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ORIGINAL ARTICLE

Effect of Extraction Solvent/ technique on the Antioxidant activity of Selected 10 herb native species south of Iran

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ABSTRACT
Currently there has been an increased interest globally to identify antioxidant compounds and low or no side effects for use in preventive medicine and food industry. Iran has great possibilities in product and export of medicinal plants. The purposed of this study was to evaluate the antioxidant activity of 10 herb native species south of Iran. The plant material was extracted using water and ethanol as solvents. The total antioxidant potential of a sample for both alcoholic and aqueous extract was determined using the ferric reducing antioxidant potential (FRAP) assay as a measure of antioxidant power. The assay was based on the reducing power of a compound (antioxidant). Antioxidant activity against the erythrocytes hemolysis was examined by using hydrogen peroxide as free radicals. Moreover, phenolic and flavonoid contents were investigated. The results of present study indicated that antioxidants properties in water extract of studied herbs were more significant than in alcohol extract and through 10 herbs studied respectively Thymus vulgaris and Teucrium polium was the significant power antioxidant properties. But in alcohol extract Lavandula stoechas L and Thymus vulgaris were shown significant antioxidant properties. Moreover RBC hemolysis in Thymus vulgaris and Teucrium polium with respect to more antioxidant properties was significant than other herbs. The present study given that some herbs are rich in natural antioxidants that can neutralize free radicals activity and recommended as prevention of various diseases of these plants, along with new synthetic drugs used.
Key words: Antioxidant, Medicinal Plants, FRAP, Hemolysis Activity

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INTRODUCTION

Reactive oxygen species (ROS) are produced in the cells by cellular metabolism and other exogenous environmental agents. They are generated by a process known as redox cycling and are catalyzed by transition metals, such as Fe^{2+} and Cu^{2+} [1]. Overproduction of ROS can damage cellular biomolecules like nucleic acids, proteins, lipids, carbohydrates, proteins and enzymes, resulting in several diseases. Living systems have specific pathways to overcome the adverse affects of various damages. However, sometimes these repair mechanisms fail to keep pace with such deleterious effects [2].

Antioxidants scavenge free radicals and are associated with reduced risk of cancer and cardiovascular diseases. Plant-derived antioxidants, especially, the phenolics have gained considerable importance due to their potential health benefits. Epidemiological studies have shown that consumption of plant foods containing antioxidants is beneficial to health because it down-regulates many degenerative processes and can effectively lower the incidence of cancer and cardio-vascular diseases [3]. In the search for plants as a source of natural antioxidants, some medicinal and aromatic plants have been frequently studied for their antioxidant activity and radical scavenging in the last few decades [4]. Recently, interest has increased notably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants such as BHT, which are being restricted due to their side effects such as carcinogenicity [5]. Among the various natural antioxidants, phenolic compounds are reported to have

the character of quenching oxygen-derived free radicals by donating a hydrogen atom or an electron to the free radical [6]. Phenolic compounds such as flavonoids, phenolic acid and tannins are considered to be a major contributor to the antioxidant activity in plants. These antioxidants also possess diverse biological activities such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic effects. These activities may be related to their antioxidant activity [7].

Recovery of antioxidant compounds from plant materials is typically accomplished through different extraction techniques taking into account their chemistry and uneven distribution in the plant matrix. For example, soluble phenolics are present in higher concentrations in the outer tissues (epidermal and sub-epidermal layers) of fruits and grains than in the inner tissues (mesocarp and pulp) [8]. Solvent extraction is most frequently used technique for isolation of plant antioxidant compounds. However, the extract yields and resulting antioxidant activities of the plant materials are strongly dependent on the nature of extracting solvent, due to the presence of different antioxidant compounds of varied chemical characteristics and polarities that may or may not be soluble in a particular solvent. Polar solvents are frequently employed for the recovery of polyphenols from a plant matrix. The most suitable of these solvents are (hot or cold) aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate [9]. Methanol and ethanol have been extensively used to extract antioxidant compounds from various plants and plant-based foods (fruits, vegetables etc.) such as plum, strawberry, pomegranate, broccoli, rosemary, sage, sumac, rice bran, wheat grain and bran, mango seed kernel, citrus peel, and many other fruit peels.

The objectives of this study were to evaluate and compare total antioxidant capacity by common antioxidant activity method, presented FRAP of selected 10 herb native species south of Iran.

MATERIALS AND METHODS

All the reagents and chemicals used in the experiments were of analytical grade. The chemicals TPTZ (2,4,6-tripyridyl-***S***-triazine) were obtained from Sigma Chemical Co., USA. All other chemicals used were obtained from Merck, Germany.

The selection of the plant materials in the present study was based on their potential medicinal uses. The plant materials were dried at room temperature and milled into uniform dry powder. The air-dried ground plant material was extracted with each of the solvents- ethanol (Merck, Germany) and water.

Sample preparation

Aqueous extract

To 1 and 4 gr of powder sample 100 ml of distilled water at 90° C was added. This was allowed to stand for 10 minutes in room temperature. The mixture was centrifuge at 3000 rpm for 5 minutes. Upper phase was transferred to another screw-capped tube and stored at -20 C for biochemical investigations. The 1% and 4% aqueous extract were prepared for FRAP and erythrocyte hemolysis assay.

Ethanolic extract

For preparation of ethanolic extract from different medicinal plant, 0.1 gr of powder sample were placed in screw–capped tubes. To all sample tube an equal volume of 10 ml of 96% ethanol (Merck, Germany) was added. All tubes were kept on the rotary shaker for an hour in room temperature. The tubes were centrifuge (10 minutes, 3000 rpm, room temperature). Upper phase was transferred to another screw-capped tube and stored at -20 C for biochemical studies.

Evaluation of Total Phenolics Content

The concentrations of phenolic compounds were assessed using the Folin- Ciocalteu reagent according to the method described by Singleton and Rossi [10], with minor modifications. 100 μL of extracts (2mg/mL) were added to 0.2mL of Folin-Ciocalteu reagent. After 3min, 1mL of Na_2CO_3 (15%) and 2mL of water were added, and the mixture was kept in the dark at room temperature for 2h. The absorbance was measured at 765nm (Shimadzu UV-160 spectrophotometer). A calibration curve of gallic acid was prepared and the results were expressed as gallic acid equivalents (mg GAE/g extract) based on five points regression curve, 0.00–0.20mg/mL, R^2 = 0.9983. Results are expressed as the mean ± SD from experiments performed in triplicate.

Evaluation of Total Flavonoids Content

The flavonoids quantification was performed according to the technique presented by Zhishen et al. [11]. Briefly, 500 μL of extracts (2mg/mL), 2mL of water and 150 μL of $NaNO_2$ 5% were mixed. After 5min incubation at room temperature, in the dark, each sample was stirred with 150 μL of $AlCl_3$ 10%, 1mL of NaOH 1M and 2mL of water. After 10min incubation in the dark at room temperature the absorbance was measured at 510 nm (Shimadzu UV-160 spectrophotometer). Rutin was used as the standard for a calibration curve. The total flavonoids content was expressed in rutin equivalents (mg RE/g extract) based on four points regression curve, 0.005– 0.080mg/mL, R^2 = 0.9923. Results are expressed as the mean ± SD from experiments performed in triplicate.

Evaluation of antioxidant activity using FRAP method

The FRAP (ferric reducing antioxidant power assay) procedure described by Benzie and Strain (1996) was followed [12]. The principle of this method is the reduction of a ferric-tripyridyl triazine complex to its colored ferrous form in the presence of antioxidants. Briefly, the FRAP reagent contained 5 ml of 10 mmol/L solution of TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mmol/L HCL plus 5 ml of a 20 mmol/L solution of $FeCl_3$ and 50 ml of a 0.3 mol/Lacetate buffer solution, pH 3.6 which was prepared freshly and warmed at 37˚C. Aliquots of 50 µL extract were mixed with 1.5 ml FRAP reagent and after incubation at 37˚C for 10 min, the absorbance of the reaction mixture was measured at 593 nm.

For construction of the calibration curve, five concentrations of $FeSO_4.7H_2O$ (1, 0.70, 0.50, 0.20, 0.10 mg /mL) were used and the absorbance values were measured as for sample solutions. The antioxidant activities were expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of µmol Fe II/mg.

Antioxidant activity (Inhibition of erythrocyte haemolysis)

The scavenging activity of aqueous extract from different plant on red blood cell (RBC) membrane hemolysis was assessed, as described by Stocks and Dormandy [13]. After a 2 h incubation period of erythrocytes (5% suspension in phosphate buffer, PH 7.4 containing 4 mM sodium azide) with different aqueous extract samples in presence of H_2O_2 (0.2M) at 37 °C the optical density was recorded at 540 nm.

Statistical analysis

The values were reported as mean ± SD. One-way ANOVA and LSD post-hoc multicomparison tests were used for the analyses.

RESULTS

The total phenolics content expressed as mg GAE/g extract and the total flavonoids content expressed as mg RE/g extract are shown in Figures 1 and 2, respectively. As can be observed, the concentration of total phenolics has a strong dependence on the solvent used on extraction. As expected, the amount of total phenolics was higher in water extracts (Fig.1). *Thymus vulgari* displayed a total phenolics content 356.23±32.4 mg GAE/g (H2O extract). Comparatively, *Lawsonia inermis Linn* (aqueous extracts) had lower content of total phenolics (16.49 ±0.02 mg GAE/g). Results obtained from this study revealed that the total phenolic content were high in *Lavandula stoechas L* (305.43 ±12.06 mg GAE/g) in ethanolic extract whereas, *Myrtus* had lower content of total phenolics (58.11 ± 0.20 mg GAE/g).

Concerning the total flavonoids content, it can be observed in Figure 2. Comparing medicinal plant extracts, *Thymus vulgaris* and *Teucrium polium* had the highest content of flavonoids in the H2O (186.93 ± 25.16 and 175.02 ± 25.13 mg rutin/g) extracts, respectively and *Lawsonia inermis Linn were low* (18.13 ±0.04 mg rutin/g). Moreover, *Lavandula stoechas L* had the highest total flavonoid contents (168.15 ±12.67 mg rutin/g) in ethanolic extract.

Ferric reducing antioxidant power:

Figure 3 shows great differences in total antioxidant capacity measured by the FRAP method between the species in two different solvent, ethanol and water, respectively. The highest level of antioxidant activity in ethanolic extract was found in *Lavandula stoechas L*, while the lowest was in *Mint*. Moreover, water extract of *Thymus vulgaris* had relatively high level of antioxidant activity whereas, *Lawsonia inermis Linn* were low.

The FRAP levels of ethanolic extract herbs ranged from 70.31 to 599.65 µmol Fe II/ mg of sample, respectively. Whereas, FRAP levels of water extract ranged from 27.49 to 698.21 µmol Fe II/mg of sample. Our results show that the water extract exhibited greater antioxidant capacity than that of ethanol.

Inhibition of erythrocyte hemolysis

Antioxidant activities of medicinal plant in inhibition of erythrocyte hemolysis were shown in figure 4. Results obtained from our study shows that aqueous extract of medicinal plants has beneficial role in membrane stability. Those aqueous extract were shown high level of polyphenol and flavonoid has antioxidant activity and scavenge the free radicals which case the erythrocyte hemolysis. As results shown in fig.4 the *Thymus vulgaris* and *Teucrium polium* reveals the highest level of antioxidant activity and inhibit the erythrocyte hemolysis. Moreover, other medicinal plants had no significant effect on inhibition of erythrocyte hemolysis.

DISCUSSION

ROS has received considerable attention in the recent past, because of its role in several pathological conditions. ROS produced *in vivo* include superoxide radical O_2^-, hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl).

Polyphenolic compounds are commonly found in both edible and inedible plants, and they have been reported to have multiple biological effects, including antioxidant activity [14]. Herbs are used in many domains, including medicine, nutrition, flavoring, beverages, dyeing, repellents, fragrances, cosmetics [15]. Many species have been recognized to have medicinal properties and beneficial impact on health, e.g. antioxidant activity, digestive stimulation action, antiinflammatory, antimicrobial, hypolipidemic, antimutagenic effects and anticarcinogenic potential [16]. Crude extracts of herbs and spices, and other plant materials rich in phenolics are of increasing interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food.

The present study investigated the antioxidant activity by FRAP method of the ethanolic and aqueous extract of 10 different medicinal plant from south of Iran.

Phenolic compounds and flavonoids are important secondary metabolites that are synthesized by plants during development. They possess an array of health-promoting benefits and have engaged a great deal of scientific interest due to their health promoting effects as antioxidants [17]. Phenols are very important plant constituents because of their scavenging ability on free radicals due to their hydroxyl groups. Therefore, the phenolic content of plants may contribute directly to their antioxidant action [18]. Phenolic compounds are ubiquitous in plants and they can act as chelators and free radical scavengers especially over hydroxyl, peroxyl, superanions, and peroxynitriles. Flavonoids are highly effective scavengers of most oxidizing molecules and of several free radicals related to various pathologies. They are also able to chelatemetals and activate some antioxidant enzymes [19]. Antioxidant activity of flavonoids depends on the structure and substitution pattern of hydroxyl groups [20].

FRAP assay, non-enzymatic antioxidants react with prooxidants and inactive them. FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe3+-TPTZ) complex and producing a coloured ferrous tripyridyltriazine (Fe2+-TPTZ) [13]. Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom [21]. FRAP assay treats the antioxidants in the sample as a reductant in a redox-linked colorimetric reaction. Medicinal plants are the best sources for chemical ingredients, antimicrobial and antioxidant agents for cure of different diseases. Thyme (*Thymus vulgaris*) belongs to the Lamiaceae family, and has been used since antiquity in traditional medicine. It is recognized by its therapeutic virtues. Most genera of the Lamiaceae are rich sources of terpenoids and they also contain a considerable amount of various iridoid glycosides, flavonoids, and phenolic acids such as rosmarinic acid and other phenolic compounds. Thyme is known to have high antioxidant capacities. Thyme like some other wild *Thymus* species possesses a wide range of biological activities including expectorant, spasmolitic, sedative [22], antifungal [23] and antioxidant properties. In most cases, investigations on biological activities were carried out on extracts, essential oils or pure compounds isolated from dried herbs of *Thymus* species. Our findings are in agreement with previous investigation of Madsen et al. [24], who reported that total phenol content in the extracts of different Lamiaceae species (including thyme) linked linearly with the antioxidant activity. The thymus polyphenol-rich extract presents a strong antioxidant activity as demonstrated by FRAP test. Significant differences between the results were likely due to genotopic and environmental differences (namely, climate, location, temperature, fertility, diseases and pest exposure) within species, choice of parts tested, time of taking samples and determination methods [25]. A number of studies have demonstrated that essential oils (e.g. thymol, thyme, rosmanol) were major components that showed high antioxidant and antimicrobial activity [25]. Thyme contains a variety of flavonoids, including apigenin, naringenin, luteolin, and thymonin. These flavonoids increase thyme's antioxidant capacity, and combined with its status as a very good source of manganese, give thyme a high standing on the list of anti-oxidant foods.

A preliminary phytochemical analysis of the ethanolic extract of thyme revealed the presence of phenolic compounds and flavonoids, to which are attributed many of the antioxidant properties, due to their hydrogen donation ability, and their structural requirement considered to be essential for effective radical scavenging, it has been reported that this activity may result from: The presence of a 3′, 4′-dihydroxy, *i.e.*, a *o*-dihydroxy group (catechol structure) in the B ring, possessing electron donating properties and being a radical target.

- The 3-OH moiety of the C ring is also beneficial for the antioxidant activity of flavonoids.
- The C2 = C3 double bond conjugated with a 4-keto group, which is responsible for electron delocalization from the B ring, enhances further the radical-scavenging capacity.
- The presence of both 3-OH and 5-OH groups in combination with a 4-carbonyl function and C2 = C3 double bond.

• The presence of hydroxyl substituents in a catechol structure on the A-ring is able to compensate the absence of the *o*-dihydroxy structure in the B-ring, and became a larger determinate of flavonoid antiradical activity [26].

In the present study, selected herb of the Lamiaceae family demonstrated significant antioxidant potential and this is in accordance with previous studies on antioxidant properties of some Lamiaceae plants [27,28]. Hall and Cuppett [29] reported that rosmarinic acid is known as the main component in Lamiaceae plants with a potent antioxidant activity and thus, the observed antioxidant properties of Lamiaceae plants could depend strongly on rosmarinic acid. Rosmarinic acid, *o*-coumaric acid, apigenin-7-Oglucoside, coumarin, herniarin, luteolin, and apigenin have been shown to be *lavander* phenolic compounds [30].

In the literature, the antioxidant effects of the methanol extracts of the *T. vulgaris* have been reported. The results showed that the selected herbs were rich in phenolics and demonstrated considerable antioxidant capacity. *Thymus vulgaris* is quoted by various authors for its polyphenol and flavonoids contents and its antioxidant activity.

Erythrocytes are highly susceptible to attack by reactive oxygen species because of the high amount of polyunsaturated fatty acid content in their membranes and the metal catalyzed oxidation reactions because of haemoglobin Fe. Oxidative attack of erythrocytes is one of the major events in some hemoglobinopathies [31], thus erythrocytes have been used as a model for studies involving free radical oxidation and antioxidant activities. Oxidative damage to erythrocytes is manifested as haemolysis and lipid peroxidation and contributes to the senescence of normal red cells and shorter lifespan for pathologic red blood cells [32]. In haemolysis test this extract is able to neutralize the free radicals liberated by the H_2O_2. This antioxidant activity protects the erythrocyte membrane from lesions and lead to an increase of the half-time haemolysis. The antioxidant activity of this extract can be linked up to the high polyphenols and flavonoids content. Divers studies mentioned an implication of the polyphenols and flavonoids in the antioxidant activity of different plants extracts [33]. Phenols have been shown to possess an important antioxidant activity toward these radicals, which is principally based on the redox properties of their phenolic hydroxyl groups and the structural relationships between different parts of their chemical structure [34]. The action of the *thymus* and *Teucrium polium* aqueous extract is not limited to inhibit the free radicals, but it also seems to have an influence on the structural stability of the erythrocyte membrane. Biological membranes can be affected by many natural products present in medicinal plants [35]. Various authors mentioned that flavonoids, the widely distributed subgroup of the polyphenol, have beneficial effect on the erythrocyte membrane stability [36-37].

Flavonoids can be incorporated into the erythrocyte membranes [36]. Furthrmore, De Freitas et al. [37] relate that the exacerbation of the van der Waals contacts inside the lipid bilayer by the flavonoids could be a source of membrane stabilization. A good part of the antioxidant activity and consequently the resistance of the erythrocytes to hemolysis inducted by the aqueous extract of *Thymus vulgaris* or *Teucrium polium* varieties can be linked up to the content of polyphenols and flavonoids.

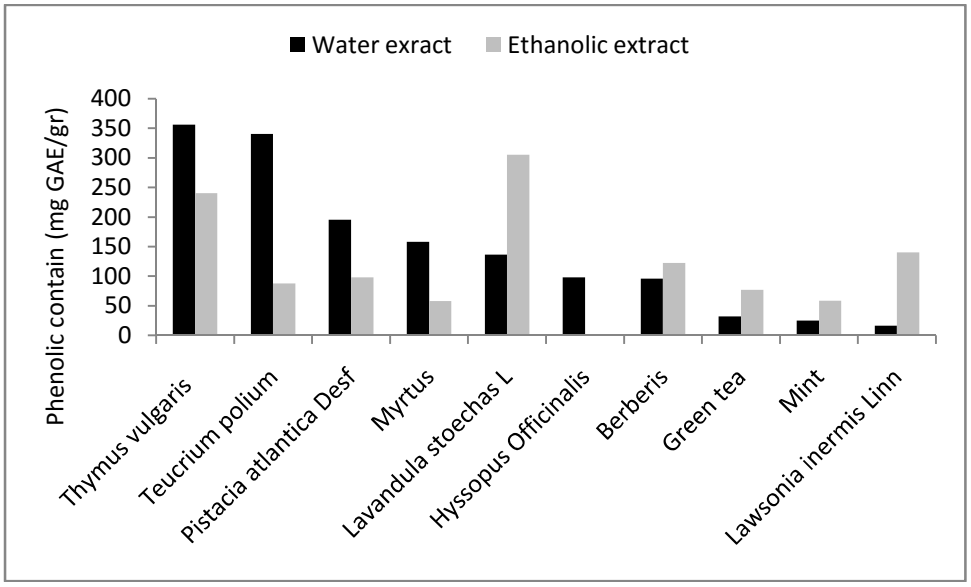

Fig.1 Total phenolics content (mg GAE/gr extract) of medicinal plants materials.

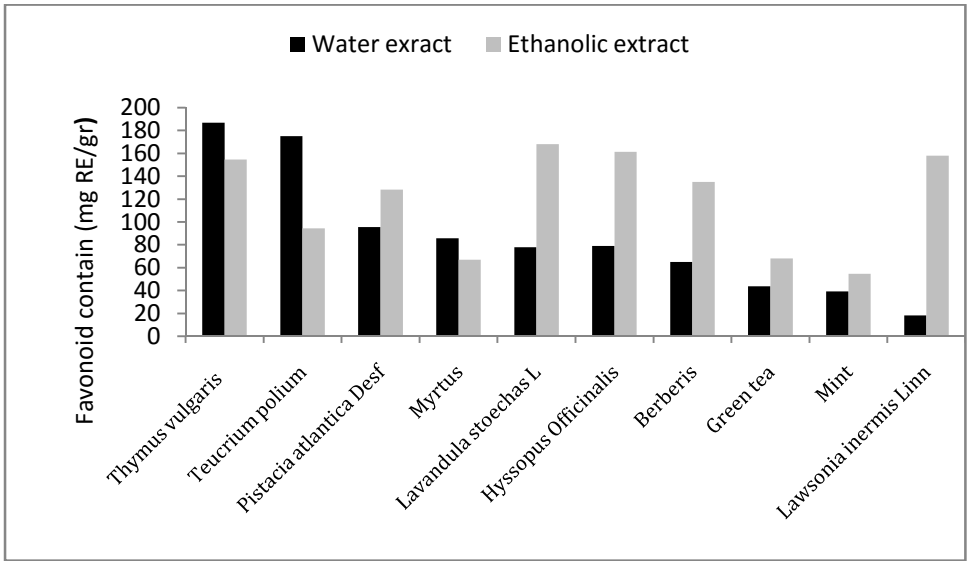

Fig .2 Total flavonoids content (mg RE/gr extract) of medicinal plants materials.

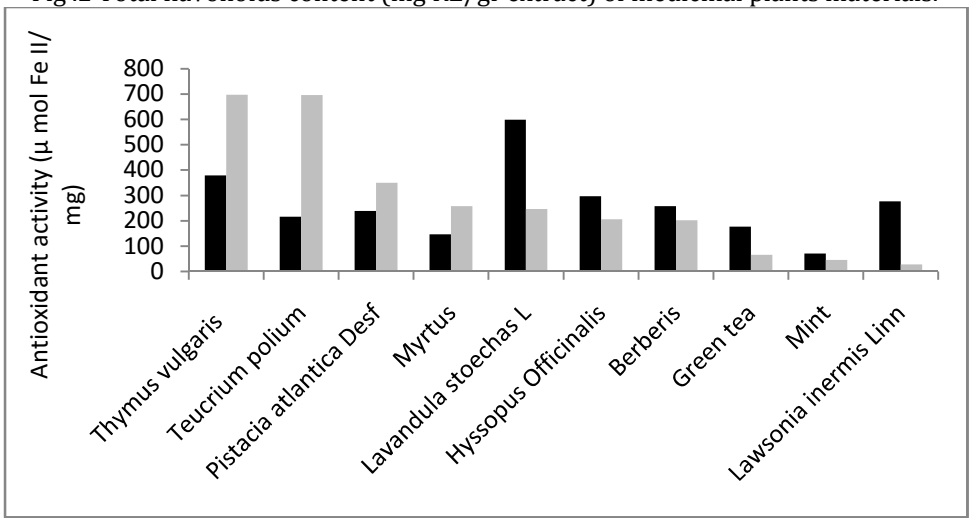

Fig.3 Antioxidant activity (FRAP) of 10 medicinal plant species south of Iran.

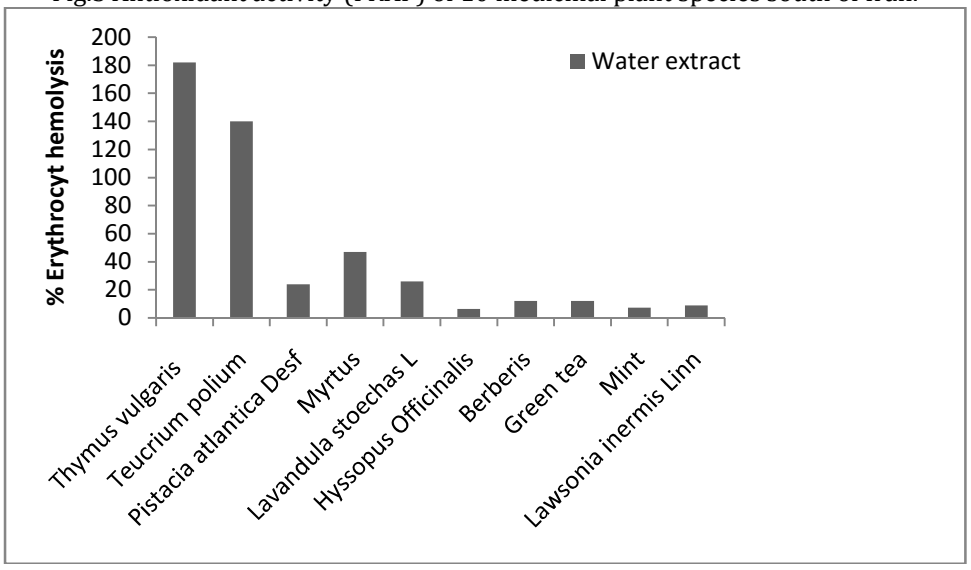

Fig.4 Inhibition of erythrocyte hemolysis by 10 species native medicinal plant south of Iran.

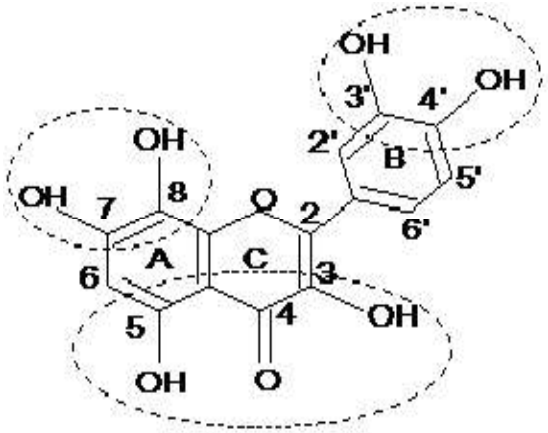

Fig.5 Structural features of flavonoids with a high radical scavenging activity.

CONCLUSIONS

In conclusion, the aqueous extract of *T. vulgaris* and *Lavandula stoechas L* are important source of phenolic compounds in water and ethanolic extract, respectively. The result of the present study showed that the extract of this plant contain high amount of flavonoids, and exhibited a great antioxidant activity. In this context, they can be used as an easily accessible source of natural antioxidants in commercial food products and drugs. However, the fact that they exert a positive interaction on the stability of the cellular membranes opens applications to the anti atherosclerotic process level. The results found are encouraging for further assessment to elucidate the mechanism of action and to identify the bioactive compounds implicated in the antioxidant effect and membrane stability.

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AUTHOR CONTRIBUTIONS

Abdollah Ramzani Ghara and Fereshteh ezzati ghadi designed the research; Fereshteh Ezzati Ghadi and Abdollah Ramzani Ghara and performed the experimental work; Abdollah Ramzani Ghara wrote the manuscript. All authors discussed, edited and approved the final version.

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