the data with the literature.

On the other side, some studies indicated that its conservation conditions are in worrying situation [12]. This report was according to the field visits from some countries (Ethiopia, Kenya, Malawi, and Mozambique) and herbarium at the British Museum and at the Royal Botanic Gardens at Kew [12]. Also our field visits in Iran (southeast of Kerman) confirmed this report, because natives uproot the plant for its traditional uses.

Therefore, *P. tomentosa* is an endangered plant now and has not a suitable ecological situation and needs to be in a better conservative situation. So, this study was carried out to investigate quantities characteristics of *P. tomentosa* according to the environmental variables and also identified chemical compounds, including flavonoids and cardenolides from its aqueous extract.

Materials and Methods

Study area and studied plant

This study was performed in southeastern Iran (rangelands of Kahnouj, 57º 42’ to 57º 46’ E; 28º 02’ to 28º 06’ N). *Pergularia tomentosa* belongs to sub-family Asclepiadaceae in Apocynaceae. This plant usually has many leaves and its branches have pale green-white stems that are ascending. Also the leaves are very short. Its flowers are small and occur in few axillary umbellate cymes [1]. *P. tomentosa* is native to the horn of Africa and Middle East [1]. In Iran, habitat of this plant is in the South of Kerman, Hormozgan, Sistan and Balochestan, South of Fars and Khozestan (Fig. 1).

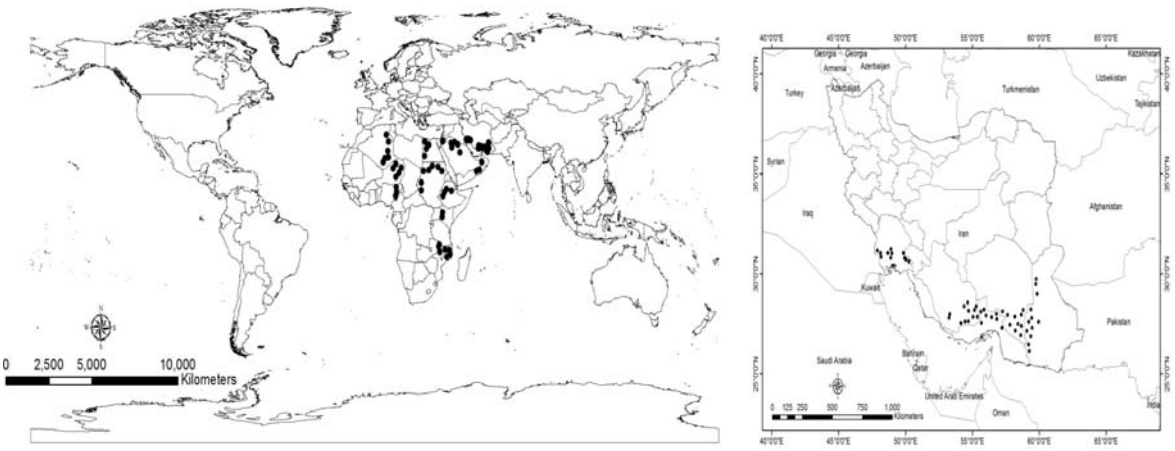

Fig. 1- distribution of *P. tomentosa* in Iran (right map) and all parts of the world (left map)

Measuring the quantity characteristics

The quantity properties of *P. tomentosa* (length of aerial parts, length of roots, length of leaves and length of fruits) measured by ruler and the number of seeds counted for each fruit. The density also measured by belt transects, 300 m long and 10 m wide. We measured these characteristics based on the physiography (in waterways, ridges and slopes of hill areas) and altitudinal gradient (550-700, 700-850, and 850-100 m).

Sampling

Leaves of *P. tomentosa* were collected in April 2016 in Kahnouj rangelands (Chahgodari), Southeast of Iran. Samples were collected from the waterways and slope in hill areas and mixed together.

Preparation the samples

After collecting the samples, Leaves were extracted at once for safekeeping the secondary metabolites. The extraction did according to the traditional method of natives. After preparation, the dried powder extracted with water in room temperature. We prepared aqueous extract based on the native method, but with just distilled water. So, 5g of dried powder were extracted with 100 ml distilled water for 25 minutes. Then, the extracts dried by using Lyophilization. For choosing the best solvent system (gradient of solvents (A and B)), we setup the method according to the TLC and analytical HPLC/UV. The concentration of the extract for the LC-MS analysis was 1 mg/2 ML [13].

LC-ESI-Orbitrap-MS analysis

To analyze the markers in Leaves of *P. tomentosa*, one method using LC-ESIOrbitrap-MS was carried out. Assessments were done by a Thermo Scientific liquid chromatography system that involved an Accela auto sampler and a quaternary Accela 600 pump, linked to a linear Trap- Orbitrap hybrid mass spectrometer banding linear trap quadrupole (LTQ) and Orbitrap mass analyser via electrospray ionization. Separation did with the XSelect CSH C18 reversed phase column. The mobile phase included by solvent

A (water with 1 mL/L of formic acid) and solvent B (acetonitrile/water (8:2 mL/ mL) with 1 mL/L of formic acid).

LC-ESI-Orbitrap-MS analyses were carried out by a Flexar UHPLC AS system (Perkin-Elmer, USA) including Flexar FX-10 pump, auto sampler, degasser, and PE 200 column oven. Finally 5 µL of the prepared extract was injected into an XSelect HSS T3 column (100 × 2.1 mm, 2.5 µm) (Waters, Milford, Massachusetts). The mass spectrometer was done in negative ion mode. In order to fragmentation investigation, one data dependent scan was applied, choosing precursor ions corresponding to most intensive peaks in LC-MS analysis [13]. Finally all the compounds were recognized based on the retention time and corresponding spectral characteristic fragmentation, using Scifinder (literature review) by evaluation with reported compounds. Acquainting and analyzing the data was done by using Xcalibur version 2.1.

Results

Associated plant species

According to the field visits in rangelands in Southeast of Kerman (Kahnouj), *P. tomentosa* usually grows under or between canopy of *Ziziphus spina-christi, Scorzonera paradoxa, Petropyrum aucheri and Pycnocycla spinosa.*

Phenology:

P. tomentosa is a perennial shrub plant. According to the results of this study, its vegetation growth begins of the mid-March and its seeding stage usually finishes in mid-June (Table 1).

Quantity characteristics:

The results showed that length of root of *P. tomentosa* was about 20-35 cm. Length of the leaves was about 2.5-3.2 cm, Length of the fruits was more than 5 cm, the height of aerial parts about 33-71 cm. The density of this medicinal plant was 0.0021 ha. Also the number of seeds in the fruits was about 18-38.

Effect of altitudinal gradient

According to results of one way ANOVA, altitudinal gradient didn't show any effect on quantity properties (length of aerial parts, length of roots, length of leaves and length of fruits) of this plant (Table 2).

Table 1- Phenological Stages of *P. tomentosa* in rangelands of Kahnouj

Phenological Stages	Start	End of session
Vegetation growth	15 March	15 April
Flowering	15 April	5 May
Seeding	5 May	30 May

Table 2- Investigation the effect of altitudinal gradient on quantity properties of *P. tomentosa*. Letter a, indicate non-significant effect of the altitudinal gradient on plant characteristics (for each column)

Altitude	Quantity properties of *P. tomentosa*					Statistics	
	aerial height	root length	length of leaves	length of fruits	density	F	Sig.
550-700	48.11 (a)	27.21 (a)	3.1 (a)	5.26 (a)	0.071 (a)		
700-850	46.07 (a)	26.44 (a)	2.9 (a)	4.99 (a)	0.069 (a)	2.06	>0.05
850-1000	45.23 (a)	25.95 (a)	2.7 (a)	5.14 (a)	0.064 (a)		

Effect of physiographic factors

Physiography was an important factor on almost of the quantity characteristics of this plant. In the other word, the borders of the waterways have the best conditions for this plant. So based on the paired sample t-test, in the ridges and slopes of the hills, length and height of this plant was smaller than the borders of the waterways ($P < 0.05$). But the density of this plant in the slopes and ridges was significantly more than borders of waterways (Fig. 2).

Effect of conapy

Results paired sample t-test exhibited that canopy cover of the associated shrubs and trees have considerable effect on the quantity characteristics of *P. tomentosa* ($P < 0.05$). In the other word, canopy cover of other plants improved the quantity properties of this plant (Fig. 3).

Phytochemical compounds:

Characterization of the Phenolic and cardenolides of *P. tomentosa* (Leaves) was carried out using mass spectrometry. We identified 19 compounds of its leaves aqueous extract. So 15 compounds were belong to the Phenolic and flavonoids and four to cardenolides structures. The results of identified compounds were shown in table 3. We also identified 3 flavonoids (Quercetin 3-*O*-galactoside (8), Kaempferol 3-*O*-glucoside (11) and Kaempferol 3-*O*-malonylhexoside (14)) and one cardenolides (17) with LCMS techniques which had been also isolated before in *P. tomentosa* [ref] and three other cardenolides (5, 18 and 19) which were not reported in this plant. Most of the identified flavonoids were belong to the flavone and flavonol [14, 15, 16, 17, 18, 10, 19, 20, 21, 5, 8] (Table 3, Fig. 4).

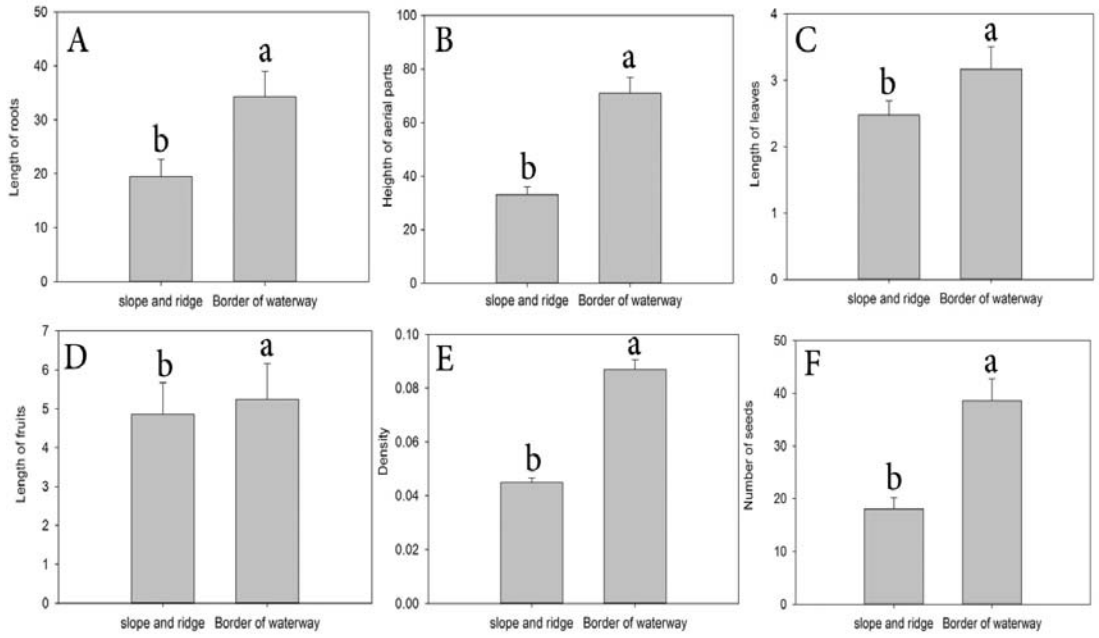

Fig. 2 (graphs A-F)- Effect of the Physiographic factors on quantity characteristics of *P. tomentosa.* From left to right, respectively, length of roots, height of aerial parts, length of leaves, length of fruits, density, and number of seeds

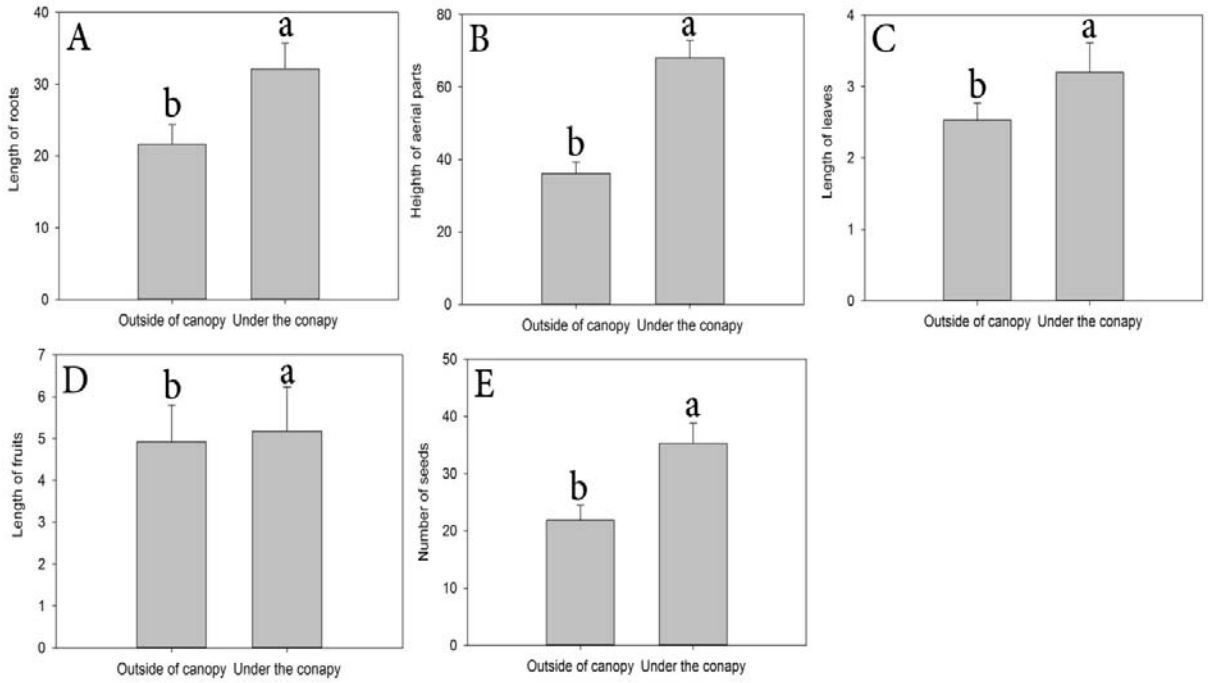

Fig. 3 (graphs A-E) - Effect of canopy cover on the quantity characteristics of *P. tomentosa.* From left to right, respectively, length of roots, height of aerial parts, length of leaves, length of fruits, and number of seeds

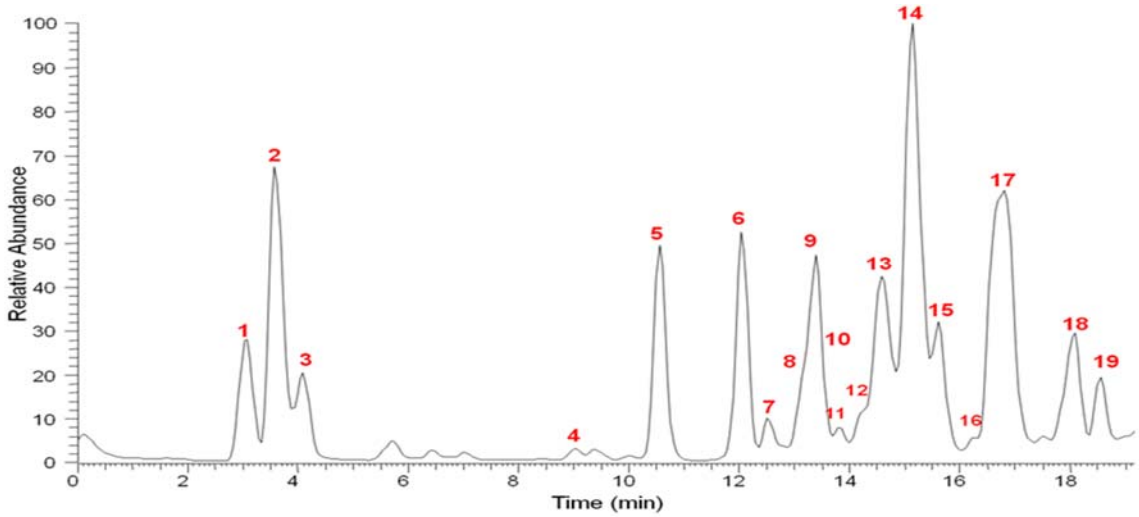

Fig. 4- Profile of LC-ESI-Orbitrap-MS Full Scan in negative ion mode of *P. tomentosa* traditional aqueous extract. The numbers are according to the identified compounds in table 3

Table 3- Identification of compounds (Phenolic and cardenolides) of leaves aqueous extract of *P. tomentosa* using HPLC-ESI-Orbitrap-MS/MS

	Identified Compounds in negative ion mode	Percentage (%)	Molecular formula	MW	[M-H]	RT (min)	MSMS	Refrences
1	Phenolic glycosid: Scrophenoside D	0.16	$C_{28}H_{34}O_{17}$	642.56	641.16956	3.17	641, 479	Li et al, 2014
2	Phenolic: Synapoyl hexoside	0.16	$C_{17}H_{22}O_{10}$	386.45	385.11212	3.58	223	Eugenia et al, 2017
3	Flavone: Luteolin-di C-lucoside	0.11	$C_{27}H_{30}O_{16}$	610.52	609.14386	4.09	591, 489, 301	Singh et al, 2015
4	Phenolic: Feruloyl Glucoside	0.17	$C_{16}H_{20}O_{9}$	356.32	355.10202	8.35	355, 193, 265, 295, 134	Friščić et al, 2016
5	Cardenolide: antiaroside G	0.24	$C_{29}H_{42}O_{12}$	582.64	581.25702	9.94	437, 185	Shi et al, 2013
6	Flavonole: Kaempferol-malonyl-dihexosid	0.11	$C_{30}H_{32}O_{19}$	696.56	695.14465	11.32	489, 285	Salced et al, 2016
7	Flavonole: Kampferol + glucose	0.17	$C_{27}H_{28}O_{17}$	624.50	623.12311	12.51	285, 447	Not reported
8	Flavonole: Quercetin 3-O-galactoside	13.74	$C_{21}H_{20}O_{12}$	464.38	463.08636	12.72	301, 300	Heneidak et al, 2006
9	Flavonole: Isorhamnetin-3-O-glucoside	3.60	$C_{22}H_{22}O_{12}$	478.40	477.10159	13.44	285, 300	Valente et al, 2016
10	Flavonole: Quercetin malonylglucoside	6.72	$C_{24}H_{22}O_{15}$	550.42	549.08643	13.70	505	Haijuan et al, 2013
11	Flavonole: Kaempferol 3-O-glucoside	1.70	$C_{21}H_{20}O_{11}$	448.38	447.09167	13.95	255, 285, 227	Heneidak et al, 2006
12	Phenolic: Ferulylmalic acid	11.05	$C_{14}H_{14}O_{8}$	310.26	309.06021	14.47	193	Hettwer, 2016
13	Phenolic: glucuronic acid	<0.01	$C_{17}H_{14}O_{10}$	378.29	377.04749	14.93	185	Song et al, 2016
14	Flavonole: Kaempferol 3-O-malonylhexoside	23.08	$C_{24}H_{22}O_{14}$	534.42	533.09137	15.60	489, 285	Heneidak et al, 2006
15	Flavonole: Kaempferol-3-O-6 -acetyl-b-Dglucopyranoside	4.62	$C_{23}H_{22}\ _{12}$	490.41	489.10165	16.27	285	Dugo et al, 2009
16	Flavonole: quercetin-3-(6″-succinoyl)-glucoside	0.10	$C_{25}H_{24}O_{15}$	564.45	563.10211	16.84	447, 301	Ojwang, 2012
17	Cardenolide: Hydroxycalactin	23.01	$C_{29}H_{40}O_{10}$	548.62	547.25317	17.81	385	Hamed et al, 2006; Piacente et al, 2009
18	Cardenolide: Antiaroside E	9.30	$C_{29}H_{42}O_{10}$	550.64	549.26831	18.35	462, 417, 387, 359, 341, 315,	Shi et al, 2010
19	Cardenolide: Antiaroside F	1.80	$C_{35}H_{52}O_{15}$	712.78	711.32043	18.89	334, 441, 577, 624, 626, 649, 667, 679, 696	Shi et al, 2010

Discussion

This study investigated the quantitative properties of *Pergularia tomentosa* according to environmental variables and its chemical compounds of aqueous extract (flavonoids and cardenolides) were identified that native people are using for different applications. According to the field visits, the vegetation growth, flowering and seeding stages of *P. tomentosa* begin, in mid-March, mid-April and early May, respectively. In addition, our results revealed that *P. tomentosa* usually grows under or between canopy of some shrubs or trees (*Ziziphus spina-christi*, *Scorzonera paradoxa*, *Pteropyrum aucheri* and *Pycnocycla spinosa*). Analytical results indicated that this association has positive effect on the quantity characteristics of this plant. Thus, these characteristics (length of aerial parts, length of roots, length of leaves, and length of fruits) under the canopy of shrubs and trees were significantly greater than those found outside canopy cover. Therefore, we can conclude that these shrubs or trees play a nurse role for *P. tomentosa*. Due to this valuable plant's low density in Iran, to conserve and develop *P. tomentosa* in the rangelands of southeastern Iran, the positive role of these protective plants should be considered. Erfanzadeh et al (2014) and Ren et al (2008) emphasized that shrubs can play an important nurse role for other plants that are under their canopy [22, 23].

In an unexpected result, we found that elevation had no considerable effect on the quantity properties of this plant, but physiography was an important variable on the quantity characteristics of *P. tomentosa*. In other words, in the slopes and ridges, almost properties (length and height) of this plant were smaller than at the borders of the waterways. The density of this plant in slopes and ridges was significantly greater than along the borders of waterways. Results of other studies [24, 25] revealed that physiographic variables are among the most important environmental factors in rangelands plants' distribution. We can conclude that due to the few suitable areas along borders of waterways, there is insufficient space for higher density. Instead, there is only a thin line in the border of waterways and despite its suitability, space is restricted.

The results of metabolite profiling showed that there are 19 compounds (phenolics and cardenolides) in the extract. Fifteen compounds belong to the phenolics and flavonoids, and 4 cardenolides were identified from leaves' aqueous extract of *P. tomentosa*. Given that *P. tomentosa* is in Asclepiadaceae, we expected that it would have contained many cardenolides, but 79% of the identified compounds are phenolic and 21% belong to the cardenolides. Other reports which investigated the aerial parts of this plant (Heneidak et al, 2006) demonstrated that aerial parts of *P. tomentosa* are a rich source of flavonoids. Almost all studies showed that the roots of *P. tomentosa* are cardenolide-bearing part [5, 8]. We identified 4 cardenolides in leaves of this plant. These results suggested that leaves of *P. tomentosa* are a rich source of flavonoids and could be suitable source for valuable cardenolides. For example, one of the cardenolides that have identified, Ghalakinoside, has potent effect on cancer cells [7, 8]. The other three identified

cardenolides (Antiaroside E-G), from results of Shi et al (2010), exhibited strong cardiotonic activity, with potent inhibitor of Na+/K+-ATPase. In addition, its phenolic compounds can play a strong antioxidant role. In this field, Yakubu et al (2015) and Al Jabri (2013) confirmed the antioxidant effect of the extract of this plant [9, 2].

Conclusion

These results indicated that metabolite profiling can provide an appropriate view about the natural products containing of the plants. Our results revealed that leaves of *P. tomentosa* are flavonoid and cardenolide bearing organ, but presence of flavonoids is more diverse than cardenolides. In regards to the conservation of this valuable rangeland plant, canopy of some nurse plants (shrubby plants) in borders of waterways could be appropriate habitat for its Reclamation in arid regions of Iran.

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