Total antioxidant capacities and reduction of ferric ions by extracts of three medicinal plants and their fractions

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Original Article

Abstract

Introduction: Medicinal plants belong to *Lamiaceae* family are potential sources of new drugs to improve the treatment of diseases which its treatment is linked to anti-oxidative agents. In this paper, antioxidant potentials of some *Lamiaceae* species have been evaluated by two methods.

Methods: phosphomolybdate assay for total antioxidant capacity and ferric reducing antioxidant potential (FRAP) were used for evaluation of the antioxidant potentials of *Zataria multiflora*, *Otostegia Persica* and *Salvia mirzayanii* extracts and their fractions.

Results: The extracts and their fractions showed noteworthy activities in all antioxidant assays which compared to the reference antioxidant, ascorbic acid in a dose dependent manner. In FRAP method, ethyl acetate fraction of *Salvia mirzayanii* (SMA) and petroleum ether fraction of *otostegia persica* (OPP) possessed the highest and the lowest antioxidant potentials, respectively. In total antioxidant capacity, butanol fraction of *Zataria multiflora* and chloroform fraction of *Otostegia persica* were the in activist and the activist samples, respectively.

Conclusion: These results may show that these plants act as free radicals scavengers and antioxidant compounds.

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Introduction:

Free radicals are formed when the cells use oxygen to generate energy. These products are generally reactive oxygen species (ROS) caused by the cellular reduction-oxidation process. ROS have beneficial effects on the cellular responses and immune functions at low or moderate concentrations (1) but at high levels, free radicals play a key role in the progress of tissue damages in various human diseases such as diabetes, aging, cancer, malaria, neurodegenerative disease, arthrosclerosis and pathological events in the living organisms (2). Antioxidants are various beneficial compounds that could naturally control free radicals formation (3,4). Butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) are examples of effective synthetic antioxidants but they have mutagenic properties in some cases (5).

Recently, herbal medicine has gained popularity in the health care. According to the World Health Organization, about 65%–80% of the population which lives in developing countries depends essentially on the plants for primary health care. In the middle of the nineteenth century, researchers isolated and characterized different classes of bioactive compounds. Many of them are used as active ingredients of modern medicine, or as basic compounds for new drugs discovery. In treatment of various degenerative ailments, several medicinal plants exist that are over filled sources of phenolic compounds, flavonoids, alkaloids and tannins (6).

Plant extracts are sources of secondary metabolites that may function as antioxidants. The most important antioxidants produced by plants are carotenoids, ascorbic acid, tocopherols, cinnamic acids, benzoic acids and folic acid (7). The *Labiatae* (*Lamiaceae*) family which is one of the largest and most distinctive families of plants, includes about 236 genera and 7000 species (8). Several species of this family like *Zataria multiflora* (*Zm*), *Salvia mirzayanii* (*Sm)* and *Otostegia persica* (*Op*) are used in traditional and modern medicine (9).

Zataria multiflora is extensively used in Iran as a flavor component in a wide variety of foods. Phenolic compounds include carvacrol, thymol and gamma terpinene are the main constituents of this plant (10). In other researches, several types of diterpenoids and flavonols including kaempferol, morin and quercitin are identified in *Op* extract (11). Crude extract of *Sm* contains different flavonoids and phenols. These compounds have antioxidant properties (12-14). In this study, we evaluated the antioxidant activities of *Zm*, *Op* and *Sm* extracts and their fractions by FRAP and TAC (Total Antioxidant Capacity) methods in order to understand the usefulness of these plants as food stuff and in the treatment of diseases.

Methods:

Chemicals

Ferric chlorides, Ascorbic acid, Ammonium per-sulfate, Sodium acetate tri-hydrate, were obtained from Sigma Chemical Co. USA. TPTZ (2,4, 6-Tri (2-pyridyl)-1, 3, 5-triazine), Ammonium molybdate and, Sodium sulfate, Sulfuric acid were purchased from Merck, Germany.

Plant materials

Specimen collection

In this study, 1kg of aerial parts of three plants includes *Sm* (MPRCM94-84)*, OP* (MPRCM94-86) were collected from Genu Mountains northeast of Bandar Abbas and *Zm* (MPRCM94-83) was obtained from Shiraz medicinal plants store. These plants identified by Dr Mahmood reza Moein, and voucher specimens have been deposited at the herbarium of Medicinal Plants Processing Research Center, Shiraz University of Medical Sciences (Table 1).

Extraction and Fractionation

The collected plants were carefully separated, washed with distilled water, and then dried under shade for two weeks. The dried plants were crushed and grounded with a blender to obtain a powder. Condensed powders (650g of *OP,* 415g of *Sm* and 522g of *Zm*) were extracted with ethanol 80% (13L, 8 L and 11 L respectively) to give ethanol extracts. The extraction was repeated three times and the extracts were concentrated by rotary evaporator under vacuum at 40°C. Then the crude extracts were suspended in (water 30%, methanol 70%) and filtered. Then fractionation was done consecutively with 3×2000 ml petroleum ether, 3×1000 ml chloroform, 3×650 ml ethyl acetate and 3×160 ml n- butanol, Figure1).

Table 1. Sampling information: Taxonomic status, gathering location, herbarium number of plants

Phylum	Family	Genius	Scientific name	Data	Locality
Magnoliphyta	Lamiaceae	*Salvia*	*Salvia mirzayanii*	20 JUN 2012	Genu Mountains
Magnoliphyta	Lamiaceae	*Otostegia*	*Otostegia persica*	15 JUN 2012	Genu Mountains
Magnoliphyta	Lamiaceae	*Zataria*	*Zataria multiflora*	25 JUN 2012	Nearest of Shiraz

Table 2. Percent efficiency of plant extracts

Samples	Amount of plant powder	Etanolic extraction	Efficiency
Zm	1 kg	700 gr	70%
Sm	1 kg	415 g	41.5%
Op	1 kg	520 gr	52%

Table 3. Total antioxidant capacities of the methanol extracts of *Sm, Op*, *Zm* and their different fractions expressed as mg ascorbic acid per mg of extract (mg of ASA/mg)

Samples	mg of ASA/mg	Samples	mg of ASA/mg	Samples	mg of ASA/mg
SMP	0.79±0.015	**ZMP**	0.31±0.005	OPP	0.6±0.025
SMC	0.53±0.02	**ZMC**	0.31±0.02	OPC	0.83±0.002
SMA	0.02±0.002	**ZMA**	0.05±0.006	OPA	0.48±0.03
SMB	0	**ZMB**	0.05±0.025	OPB	0
SME	0.82±0.009	**ZME**	0	OPE	0.62±0.001

All the values are mean±SD of tree individual observations

Table 4. Ferric reducing antioxidant potential of samples expressed as μg quercetin per mg of extract (μg of QUE/mg)

Samples	0.05mg/ml	0.1mg/ml	0.2mg/ml
SMA	34.32±0.78	64.31±3.44	92.7±2.41
SMB	5.88±0.17	8.15±0.54	11.05±0.72
SMC	4.83±0.08	5±0.27	5.49±0.02
SME	4.85±0.62	5.77±0.16	6.85±1
SMP	4.72±0.2	5±0.14	5.25±0.14
ZMA	11.21±0.23	21.88±1.05	42.94±2.31
ZMB	7.9±0.1	11.01±0.56	18.75±0.43
ZMC	0	0	5.14±0.3
ZME	4.65±0.44	7.41±0.29	11.08±0.04
ZMP	4.87±0.1	8.01±0.23	11.75±0.23
OPA	5.71±0.02	6.83±0.28	9.17±0.18
OPB	5.21±0.08	6.36±0.14	10.64±0.34
OPC	0	4.75±0.08	5.83±0.12
OPE	4.39±0.57	5.96±0.14	8.05±0.16
OPP	0	0	5.83±0.37

All the values are mean, ± SD of tree individual observations

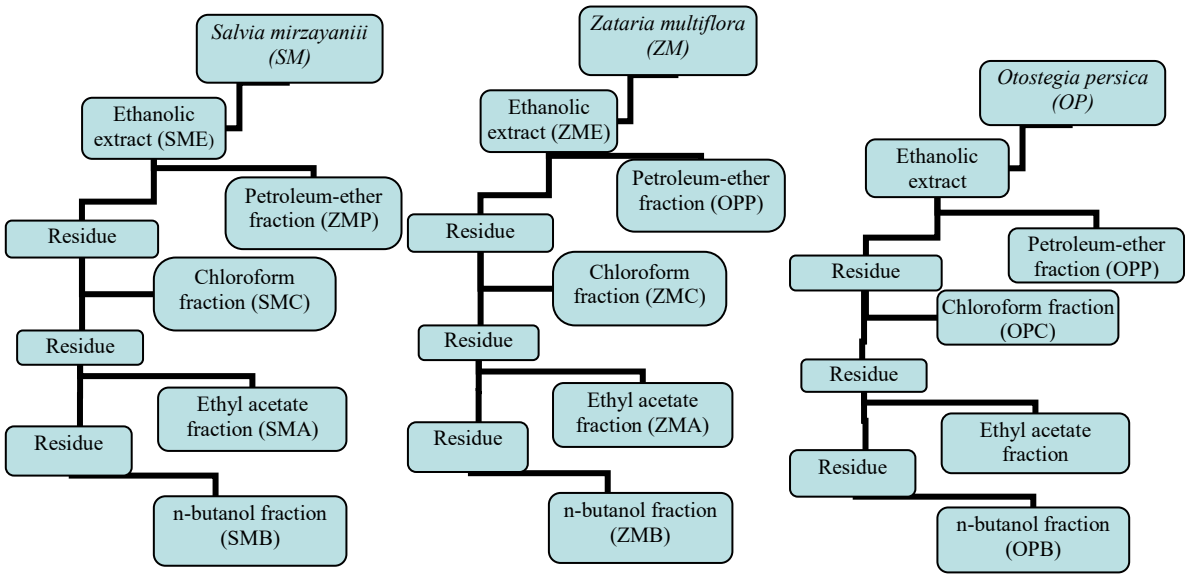

Figure1. Scheme of fractionation of plants extracts

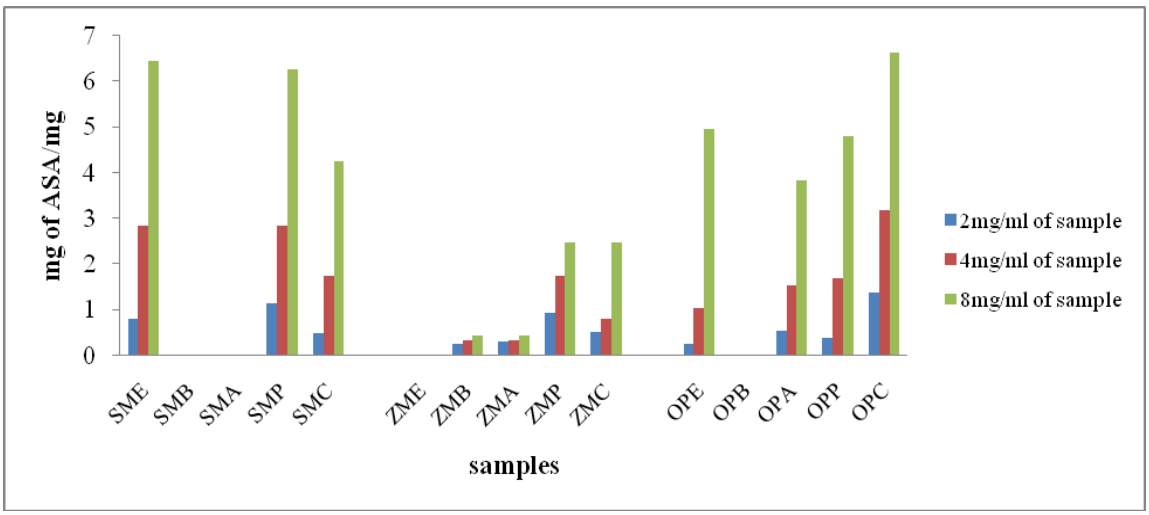

Figure 2. Total antioxidant capacities of samples in different concentrations
Blue, red and green colors showed 2 mg/ml, 4 mg/ml and 8 mg/ml of samples respectively

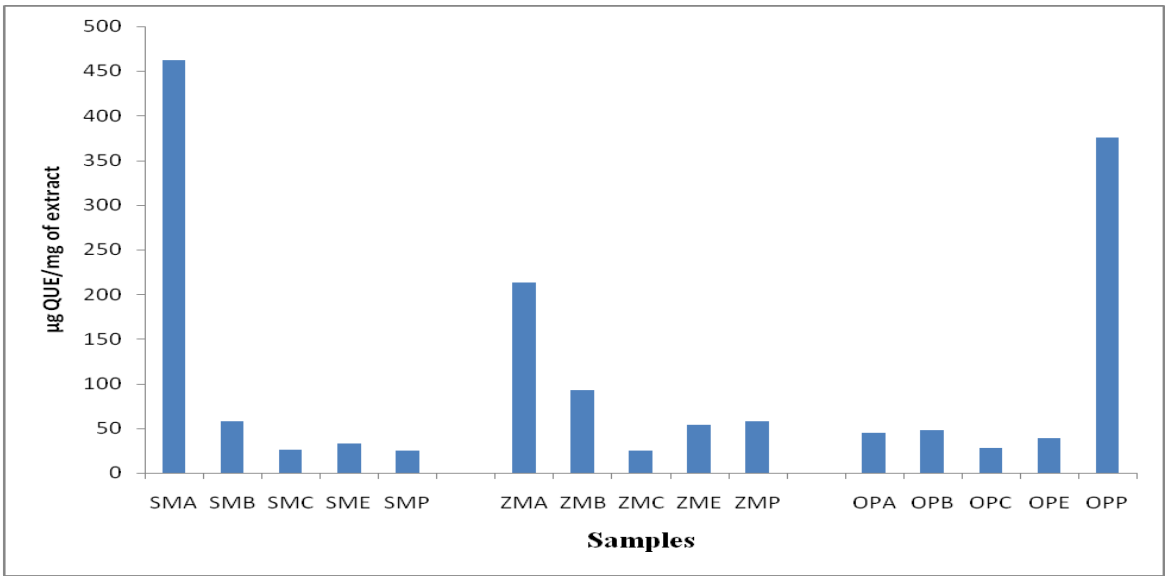

Figure 3. Antioxidant potentials of samples by FRAP method expressed as μg quercetin per mg extract

Phospho Molybdate Assay for Total Antioxidant Capacity

The antioxidant activities of *Zm*, *Op* and *Sm* extracts and their fractions were evaluated by the phosphor - molybdenum method according to the procedure describe by Vijayabasker et al (15) with some modifications. For sample preparation, 0.1ml of the extract was combined with 1ml of reagent solution (sulfuric acid 0.6 M, sodium sulfate 28 mM and ammonium molybdate 4mM). The tubes contain the reaction solution were incubated at room temperature for 15min. Then the absorbance of the solution was measured at 695nm using a spectrophotometer, against blank. Blank contains 0.1ml of methanol instead of plant extract. The antioxidant activity is expressed as the number of mg/ml equivalents of ascorbic acid. All determinations were performed in triplicates.

Ferric Reducing Antioxidant Potential

Ferric reducing antioxidant potential (16) of *Zm*, *Op* and *Sm* extracts and their fractions were measured according to the method described by Benzie and Strain (17) with some modifications. FRAP reagent was prepared by mixing in 25ml acetate buffer (300mM; pH 3.6), 2.5 ml TPTZ solution (10 mM) and 2.5ml ferric chloride solution (20 mM). Quercitin was used as a standard in this assay, and its calibration curve was obtained by using its concentrations range from 0. 78μg/ml to 50μg/ml. To 1.5ml of FRAP reagent, 50µL of various concentrations of samples or standard were added. The mixture was incubated for 10 min in the dark (37 °C) and its absorbance was measured at 593 nm. The blank contained an equal volume of methanol instead of the plant extract. All determinations were performed in triplicates. Reducing power is expressed as the number of equivalence of quercitin. A calibration curve of quercitin (its range from 0 to 50μg/ml) was prepared. The reducing power in FRAP method was expressed as μg quercitin per mg extract.

Statistical analysis

The data were expressed as the mean±SD of three replicates. Analysis was performed using Graph pad and Excel 2010. One-way analysis of variance (ANOVA) was used to determine the differences among the means. Values of $P \leq 0.05$ were considered as significant differences.

Results:

Sampling information: taxonomic status, scientific name, date and location of gathering of plants are showed in Table 1.

Efficiency of extraction and fractionation

The efficiency of herbal extracts showed that *Zm* and *Sm* extracts possessed the highest (70%) and the lowest (41.5%) efficiency (Table 2),

respectively. Procedure of plants extracts fractionation is illustrated in Figure 1.

Phosphomolybdate Method for Total Antioxidant Capacity

Total antioxidant capacities of the ethanol extracts of *Sm*, *Op*, *Zm* and their fractions were determined using Phospho molybdate method (Table 3 & Figure 2).

In comparison with other samples, *ZMB* had the lowest total antioxidant capacity, while the chloroform fraction of *OP (OPC)* showed the highest total antioxidant capacity. Butanol fractions of *Sm*, *ZME* and *OPB* didn't show any antioxidant activities (Table 3 & Figure 2).

Ferric Reducing Antioxidant Potential

Reducing antioxidant potential of extracts was determined using FRAP assay. As Table 4 shows among all the samples, ethyl acetate fraction of *Sm* (SMA) and alcoholic extract of *Op* (OPE) had the highest and the lowest reducing antiozidant potential, respectively. All the three extracts showed different increasing trends in activity with increase in extract concentrations ($P<0.05$), (Figure 3 & Table 4).

Conclusion:

Oxidative stress has been known as the cause of the development and progression of several diseases. Supplementation of antioxidants neutralizes the effects of reactive oxygen species (ROS) which induce oxidative damages. Antioxidants can be synthesized in vivo such as reduced glutathione (18) and superoxide dismutase (SOD) or take as dietary antioxidants. Plants have long been used as a source of exogenous (i.e., dietary) antioxidants. It is believed that two-thirds of the world's plant species have medicinal importance, and almost all of them have excellent antioxidant potentials. The interest in the exogenous antioxidant plants was firstly evoked by the discovery and subsequent isolation of ascorbic acid from the plants. Then, the antioxidant potentials of plants have received a great deal of attentions, because increase of the oxidative stress has been identified as a major causative factor in the development and progression of several life threatening diseases, such as neurodegenerative and cardiovascular diseases.

In addition, supplementation with exogenous antioxidants or boosting the endogenous antioxidants are promising methods for counter the undesirable effects of oxidative stress (19,20).

The antioxidants may be classified into repairing, preventing and scavenging antioxidants. The preventing antioxidants are one of the most important types that control the capacity of antioxidants and the subject of many researches (21). The free radical scavengers (antioxidants) donate one hydrogen or an electron (22).

In this study, we evaluated the antioxidant activities of *Zm*, *Op* and *Sm* extracts and their fractions by two methods include FRAP and TAC. The TAC assay is based on the reduction molybdenum (VI) to molybdenum (V) in the presence of a reducing agent (antioxidant) which forms a green Phospho- molybdate (V) complex. The absorbance of this complex is read by spectrophotometer at 765 nm (23). In this method**,** among different fractions, petroleum ether as a non-polar fraction showed the highest total antioxidant capacity and polar fractions showed the lowest total antioxidant capacities. It is assumed, that this is due to the presence of some active compounds in non-polar fraction, also other researchers report that non- polar compounds have antioxidant activities (24,25). But some researchers report that polar fractions possess the highest total antioxidant capacities (23,26). This difference may be caused by phytochemical properties of the plants, ecology of their growth environment and experimental condition that previously pointed (27).

Phenols and flavonoids are known as the main plant antioxidant compounds (28) and in other hand several studies have been reported that phenolic compounds are polar (29). According to this point in some studies, significant correlations between the antioxidant properties and the total phenolic content (30) are not found. Based on our results from TAC method, it seems that non-phenolic compounds may be involved in the antioxidant properties of these plants (30).

In FRAP assay, ferric ions are reduced to ferrous ions in the presence of an antioxidant which form a blue-colored ferrous tripyridyl triazine

complex (Fe^{2+}-TPTZ) at pH 3.6. This method is based on the transfer of one electron (ET) (31).

According to Table 4, *SMA*, *ZMA* and *OPA* showed the highest reducing antioxidant potentials ($P<0.05$) and after them *SMB*, *ZMB* and *OPB* showed the great reducing antioxidant potentials in comparison with other samples. Hosseini et al have reported that the presence of phenolic compounds in ethyl acetate fraction of *Zm*, is the reason of high antioxidant effect of this fraction (32). Also, Moein et al have reported that the ethyl acetate fraction of *Sm* extract exhibited interesting antioxidant properties (12,33). Our findings are agreed with the results of the other study that reported by Alwash et al. They also found high antioxidant activities in the ethyl acetate and butanol extracts of the aerial parts of *Teucrium barbeyanum* Aschers (34). Petroleum ether and chloroform fractions of *Op*, *Sm* and *Zm* showed the lowest antioxidant potentials. Generally in FRAP method, polar fractions showed the highest radicals reduction (antioxidant potential) and non- polar fractions showed the lowest radicals reduction. It is assumed that the presence of active compounds in polar fractions, inhibit the oxidation of macromolecules.

In present study, results which achieved from these two methods were different, this means that TAC method is used for antioxidant potentials of non- polar fractions, while FRAP method is used for antioxidant potentials of polar and semi-polar fractions.

According to our study and other studies (35) it is seen that ethanol is better for extraction of *salvia* species than methanol.

In other researches, significant antioxidant activity of *Zm* extract was reported by evaluation of scavenging DPPH radical, total antioxidant power (TAP) and thiobarbituric acid reactive substances (TBARS) in the serum of treated rats (36-38). Phytochemical screening of *Zm* extract supports the presence of monoterpens as phenolic compounds in this plant with significant antioxidant properties (39). Therefore, the beneficial effects of *Zm* in neutralization of free radicals turn back to its compounds.

Sharififar et.al reported that the extract of *Op* exhibited strong antioxidant activity and two compounds were responsible for antioxidant activities. By using UV, IR, MS, and ^{1}H and ^{13}C NMR techniques, it was found that these two compounds are morin and quercetin (40). In other research, it is reported that some flavonoids such as morin, kaempferol, quercitin, and isovitexin which found in *Op* extract, may be responsible for the antioxidant activity of this plant.

Since nonphenolic compounds may decrease the antioxidant activity (41) it seems that use of non-purification extracts and their fractions was a limitation in this study and also use of just two methods for evaluation antioxidant activities was another limitations. Thus further research, on isolated compounds from these species seems necessary.

Present study indicates that ethanol extracts of three genius of Lamiaceae consists of *Salvia mirzayanii*, *Otostegia persica* and *Zataria multiflora* and some of their fractions possessed remarkable reducing power and total antioxidant capacity. These plants can be used for decreasing blood sugar and inhibition of α-glucosidase.

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