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Research Article

In Vitro Antioxidant Properties of *Salvia coccinea* Buc'hoz ex etl. and *Salvia officinalis* L.

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ABSTRACT

Free radical induced oxidative stress is involved in the pathogenesis of various diseases and disorders. Antioxidants play an important role in protecting the body against this oxidative stress. Research in the past few decades has contributed evidences related to the enrichment of the body with antioxidant principles from plants. The present study was carried out to evaluate the *in vitro* antioxidant activity of methanolic extracts of leaves of *Salvia coccinea* Buc'hoz ex etl. and *Salvia officinalis* L. The methanolic leaf extracts of *S. coccinea* and *S. officinalis* were assayed using different *in vitro* free radical systems like superoxide, nitric oxide, hydroxyl and 2, 2-diphenyl-1-picrylhydrazyl (DPPH). Butylated hydroxy toluene (BHT) and butylated hydroxyl anisole (BHA) were used as standard antioxidants. The results of the *in vitro* antioxidant assays revealed potent antioxidant and free radical scavenging activity of the methanolic extracts of leaves of *Salvia coccinea* and *Salvia officinalis*. The IC_{50} (Inhibition concentration 50) value of the extracts ranged from 5.52 mg/mL to 8.79 mg/mL, suggesting to their possible use as natural sources in the development of free radical scavengers and antioxidant agents.

Key Words: Free radical, Antioxidants, Superoxide, Nitric oxide, Hydroxyl, DPPH.

INTRODUCTION

Oxygen is essential for the life of all aerobic organisms as it is involved in the vital process to liberate energy. However, along with the release of energy, free radicals which are capable of damaging various biomolecules such as proteins, deoxyribose nucleic acid or polyunsaturated fatty acids are also formed (Davies, 1995). A free radical is a molecule with one or more unpaired electron(s) in its outer orbital (McCord, 2000). The unpaired or odd electron is highly reactive as it seeks to pair with another free electron. These are continuously produced during respiration and some cell-mediated immune functions. Environmental pollutants, cigarette smoke, automobile exhaust fumes, radiation, pesticides etc. also contribute towards free radical formation (Tiwari, 2004). Most free radicals are derived from molecular oxygen and are commonly referred to as Reactive Oxygen Species (ROS). Also, Reactive Nitrogen and Chlorine Species of clinical significance exist, and some species without real radical nature i.e. unpaired electrons are usually included in the concept of free radicals due to their reactive nature eg. Hydrogen peroxide, H_2O_2 (Halliwell and Gutteridge, 1999).

Free radical formation is checked naturally by various beneficial compounds known as antioxidants. An antioxidant is any substance which when present at low concentrations as compared to that of an oxidizable substrate prevents the oxidation of that particular substrate. These are our first line of defence against free radical damage and are critical for maintaining optimum health and well being (Percival, 1998). A critical balance usually exists between the generation and detoxification of free radicals in the cells. But certain endogenous or exogenous factors could lead to excess load of free radicals in the body causing an imbalance between the oxidants (free radicals) and antioxidants. This imbalance creates an oxidative stress that has been suggested to be the root cause of various diseases such as atherosclerosis, stroke, diabetes, cancer and neurodegenerative diseases such as Alzheimer's and Parkinson's (Halliwell *et al.*, 1992).

Research in the recent past has accumulated enormous evidences revealing that the enrichment of body systems with natural antioxidants may prevent, delay or ameliorate many of the disorders caused due to oxidative stress (Havsteen, 2002). Plants constitute an important source of natural antioxidants that differ widely in terms of their structures, biological properties and mechanism of action. Apart from this, there is limited toxicity

associated with plants as seen in case of synthethic antioxidants such as BHT, BHA, Propyl galate (PG) etc. (Grice, 1986; Wichi, 1988; Oktay *et al*., 2003).

Plants belonging to the genus *Salvia* are widely used in traditional systems of medicine, food, drugs, cosmetics and perfumeries and thus are of great economic importance (Miliauskas *et al*., 2004; Tepe *et al*., 2006). The infusion and decoction of the aerial parts of plants belonging to this genus have been used as tonic, carminative, digestive, antispasmodic and anti-inflammatory in Iranian traditional medicine (Nickavar *et al*., 2007). The present investigation was aimed at examining the antioxidant activity of methanolic extracts of *Salvia coccinea* and *Salvia officinalis* leaves through various *in vitro* models.

MATERIALS AND METHODS

Plant material and Preparation of extract

Two plant species of *Salvia* namely; *Salvia coccinea* and *Salvia officinalis* belonging to the family Lamiaceae were selected for the study. Fresh leaves of the two plants were collected from their respective plants grown in the green house of Ramniranjan Jhunjhunwala College (R. J. C.). The plants were authenticated by the department of Botany of R. J. C., Mumbai.

The leaves collected were air dried. The methanolic extracts were prepared by grinding two grams of leaf to a fine powder, followed by cold extraction with 60% methanol for 24 hours. The methanolic leaf extracts were then centrifuged at room temperature. The supernatant that was collected was evaporated to dryness and used directly for the assessment of antioxidant activity. BHT and BHA were used as the standard compounds throughout.

Chemicals

BHT, BHA and DPPH were obtained from Sigma Chemicals Co. (St. Louis, MO, USA); Phenazine methosulphate (PMS) and reduced Nicotinamide adenine dinucleotide (NADH) from Sisco Research Laboratories Pvt. Ltd. (India); Nitroblue tetrazolium (NBT) and Sodium azide were obtained from Central drug house (India); Sulphanilamide, Napthyl ethylene diamine dihydrochloride (NEDD) and Ethylene diamine tetra acetic acid (EDTA) from Loba Chemie (India). All other chemicals and reagents used were of analytical grade.

Superoxide radical scavenging ability

Superoxide radical scavenging activity was assessed by using the method of Nishikimi *et al*., (1972) modified by Yamauchi *et al*., (2000). Superoxide radicals were generated in a system with 3.0 mL of tris-HCl buffer (16 µM, pH 8.0), which contained 78 µM NADH, 50 µM NBT, 10 µM PMS. The radical scavenging potential was assayed by adding different amounts of plant extracts to the reaction mixture. A blank without the plant extract or standard antioxidant was also prepared. The radical scavenging activity was monitored by a decrease in an absorbance at 560 nm. In the PMS/NADH-NBT system, superoxide anions reduce NBT to a blue coloured compound called formazan. The decrease in the absorbance at 560 nm indicates the consumption of superoxide radicals by the plant extracts and the standard antioxidant compounds. The results were expressed as percentage superoxide radical scavenging activity and calculated using the equation (Equation 1).

Equation 1:

$$\% \text{ Scavenging activity} = [A_{control} - A_{test} / A_{control}] \text{ X } 100$$

where, A_{test} is the absorbance of the test solution with the extract or standard compound added, and $A_{control}$ is the absorbance of the solution without extract.

Nitric oxide radical scavenging ability

In an aqueous solution at physiological pH, sodium nitroprusside spontaneously generates nitric oxide radicals which interact with oxygen to produce nitrite ions. The concentration of these nitrite ions was estimated using Greiss reagent (Sreejayan and Rao, 1996). Scavengers of nitric oxide radicals compete with oxygen thereby reducing the production of nitrite ions (Marcocci, 1994). Sodium nitroprusside (5 mM) in phosphate buffer was mixed with the plant extracts and incubated at 25°C for 150 minutes. A blank was treated similarly, but without the plant extracts. The reaction mixtures were then allowed to react with Greiss reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% NEDD). The diazotization of nitrite with sulphanilamide and its subsequent coupling with NEDD yields a chromophore, the absorbance of which was read at 546 nm. The results were compared with identical systems containing standard antioxidants; BHT and BHA instead of the plant extracts. The results were expressed as percentage nitric oxide radical scavenging activity.

Hydroxyl radical scavenging ability

The hydroxyl radical scavenging ability of plant extracts was estimated according to the method of Halliwell *et al*., (1987). The method is based on the specific reaction of 2-deoxyribose with hydroxyl radicals generated in the medium. It is measured as the formation of a complex of TBA (Thio barbituric acid) and the degraded product of

2-deoxyribose. Reaction mixture was prepared by adding different concentrations of the plant extracts to a mixture of 1 mM EDTA, 10 mM H_2O_2, and 10 mM 2-deoxyribose. The mixture was then incubated at 37°C for 60 minutes and subsequently quenched by the addition of TBA (1% in 0.05 M sodium hydroxide) and 2.8% TCA (Tri carboxylic acid) (w/v in distilled water) to develop a pink chromophore. It was then centrifuged at 10,000 g for 15 minutes at 4°C. A blank without the plant extract was also prepared and treated in the same manner. The absorbance of the blank as well as the test mixtures was measured at 532 nm.

The hydroxyl radical scavenging activity of the extract was reported as percentage hydroxyl radical scavenging activity.

DPPH radical scavenging ability

The ability of the samples to scavenge the free radical DPPH was determined spectrophotometrically by measuring a decrease in the absorbance caused by the radical at 517 nm by the method of Blois (1958). The antioxidant activity of the methanolic extracts of the plants was tested using a stable radical DPPH. Plant extracts at different concentrations were allowed to react with methanolic solution of DPPH (100 µM) and the scavenging activity was monitored by recording a decrease in an absorbance at 517 nm. The results were expressed as percentage scavenging of DPPH radical.

Statistical analysis

All data represent an average of 3 replicates. Mean values and standard errors were calculated from the results. P value greater than 0.05 was regarded as significant. The standard deviations, wherever necessary are indicated as error bars.

RESULTS

Several experimental systems of generating free radicals *in vitro* have been reported and used to assess the radical scavenging and antioxidant capacity of individual compounds and plant extracts. Same plant extracts may exhibit varying measures of scavenging different radicals and these measures add up to give the overall antioxidant potential of the plant (Cao *et al*., 1998).

Superoxide radical scavenging activity

The superoxide radicals derived from dissolved oxygen by the PMS/NADH coupling reaction reduces NBT and the decrease in the absorbance at 560 nm in presence of different plant extracts indicates the capacity of the respective plant extract to scavenge the superoxide radicals from the medium. Between the two species of *Salvia*, the methanolic leaf extracts of *Salvia officinalis* exhibited the maximum potential to scavenge the superoxide radicals (74.22 ± 1.37%) whereas, the methanolic leaf extracts of *Salvia coccinea* showed comparatively less superoxide radical scavenging capacity (43.25 ± 1.94%). It was observed that BHT possesses the highest capacity to scavenge superoxide radicals (90.32 ± 1.71%) closely followed by BHA with 89.65 ± 1.17% inhibition (Table 1, Fig. 1).

The IC_{50} values of the leaf extracts of *Salvia coccinea* and *Salvia officinalis* were found to be 11.56 mg/mL and 6.73 mg/mL respectively as against the IC_{50} values of the standard compounds, BHT and BHA which were 5.53 mg/mL and 5.57 mg/mL respectively (Table 1, Fig. 2).

Nitric oxide radical scavenging ability

Nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be scavenged by methanolic leaf extracts of both the *Salvia* species used in the study. The methanolic leaf extracts of *Salvia officinalis* were found to be slightly more effective in scavenging the nitric oxide radicals (67.36 ± 1.35%) than *Salvia coccinea* (63.83 ± 1.42%). As seen from the graph (Table 1, Fig. 1), the standards; BHA and BHT were capable of scavenging the nitric oxide radical very effectively (90 ± 1.52% and 89.45 ± 1.45% respectively).

The IC_{50} values of the methanolic leaf extracts of *Salvia coccinea* and *Salvia officinalis* were found to be 7.83 mg/mL and 7.42 mg/mL respectively and those of the standard compounds, BHT and BHA, it was found to be 5.59 mg/mL and 5.55 mg/mL respectively (Table 1, Fig. 2). **Hydroxyl radical scavenging ability**

In the present study, the hydroxyl radical scavenging capacity of the plant extracts was assayed as the extent of inhibition of ferrous ion dependent, hydroxyl radical mediated damage to deoxyribose. The hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxyribose into fragments that formed a pink chromogen upon heating with TBA at low pH (Aruoma, 1991). When the test compounds were added to the reaction mixture, they removed hydroxyl radicals from the sugar and prevented their degradation.

With regards to the hydroxyl radicals, the two standard antioxidants used (BHT and BHA) proved to be strong hydroxyl radical scavengers (90.62 ± 1.11% and 88.94 ± 1.36% respectively) (Table 1, Fig. 1). Moderate hydroxyl radical scavenging activity was exhibited by the methanolic leaf extracts of *Salvia officinalis* and *Salvia coccinea* (57.28 ± 1.04% and 49.34 ± 1.17%

respectively). The IC_{50} values of the methanolic leaf extracts of *Salvia coccinea* and *Salvia officinalis* were found to be 10.13 mg/mL and 8.72 mg/mL respectively whereas, the IC_{50} values of the standard compounds, BHT and BHA were found to be 5.51 mg/mL and 5.62 mg/mL respectively (Table 1, Fig. 2).

Table 1: Effect of methanolic leaf extracts of *Salvia officinalis* and *Salvia coccinea* on different antioxidant systems

Sample	Superoxide radical		Nitric oxide radical		Hydroxyl radical		DPPH radical		Mean IC_{50} (mg/mL)
	% scavenging	IC_{50} (mg/mL)	% scavenging	IC_{50} (mg/mL)	% scavenging	IC_{50} (mg/mL)	% scavenging	IC_{50} (mg/mL)	
S. officinalis	74.22 ± 1.37	6.73	67.36 ± 1.35	7.42	57.28 ± 1.04	8.72	73.57 ± 1.19	6.79	7.34
S. coccinea	43.25 ± 1.94	11.56	63.83 ± 1.42	7.83	49.34 ± 1.17	10.13	71.05 ± 1.54	7.03	8.79
BHT	90.32 ± 1.71	5.53	89.45 ± 1.45	5.59	90.62 ± 1.11	5.51	94.11 ± 1.47	5.31	5.48
BHA	89.65 ± 1.17	5.57	90 ± 1.52	5.55	88.94 ± 1.36	5.62	93.36 ± 1.08	5.35	5.52

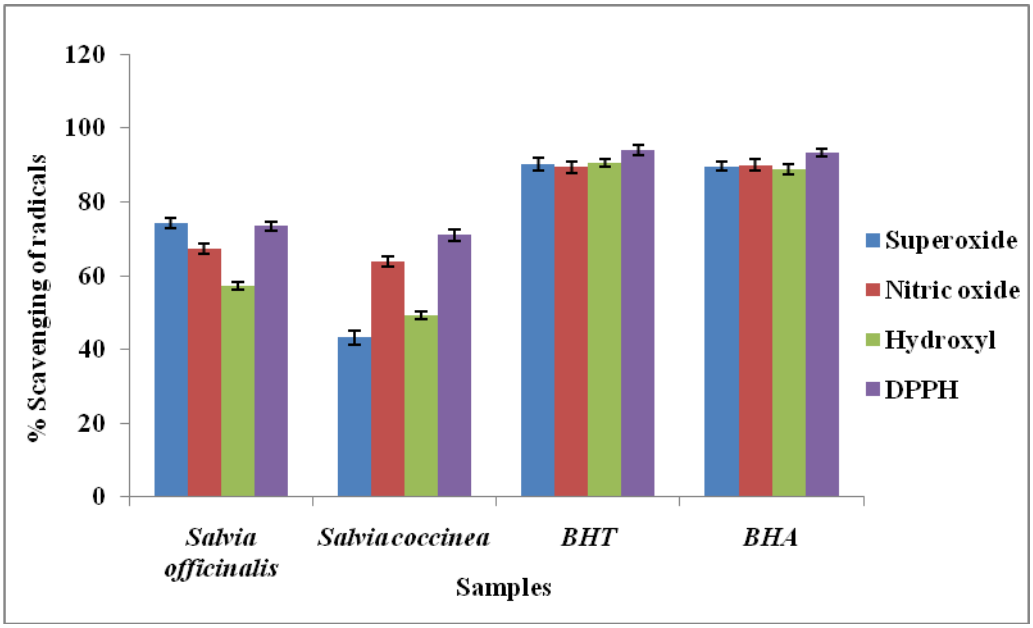

Figure 1: Percentage scavenging of different radicals by methanolic leaf extracts of *S. officinalis and S. coccinea*

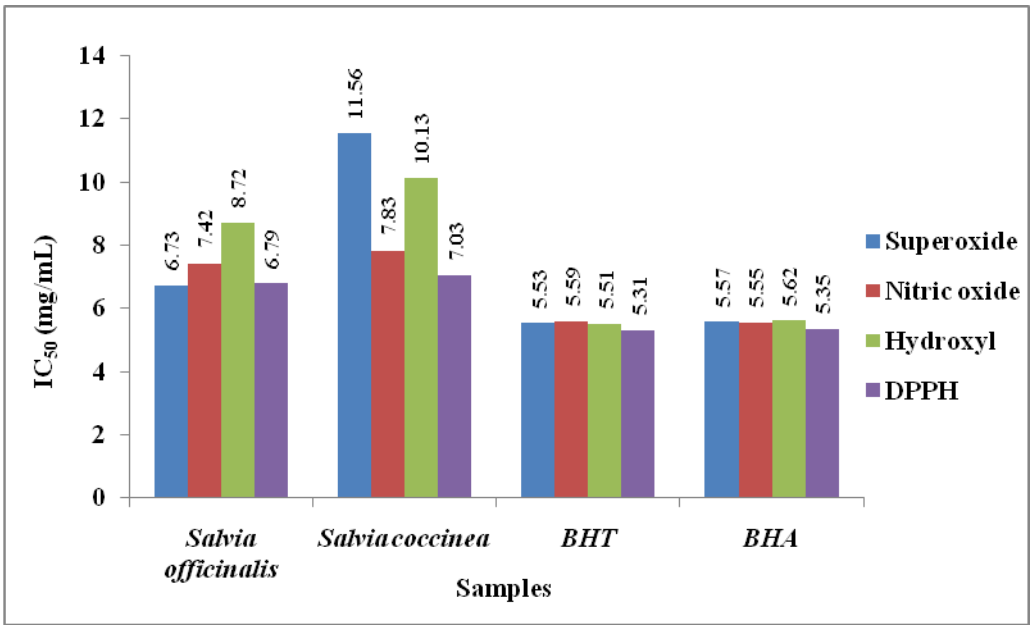

Figure 2: IC_{50} values of methanolic leaf extracts of *S. officinalis and S. coccinea* against different radicals

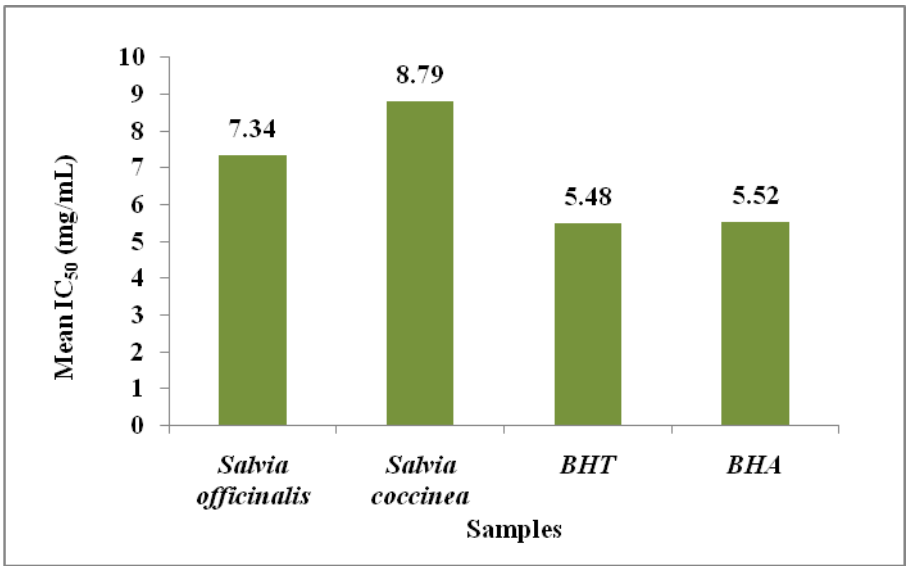

Figure 3: Mean IC_{50} values of methanolic leaf extracts of *S. officinalis and S. coccinea*

Total antioxidant activity using DPPH

The molecule of DPPH is characterized as a stable free radical, by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerize, as would be the case with most other free radicals. The decolourization also gives rise to a deep violet colour, characterized by an absorption measured at 517 nm in an ethanol or methanol solution.

Both the plant extracts tested, exhibited more than 70% scavenging activity of the DPPH radical (Table 1, Fig. 1). The methanolic leaf extracts of *Salvia officinalis* and *Salvia coccinea* were capable of scavenging the DPPH radical significantly (73.57 ± 1.19% and 71.05 ± 1.54% respectively). The standards; BHT and BHA were found to possess very high DPPH radical scavenging ability (94.11 ± 1.47% and 93.36 ± 1.08% respectively).

The IC_{50} values of the methanolic leaf extracts of *Salvia coccinea*, *Salvia officinalis* and those of the standard compounds, BHT and BHA were found to be 7.03 mg/mL, 6.79 mg/mL, 5.31 mg/mL and 5.35 mg/mL respectively (Table 1, Fig. 2).

DISCUSSION

In the present study, the antioxidant activity of *Salvia officinalis* and *Salvia coccinea* was studied by measuring their ability to scavenge superoxide anion, nitric oxide, hydroxyl as well as DPPH radical. The methanolic extracts of leaves of *Salvia officinalis* and *Salvia coccinea* exhibited potent antioxidant activity by strongly scavenging these radicals when compared with the standards; BHT and BHA. The scavenging activity of both the plant extracts tested using different *in vitro* systems, was observed to be more than 40%. The mean IC_{50} values of the extracts ranged from 5.52 mg/mL to 8.79 mg/mL. There was a variation observed in the *Salvia* species with respect to their ability in scavenging the different types of free radicals. This variation appears to be mainly due to the variation in the nature of flavonoids and phenolics. The ability of the phenolics to act as antioxidant depends on the redox properties of their phenolic hydroxyl groups, that allow them to act as reducing agents, hydrogen donating antioxidants and oxygen quenchers (Rice-Evans and Miller, 1996).

Among the two species of *Salvia* tested, *Salvia officinalis* exhibited maximum activity against all the radicals, which could be attributed to its higher phenolic and flavonoid content than *Salvia coccinea*. Pourmorad and his co-workers (2006), while working on *Mellilotus officinalis* have also observed that the antioxidant activity of the plant is directly proportional to its phenolic content.

The findings of this study support the view that *Salvia officinalis* and *Salvia coccinea* are promising sources of potential antioxidants and may be efficient as preventive agents in diseases caused due to oxidative stress. The study will also help in enriching the existing comprehensive data of the antioxidant activity of these *Salvia* species. In addition, these plant extracts can be used as easily accessible sources of natural antioxidants and as possible food supplements or in pharmaceutical industries. However, there is a need for further studies to be carried out to isolate and identify the components of these two *Salvia* species responsible for endowing them with their antioxidant activity.

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