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In-vitro Antioxidant Studies of Various Extracts of *Salvia hypoleuca*

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Abstract: The present study was undertaken to determine the antioxidant activities of various extracts of whole plant of *Salvia hypoleuca* in different *in vitro* methods. The antioxidant activity was evaluated by Total antioxidant activity (Phosphomolybdic acid method), FRAP assay with reference standard Ascorbate and total phenol content, respectively. The methanolic extract of *Salvia hypoleuca* was the most effective total antioxidant activity among the three extracts. The IC50 values of the methanolic extract of *Salvia hypoleuca* and ascorbate were found to be 160 and 410 µg/mL, respectively. The methanolic extract of *Salvia hypoleuca* was found more effective in FRAP assay than that of petroleum ether and ethyl acetate extracts. But when compare to the all the three extracts with ascorbate (standard), the methanolic extract of the *Salvia hypoleuca* showed the significant result. The methanolic extract of *Salvia hypoleuca* contains high amount of phenolic compounds than that of other two extracts. So, the *in vitro* study clearly shows that the methanolic extract of *Salvia hypoleuca* has a strong antioxidant activity. This study indicated that methanolic extract of *Salvia hypoleuca* comprise effective potential source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses.

Key words: Antioxidant activity, FRAP assay, phosphomolybdic acid method, *Salvia hypoleuca*

INTRODUCTION

Oxygen is vital for aerobic life processes. However, about 5% or more of the inhaled O_2 is converted to Reactive Oxygen Species (ROS) such as, O^{2-} H_2 O_2, and OH by univalent reduction of O_2. Thus cells under aerobic condition are always threatened with the insult of ROS, which however are efficiently taken care of by the highly powerful antioxidant systems of the cell without any untoward effect. When the balance between ROS production and antioxidant defenses is lost, 'oxidative stress' results which through a series of events deregulates the cellular functions leading to various pathological conditions including cardiovascular dysfunction, neurodegenerative diseases, gastroduodenal pathogenesis, metabolic dysfunction of almost all the vital organs, cancer, and premature aging (Thomas and Kalyanaraman, 1997). Reactive Oxygen Species (ROS), sometimes called active oxygen species, are various forms of activated oxygen, which include free radicals such as superoxide ions (O^{2-}) and hydroxyl radicals (OH), as well as nonfree-radical species such as hydrogen peroxide (H_2O_2) (Halliwell, 1997; Beckman and Ames, 1998) . There is extensive evidence to implicate free radicals in the development of degenerative diseases (Gerard and Sandra, 1993). Free radical induced oxidative stress, which involve preventive mechanisms, repair mechanism, physical defenses and antioxidant defenses (Olga *et al*., 2003). It is commonly recognized that antioxidants radicals can neutralize potentially harmful reactive free radicals in body cells before they cause lipid and protein oxidation and may reduce potential mutation and therefore, help prevent cancer or heart diseases (Vaidyaratnam and Varier's, 1994). In response to the increased popularity and greater demand for medicinal plant, a number of conservation groups are recommending that wild medicinal plant be brought into cultivation. A large number of medicinal plants and their purified constituents have shown beneficial therapeutic potentials. Various herbs and spices have been reported to exhibit antioxidant activity. The plant often contain substantial amount of antioxidants, such as flavonoids, carotenoids and tannins, flavones, isoflavones, anthocyanins, coumarin lignans, catechins and isocatechins (Nadkarni, 2001). Antioxidant-based drug formulations are used for the prevention and treatment of complex diseases like Diabetes, Atherosclerosis, Stoke, Alzheimer's disease and Cancer (Thomas and Kalyanaraman, 1997). The genus Salvia, one of the most important genus of Lamiaceae family, is widely used in flavouring and folk medicine all around the world (Rustayan *et al*., 1999). Fifty-eight species of this genus are documented in the Flora of Iran; 17 of them are endemic (Rustayan *et al*., 1999). The plants of the genus Salvia, which consist about 900 species are generally known for their multiple pharmacological effects such as analgesic and anti-inflammatory (Brickell, 1996), hepatoprotective (Cuppett and Hall, 1998), hypoglycemic activities (Wasser *et al*.,

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Table 1: Total antioxidant activity of petroleum ether extract of *salvia hypoleuca* by phosphomolybdic acid method

Concentration (µg/mL)	Sample (Petroleum ether extract)	Standard (Ascorbate)
125	13.69±0.070	26.87±0.076
250	25.61±0.014	30.30±0.054
500	41.09±0.096	60.64±0.022
1000	48.83±0.043	55.23±0.014
	IC50 = 1150 µg/mL	IC50 = 410 µg/mL

All values are expressed as mean±SEM

Table 2: Total antioxidant activity of ethyl acetate extract of *Salvia hypoleuca* by phosphomolybdic acid method

Concentration (µg/mL)acetate	Sample (Ethyl extract)	Standard (Ascorbate)
125	39.72±0.066	26.87±0.076
250	49.68±0.047	30.30±0.054
500	58.08±0.072	60.64±0.022
1000	80.01±0.039	55.23±0.014
	IC50 = 260 µg/mL	IC50 = 410 µg/mL

All values are expressed as mean±SEM

Table 3: Total antioxidant activity of methanolic extract of *Salvia hypoleuca* by phosphomolybdic acid method

Concentration (µg/mL)	Sample(Extract) Methanolic extract)	Standard (Ascorbate) (Ascorbate)
125	41.91±0.012	26.87±0.076
250	69.17±0.049	30.30±0.054
500	75.47±0.036	60.64±0.022
1000	84.75±0.024	55.23±0.014
	IC50 = 160 µg/mL	IC50 = 410 µg/mL

All values are expressed as mean±SEM

Table 4: Reduction ability of petroleum ether extract of *Salvia hypoleuca* on FRAP assay

Concentration (µg/mL)	Sample (Petroleum ether extract)	Standard (Ascorbate)
125	15.49±0.077	72.04±0.014
250	24.21±0.027	82.05±0.034
500	39.97±0.022	86.04±0.026
1000	51.02±0.041	98.07±0.041
	IC50 = 970 µg/mL	IC50 = 50 µg/mL

All values are expressed as mean±SEM

1998), and antiischemia (Jimenez *et al*., 1996). The literature survey showed that no study has been done on antioxidative stress activity of *Salvia hypoleuca*. Therefore, we were interested in studying free radical scavenging potential of various extracts of this plant by different in- vitro models.

MATERIALS AND METHODS

Collection and identification of plant materials: *Salvia hypoleuca* was collected from Guilan province (Iran) on January 2011, and authenticated at Medicinal Plants and Drugs Research Institute, Shahid-Beheshti University, Tehran, Iran. Its leaves and fruits were dried, under shade and powdered and then brought to department of biology, Science and Research Branch, Islamic Azad University, Fars, Iran for future studies.

Preparation of extracts: The above powered materials were successively extracted with Petroleum ether (40-60ºC) by hot continuous percolation method in Soxhlet apparatus for 24 h. Then the marc was subjected to Ethyl acetate (76-78ºC) for 24 h and then marc was subjected to Methanol for 24 h. The extracts were concentrated by using a rotary evaporator and subjected to freeze drying in a lyophilizer till dry powder was obtained.

Evaluation of antioxidant activity by in-vitro techniques:

Total antioxidant activity (Phosphomolybdic acid method): The antioxidant activity of the sample was evaluated by the transformation of Mo (VI) to Mo (V) to form phosphomolybdenum complex. An aliquot of 0.4 mL of sample solution was combined in a vial with 4 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The vials were capped and incubated in a water bath at 95ºC for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. The antioxidant activity was expresses relative to that of ascorbic acid.

FRAP assay: A modified method of Benzie and Strain was adopted for the FRAP assay. The stock solutions included 300 mM acetate buffer, pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-S-triazine) solution in 40 mM HCl and 20 mM $FeCl_3$. $6H_2O$. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ and 2.5 mL $FeCl_3$.$6H_2O$. The temperature of the solution was raised to 37ºC before using. Plant extracts (0.15 mL) were allowed to react with 2.85 mL of FRAP solution for 30 min in the dark condition. Readings of the colored product (Ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 200 and 1000 µM $FeSO_4$. Results are expressed in µM Fe (II) /g dry mass and compared with that of ascorbic acid.

Total phenol: To 0.25 g of sample, added 2.5 mL of ethanol and centrifuged at 2ºC for 10 min. The supernatant was preserved. Then, the sample was re-extracted with 2.5 mL of 80% ethanol and centrifuged. The pooled supernatant was evaporated to dryness. Then, added 3 mL of water to the dried supernatant. To which added 0.5 mL of Folins phenol reagent and 2 mL of sodium carbonate (20%). The reaction mixture was kept in boiling water bath for 1 min. the absorbance was measured at 650 nm in a spectrophotometer.

RESULTS

Total antioxidant activity (phosphomolybdic acid method): The percentage of total antioxidant activity of petroleum ether extract of *Salvia hypoleuca* was estimated and the results are presented in Table 1. The petroleum

Table 5: Reducing ability of ethyl acetate extract of *Salvia hypoleuca* on FRAP assay

Concentration (µg/mL)	Sample(Ethyl acetate extract)	Standard (Ascorbate)
125	40.20±0.016	72.04±0.014
250	58.28±0.011	82.05±0.034
500	71.68±0.029	86.04±0.026
1000	76.46±0.021	98.07±0.041
	IC50 = 180 µg/mL	IC50 = 50 µg/mL

All values are expressed as mean±SEM

Table 6: Reducing ability of methanolic extract of *Salvia hypoleuca* on FRAP assay

Concentration (µg/mL)	Sample (Methanolic extract)	Standard (Ascorbate)
125	60.42±0.044	72.04±0.014
250	67.65±0.029	82.05±0.034
500	75.81±0.036	86.04±0.026
1000	80.87±0.013	98.07±0.041
	IC50 = 65 µg/mL	IC50 = 50 µg/mL

All values are expressed as mean±SEM

Table 7: The total phenolic content of various extracts *Salvia hypoleuca*

Extracts	Total phenol content (mg/g of Catechol) (±SEM)*
Petroleum ether extract of *Salvia hypoleuca*	2.8±0.087
Ethyl acetate extract of *Salvia hypoleuca*	3.2±0.045
Methanolic extract of *Salvia hypoleuca*	4.8±0.073

All values are expressed as mean±SEM

ether extract of *Salvia hypoleuca* exhibited a maximum total antioxidant activity of 48.83% at 1000 µg/mL whereas for ascorbate (standard) was found to be 55.23% at 1000 µg/mL. The IC50 values of the petroleum ether extract of *Salvia hypoleuca* and ascorbate were found to be 1150 and 410 µg/mL, respectively.

The percentage of total antioxidant activity of ethyl acetate extract of *Salvia hypoleuca* was estimated and the results are presented in Table 2. The ethyl acetate extract of *Salvia hypoleuca* exhibited a maximum totalantioxidant activity of 80.01% at 1000 µg/mL whereas for ascorbate (standard) was found to be 55.23% at 1000 µg/mL. The IC50 values of the ethyl acetate extract of *Salvia hypoleuca* and ascorbate were found to be 260 and 410 µg/mL, respectively.

The percentage of total antioxidant activity of methanolic extract of *Salvia hypoleuca* was estimated and the results are presented in Table 3. The methanolic extract of *Salvia hypoleuca* exhibited a maximum total antioxidant activity of 84.75% at 1000 µg/mL whereas for ascorbate (standard) was found to be 55.23% at 1000 µg/mL. The IC50 of the methanolic extract of *Salvia hypoleuca* and ascorbate were found to be 160 and 410 µg/mL, respectively. Based on the result showed the methanolic extract of *Salvia hypoleuca* was most effective among the extracts. But when compare all the extracts with standard the methanolic extract of *Salvia hypoleuca* was found strong antioxidant activity. The IC50 of the methanolic extract of *Salvia hypoleuca* and Ascorbate were found to be 160 and 410 µg/mL, respectively.

FRAP assay: The antioxidant potential of *Salvia hypoleuca* was ascertained from FRAP assay based on their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II). The reducing ability of the petroleum ether extract of *Salvia hypoleuca* and ascorbate at various concentrations (125, 250, 500, 1000 µg/mL) were examined and the values are presented in Table 4. The maximum reducing ability at 1000 µg/mL for plant extract and ascorbate was found to be 51.02 and 98.07%, respectively. The IC50 values of plant extract and ascorbate was recorded as 970 and 50 µg/mL, respectively.

The reducing ability of the ethyl acetate extract of *Salvia hypoleuca* and ascorbate at various concentrations (125, 250, 500, 1000 µg/mL) were examined and the values are presented in Table 5. The maximum reducing ability at 1000 µg/mL for plant extract and ascorbate was found to be 76.46 and 98.07%, respectively.
The IC50 values of plant extract and ascorbate was recorded as 180 and 50 µg/mL, respectively.

The reducing ability of the methanolic extract of *Salvia hypoleuca* and ascorbate at various concentrations (125, 250, 500, 1000 µg/mL) were examined and the values are presented in Table 6. The maximum reducing ability at 1000 µg/mL for plant extract and ascorbate was found to be 80.87 and 98.07%, respectively. The IC50 values of plant extract and ascorbate was recorded as 65 and 50 µg/mL, respectively.

Total phenol: Phenolic compounds are known as powerful chain breaking antioxidants. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups. The phenolic compounds may contribute directly to antioxidative action. The total amount of phenolic content of various extract of whole plant of *Salvia hypoleuca* was estimated and the results are present in Table 7.

DISCUSSION

Free radical is a molecule with an unpaired electron and is involved in bacterial and parasitic infections, lung damage, inflammation, reperfusion injury, cardiovascular disorders, atherosclerosis, aging and neoplastic diseases (Thomas and Kalyanaraman, 1997). They are also involved in autoimmune disorders like rheumatoid arthritis etc (Halliwell, 1997; Beckman and Ames, 1998). There fore, research for the determination of the natural antioxidants source is important. Based on the above results indicated, the methanolic extract of *Salvia hypoleuca* was found to most effective than that of petroleum ether and ethyl acetate extract. But when compare to the all the three extracts with ascorbate (standard), the methanolic extract of the *Salvia hypoleuca* showed the similar result.

According to the result the methanolic extract of *Salvia hypoleuca* was found higher content of phenolic components than that of petroleum ether and ethyl acetate extracts of *Salvia hypoleuca*. In conclusion, the results of the present study show that the methanolic extract of *Salvia hypoleuca* contains the high amount of flavonoids. So, the present study suggests that the whole plant of *Salvia hypoleuca* might be a potential source of natural antioxidant. Therefore the plant can be further harnessed for novel antioxidant/ bioactive compound which is very well evidence by the present studey.

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