In Vitro Antioxidant and Free Radical Scavenging Activity of Different Extracts of *Boerhavia diffusa* and *Boswellia serrata*

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Abstract:

The antioxidant activity of aqueous and ethanolic extracts of *Boerhavia diffusa* (leaves) and *Boswellia serrata* (bark) was evaluated by using DPPH method, NO scavenging method, reducing ability method and total polyphenols content by Folin Ciocalteu method. Preliminary phytochemical screening revealed that the extract of the leaves of *Boerhavia diffusa* possesses alkaloids, carbohydrates, terpenoids, phenolic compounds, flavonoids and tannins in the order of ethanol > aqueous extract and the extracts of *Boswellia serrata* indicated the presence of various chemical compounds like alkaloids, carbohydrates, terpenoids, phytosterols, phenolic compounds, flavonoids, and tannins except saponins. The extracts of both plants showed significant activities in all antioxidant assays compared to the standard antioxidant in a dose dependent manner and remarkable activities to scavenge reactive oxygen species may be attributed to the high amount of hydrophilic phenolics. The IC50 values indicated that the extract of *Boswellia serrata* has more scavenging activity than extract of *Boerhavia diffusa*.

Keywords: Antioxidant; Free radical; Radical scavenging; DPPH; NO Scavenging, *Allium sativum* Linn**.**

Introduction:

The generation of reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) at low/ moderate concentration play key roles in normal physiological processes, including cellular life/death processes, protection from pathogens, various cellular signaling pathways, and regulation of vascular tone. But the high concentration of ROS and RNS or its ineffective removal by body's own antioxidant system causing potential biological damage is termed oxidative stress and nitrosative stress.[1-3]

Oxidative stress is one of major causative factors in induction of many chronic and degenerative diseases including atherosclerosis, diabetes mellitus, cancer, Parkinson's disease, Alzheimer's disease, chronic renal failure, immune dysfunction, chronic inflammatory diseases and is involved in aging.[1] The most effective way to eliminate free radicals which cause the oxidative stress is with the help of antioxidants. Antioxidants are those substances which possess free radical chain reaction breaking properties.

Although synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole, and tertbutylhydroquinone have been commonly used as antioxidants in foods for years, their safety has long been questioned (Branen, 1975; Ito et at., 1983). This has led to an increased interest in natural antioxidants (Lim et al., 2002; Kayano et al., 2002; Gyamfi and Aniya, 2002; Braca et al., 2002). Spices and herbs are recognized as sources of natural antioxidants that can protect from oxidative stress and thus play an important role in the chemoprevention of diseases that has their etiology and pathophysiology in reactive oxygen species. The medicinal properties of folk plants are mainly attributed to the presence of flavonoids, but may also be influenced by other organic and inorganic compounds such as coumarins, phenolic acids and antioxidant micronutrients, e.g., Cu, Mn, Zn (Repetto and Llesuy, 2002)[4].

Boerhaavia diffusa, commonly known as punarnava in Sanskrit, is a herbaceous plant of the family Nyctaginaceae and widely distributed in the tropics and subtropics. The whole plant or its specific parts (leaves, stem, and roots) are known to have medicinal properties and have a long history of use by indigenous and tribal people in India. The medicinal value of this plant in the treatment of a large number of human ailments is mentioned in Ayurveda, Charaka Samhita, and Sushrita Samhita[5].

Boswellia serrata (frankincense) is a moderate-to-large branching tree (growing to a height of 12 feet) found in India, Northern Africa, and the Middle East. Strips of Boswellia bark are peeled away, yielding a gummy oleo-resin. Extracts of this gummy exudate have been traditionally used in the Ayurvedic system of medicine as an antiarthritic, astringent, stimulant, expectorant, and antiseptic[6].

The present study evaluated the ant oxidative potential and radical scavenging activity of aqueous and ethanolic extracts of *Boerhaavia diffusa* and *Boswellia serrata***.**

Materials and Methods:

Collection of the plant material:

The leaves of punarnavaa (*Boerhavia diffusa*) were collected from the rural areas of Gorakhpur district of eastern Uttar Pradesh. The bark of salai (*Boswellia serrata*) was collected from the local market and authenticated by Dr. M.B. Singh, Programme Coordinator, KVK Sultanpur,U.P.

Extraction:

Preparation of extracts of different parts of medicinal plants viz; leaves and bark using different solvents: aqueous, alcoholic (ethanol) by methods such as soxhlation, stirring and soaking.

Extraction with alcohol

Defined quantities of plant material were collected, shade dried at room temperature, pulverized and extracted with 95% ethanol in a Soxhlet extractor. The extract was concentrated and dried using rotary flash evaporator. It was kept in dessicator until further used.

Aqueous Extraction by Maceration:

Defined quantities of plant material were collected were collected; shade dried at room temperature, pulverized and was macerated with 3% chloroform water for seven days with occasional shaking to get aqueous extract. The aqueous extract was concentrated and dried using rotary flash evaporator. It was kept in dessicator until further used.

Phytochemical analysis of different extract:

Different chemical tests were carried out for both types of extracts to identify the presence of various chemical constituents like alkaloids, phytosterols, carbohydrates, terpenoids, saponins, flavanoid, phenolic compounds etc.

Determination of Antioxidant activity

DPPH Assay[7-9]

Free radical scavenging activity of different extracts was tested against a methanolic solution of 1, 1-diphenyl-2-picryl hydrazyl (DPPH). Antioxidants react with DPPH and convert it to 1-1-diphenyl -2-picryl hydrazine. The degree of discoloration indicates the scavenging potential of the antioxidant extract. The change in the absorbance produced at 517nm has been used as a measure of antioxidant activity.

The samples of different extracts were prepared in various concentrations viz. 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 µg/ml in AR grade methanol. 1 ml samples of above concentrations were mixed with equal volume of 0.1mM methanolic solution of DPPH (0.39mg in 10 ml methanol). An equal amount of methanol and DPPH was added and used as a control. Ascorbic acid solution of various concentrations viz. 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 µg/ml in distilled water were used as standard. After incubation for 20 minutes in dark, absorbance was recorded at 517 nm. Experiment was performed in triplicates. % scavenging was calculated by using the following formula:

$$\% \text{ Scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

A graph was plotted with concentration (µg/ml) on X axis and % scavenging on y axis and IC50 values were calculated, which represents the concentration of the scavenging compound that caused 50% neutralization.

Nitric oxide radical scavenging (NO) assay[10-11]

This procedure is based on the principle that sodium nitropruside in aqueous solution at physiological pH, spontaneously generates nitric oxide, which interact with oxygen to produce nitrite ions, which can be measured using a Griess reagent. Scavengers of NO compete with oxygen leading to reduced production of NO.

The samples of different extracts were prepared in various concentrations viz. 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml in AR grade methanol. 2 ml samples of above concentrations were mixed with 3 ml of solution of 10 mM sodium nitroprusside. The same reaction mixture without the methanolic extract of sample but with equivalent amount of methanol served as control. The reaction mixture was allowed to incubate at room temperature for 180 minutes. Ascorbic acid solution of various concentrations viz. 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml in distilled water were used as standard for comparison. After incubation the samples were reacted with Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 2% phosphoric acid). The absorbance of the chromophores formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine dichloride was read at 546 nm and referred to the absorbance of ascorbic Acid, used as a positive control treated in the same way with Griess reagent. Experiment was performed in triplicates. % scavenging was calculated by using the following formula:

$$\% \text{ Scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

A graph was plotted with concentration (µg/ml) on X axis and % scavenging on y axis and IC50 values was calculated.

Determination of Reducing Power[12-13]

The reducing power (or ability) describes how easily one substance can give electrons to another. A powerful reducing agent is keen to donate electrons. This method measures the ability of antioxidants to reduce ferric ion. Reducing power was investigated using the method developed by Oyaizu.

The samples of different extracts were prepared in various concentrations viz. 250, 500 and 1000 µg/ml in distilled water. 1.25 mL of sample aliquots was mixed with 1.25 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 1.25 mL of 1% potassium ferricyanide ($K_3Fe(CN)_6$). The mixtures were incubated at 50 ºC for 20 minutes. The resulting solution was cooled rapidly, mixed with 1.25 mL of 10% trichloroacetic acid and centrifuged at 3,000rpm for 10min. The supernatant (2.5 mL) was taken out and immediately mixed with 2.5 mL of distilled water and 500 µL of 1.0 % ferric chloride ($FeCl_3$) was then added. After incubation for 10 min, the absorbance (abs) against blank was determined at 700 nm. All samples were assayed in triplicate. Ascorbic acid standard was utilized for comparison.

Folin-Ciocalteau Total Phenolic Assay [14-15]

This assay measures the change in colour metal oxides are reduced by polyphenolic antioxidants such as gallic acid and catechin, resulting in a blue solution with maximal absorption at 765 nm. The standard curve is prepared using gallic acid, and results are reported as gallic acid equivalents. Total phenols were determined by Folin-Ciocalteu reagent. The Folin-Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenolic and polyphenolic antioxidants. However, this reagent does not only measure total phenols and will react with any reducing substance. The reagent therefore measures the total reducing capacity of a sample, not just the level of phenolic compounds.

A dilute sample of diffrent extract (0.5 ml of 1:10 g/ml) or gallic acid (standard phenolic compound) was mixed with Folin-Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined by colorimetry at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250, 300 mg /L solutions of gallic acid in methanol: water (50:50, v/v). The total phenolic content was expressed as mg/g equivalents of gallic acid using the following equation based on the calibration curve: $y = mx + c$ (y= absorbance, m = slope, x = concentration, c = intercept), which is a common reference compound.

Results and Discussion:

The average value of extractive of aqueous and ethanolic extract of *Boerhavia diffusa* and *Boswellia serrata* was found to be 4.8 % and 3.2% and 6.21% and 7.4% respectively.

The obtained extracts were subjected to phytochemical screening for its constituents by standard methods and the results were tabulated in table 1.

Table 1:- Phytochemical screening of leaves of *Boerhavia diffusa* and bark of *Boswellia serrata*

Test	*Boerhavia diffusa*		*Boswellia serrata*	
	Ethanolic Extract	**Aqueous Extract**	**Ethanolic Extract**	**Aqueous Extract**
Alkaloids	+	+	++	++
Carbohydrates	+	+	++	++
Phytosterols	-	+	+	+
Terpenoids	+	+	+	+
Saponins	-	-	-	-
Phenolic compounds	++	+	++	++
Flavonoids	++	+	++	++
Tannins	+	+	+++	++

(+ = Present, - = Absent)

In vitro tests of alcoholic and aqueous extracts of the plant materials evaluated for its antioxidant property revealed DPPH, Nitric oxide free radical, reducing power and total phenolic content effect.

The ability to scavenge the stable free radical DPPH was measured by decrease in the absorbance at 517 nm. The ethanol and water extracts of plant materials exhibited a significant dose dependent inhibition of DPPH activity (Table 2 & 3). A concentration dependent assay was carried out with these extracts and the results are presented in Fig.1 & 2. The amount of extract needed for 50% inhibition of DPPH free radical is known as IC50 value of the extract. Lower the IC50 value shows better scavenging ability of the sample. The IC50 value of aqueous extract of *Boerhavia diffusa* was not found because it unpredictable in the selected concentration range and for alcoholic extract, it was found to be 96.70µg/ml. The IC50 value of aqueous and alcoholic extract of *Boswellia serrata* was found to be 23.53 and 91.97µg/ml respectively. Hence, the aqueous extract of *Boswellia serrata* has more prominent scavenging activity rather than other extracts.

Table 2:- DPPH radical scavenging of leaves of *Boerhavia diffusa*.

S. No.	Conc. (µg/ml)	% Scavenging		
		Aqueous extract	**EtOH extract**	**Ascorbic acid**
1	2	0.31±0.52	0.32±0.95	9.56±0.47
2	4	3.2±0.28	12.22±0.35	28.24±0.28
3	8	4.25±0.37	13.66±0.37	52.08±0.37
4	16	4.61±0.57	14.39±0.46	89.76±0.43
5	32	6.43±0.62	32.99±0.54	93.27±0.72
6	64	11.25±0.37	33.85±0.39	95.73±0.37
7	128	22.31±0.94	61.94±0.59	95.92±0.81
8	256	26.42±0.12	71.46±0.34	95.81±0.49
9	512	32.11±0.61	72.24±0.66	87.17±0.43
10	1024	34.24±0.28	55.03±0.58	83.58±0.28

All values in this table represent the mean ±SD (n=3).

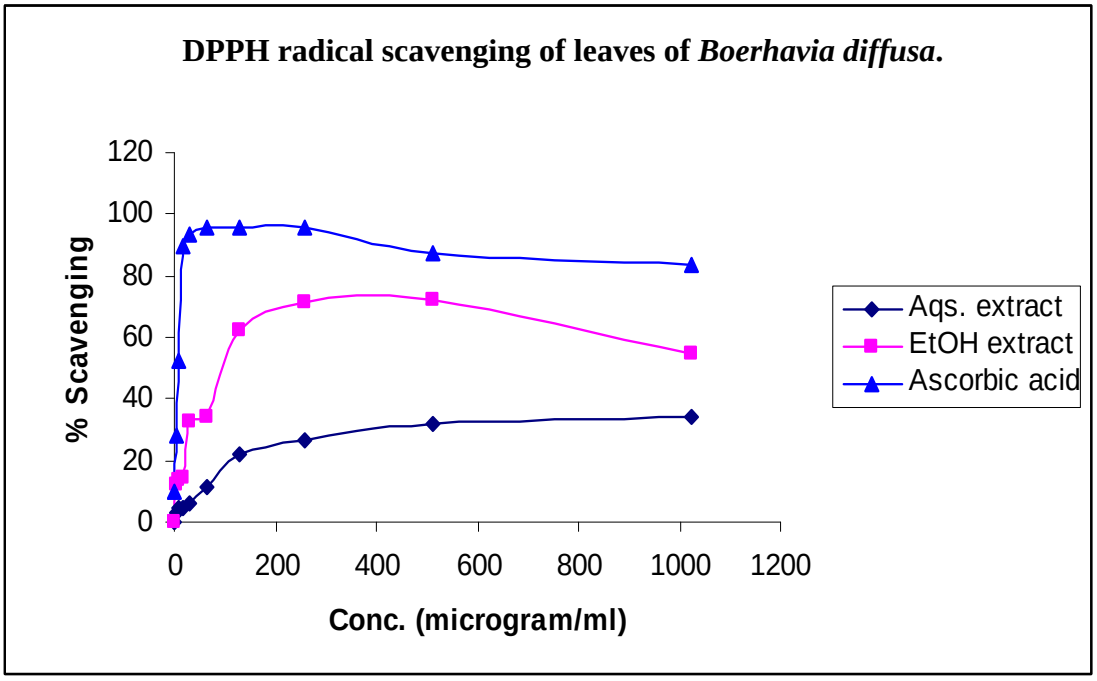

Fig. 1:- DPPH radical scavenging of leaves of *Boerhavia diffusa*.

Table 3:- DPPH radical scavenging of bark of *Boswellia serrata*.

S. No.	Conc. (µg/ml)	% Scavenging		
		Aqueous extract	EtOH extract	Ascorbic acid
1	2	7.25±0.23	4.22±0.51	9.56±0.47
2	4	22.56±0.51	12.34±0.56	28.24±0.28
3	8	38.41±0.55	21.24±0.37	52.08±0.37
4	16	44.22±0.37	31.11±0.66	89.76±0.43
5	32	57.64±0.35	39.64±0.52	93.27±0.72
6	64	71.28±0.47	44.56±0.23	95.73±0.37
7	128	95.26±0.44	57.12±0.38	95.92±0.81
8	256	96.24±0.64	66.31±0.29	95.81±0.49
9	512	96.57±0.28	71.22±0.51	87.17±0.43
10	1024	96.71±0.94	72.45±0.18	83.58±0.28

All values in this table represent the mean ±SD (n=3).

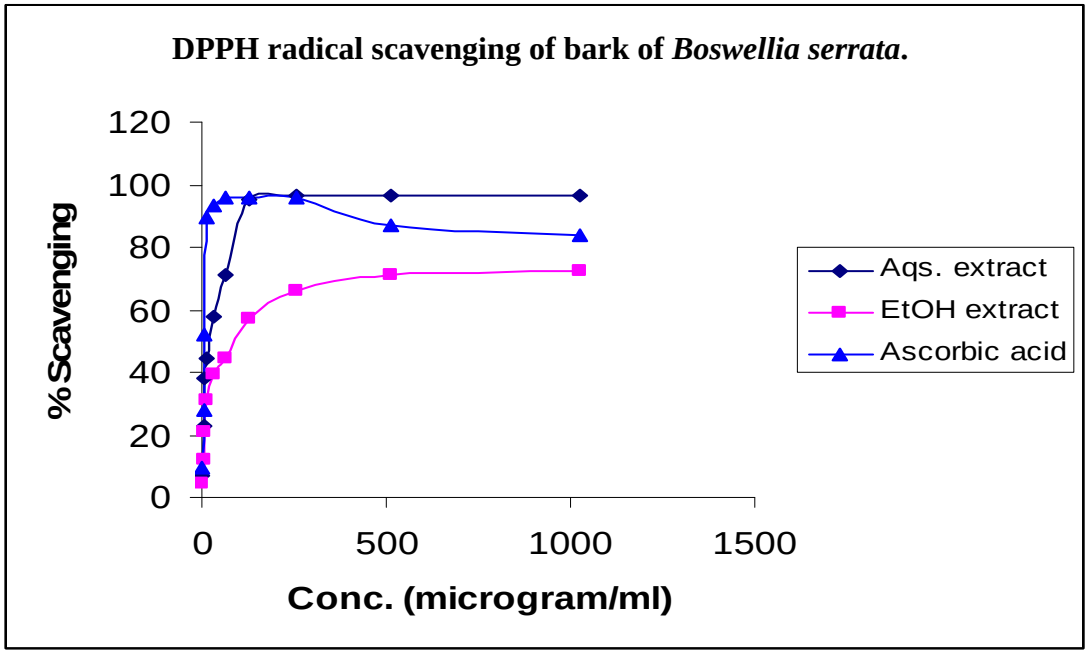

Fig. 2:- DPPH radical scavenging of bark of *Boswellia serrata*.

Nitric oxide (NO) is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities. Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with superoxides, such as NO_2, N_2O_4, N_3O_4, NO_3, and NO_2 are very reactive. These compounds are responsible for altering the structural and functional behavior of many cellular components[11]. Table 4 & table 5 illustrate the percentage inhibition of nitric oxide generation by different extracts of *Boerhavia diffusa* and *Boswellia serrata* respectively. A dose dependent scavenging of NO free radicals by different extracts of *Boerhavia diffusa* and *Boswellia serrata* was shown in fig. 3 & 4 respectively. The IC_{50} value for NO inhibition of aqueous extract of *Boswellia serrata* was found to be lowest among the extracts (69.67µg/ml). Hence, the aqueous extract of *Boswellia serrata* has more prominent scavenging activity of NO free radicals rather than other extracts.

Table 4:- Nitric oxide scavenging activity of leaves of *Boerhavia diffusa*.

S. No.	Conc. (µg/ml)	% Scavenging		
		Aqueous extract	EtOH extract	Ascorbic acid
1	20	14.67±0.76	9.87±0.62	18.64±0.62
2	30	26.87±0.52	14.79±0.67	28.24±0.52
3	40	21.24±0.11	18.84±0.59	57.08±0.31
4	50	31.11±0.32	22.78±0.27	86.76±0.94
5	60	39.64±0.19	32.54±0.15	93.27±0.27
6	70	44.56±0.28	40.97±0.26	95.73±0.26
7	80	57.12±0.76	54.33±0.39	95.92±0.19
8	90	66.31±0.52	69.72±0.19	95.89±0.54
9	100	71.22±0.46	74.66±0.52	96.06±0.67

All values in this table represent the mean ±SD (n=3).

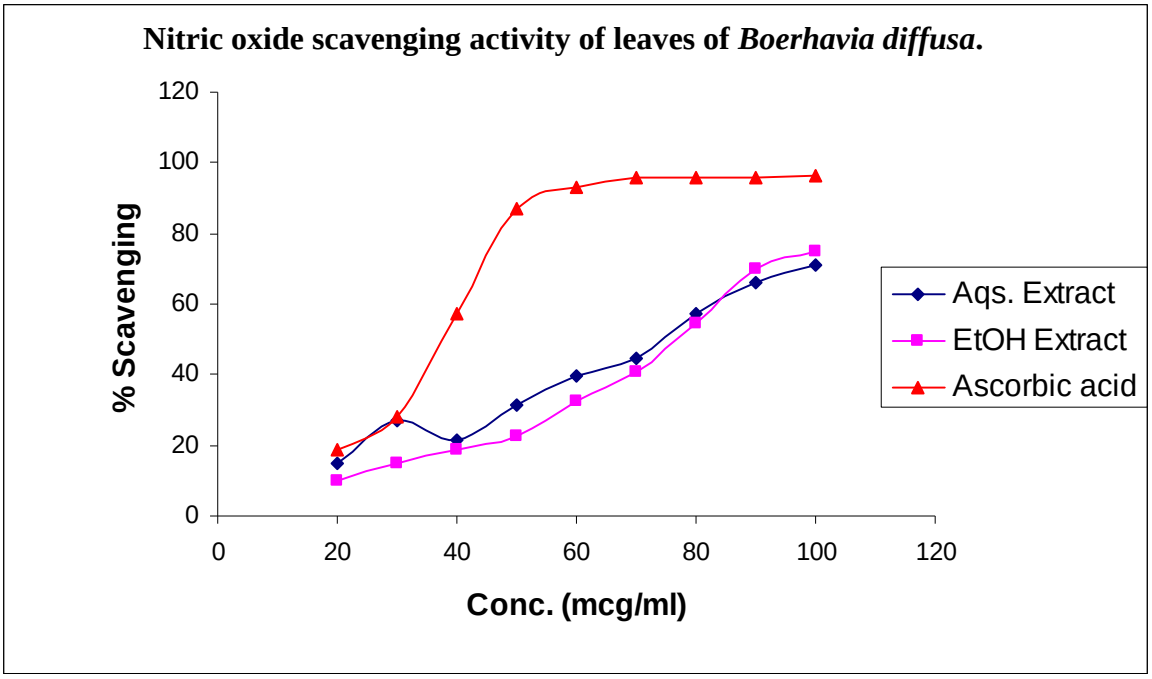

Fig. 3:- Nitric oxide scavenging activity of leaves of *Boerhavia diffusa*.

Table 5:- Nitric oxide scavenging activity of bark of *Boswellia serrata*.

S. No.	Conc. (µg/ml)	% Scavenging		
		Aqueous extract	EtOH extract	Ascorbic acid
1	20	14.87±0.37	12.82±0.35	18.64±0.62
2	30	18.74±0.34	17.22±0.48	28.24±0.52
3	40	26.97±0.66	25.39±0.75	57.08±0.31
4	50	33.47±0.38	36.21±0.95	86.76±0.94
5	60	39.95±0.25	40.28±0.56	93.27±0.27
6	70	53.83±0.25	47.29±0.66	95.73±0.26
7	80	65.22±0.39	53.27±0.37	95.92±0.19
8	90	71.09±0.48	63.82±0.26	95.89±0.54
9	100	78.27±0.26	70.11±0.55	96.06±0.67

All values in this table represent the mean ±SD (n=3).

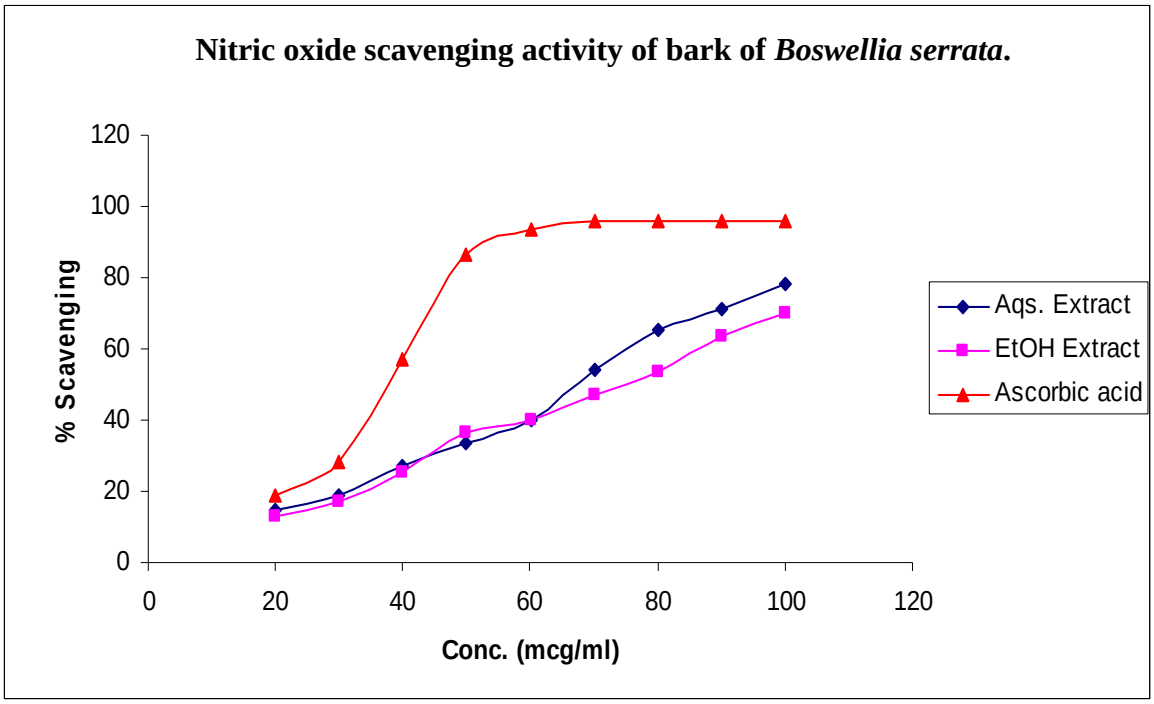

Fig. 4:- Nitric oxide scavenging activity of bark of *Boswellia serrata*.

The reducing power of the plant extracts involved the transformation of Fe^{3+} to Fe^{2+}. The reducing ability of a molecule may serve as an indicator of its potential antioxidant ability[12]. Table 6 showed the reducing capacity of aqueous and ethanolic extracts of leaves of *Boerhavia diffusa* and bark of *Boswellia serrata* in comparison with ascorbic acid used as a standard. The ethanol extracts of leaves of *B. diffusa* showed higher reducing power as compared to water extracts and reducing capability increases as the concentration increased. At concentration 1 mg/mL *Boswellia serrata* extracts shows poor reducing ability, but we can get better reducing capability at higher concentrations.

Table 6:- Reducing power ability of leaves of *Boerhavia diffusa*.

Conc. (mg/ml)	***Boerhavia diffusa* (Absorbance)**		***Boswellia serrata* (Absorbance)**		**Ascorbic acid (Absorbance)**
	Aqueous extract	**EtOH extract**	**Aqueous extract**	**EtOH extract**	
0.25	0.1701±0.22	0.2016±0.39	0.0701±0.34	0.1241±0.28	0.4122±0.94
0.50	0.3401±0.56	0.3864±0.17	0.1433±0.29	0.2395±0.94	0.8172±0.35
1.0	0.6804±0.48	0.7893±0.28	0.2862±0.33	0.4875±0.17	1.6545±0.49

All values in this table represent the mean ±SD (n=3).

Phenolic compounds are commonly found in both edible and inedible plants and plant parts. They have been reported to have multiple biological effects, including antioxidant activity. The content of phenolic compounds (mg/g GAE) in ethanolic and water extract was determined from regression equation of calibration curve ($y = 0.0029x + 0.0903$, $R^2 = 0.9725$) and expressed in gallic acid equivalents (GAE). The total phenolic content was found 25.37 and 25.23 mg/g GAE for *B. diffusa* extracts in ethanol and water respectively. The in vitro antioxidative effect of the aqueous extract of *B. diffusa* may be due to the phenolic components. The aqueous and ethanolic extracts of *Boswellia serrata* showed the presence of phenolic contents (28.46 and 12.73 mg/g GAE respectively) that supports their antioxidant activity.

Conclusion:

Finally, it could be concluded that studied plant extracts possessed variable but interesting antioxidant properties. These properties were significantly correlated to their total phenolics content and they could be used as a source of natural antioxidants in food, cosmetic and pharmaceutical industries. From the general point of view, the activity of these plants must be tested individually in different food systems and breakdown products under different conditions must be investigated. It could also be necessary that full structural identification of the active components of antioxidant compounds of plants is, therefore, required and their toxicological properties be investigated.

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