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Research Article

IN-VITRO ANTIOXIDANT ACTIVITY AND FREE RADICAL SCAVENGING POTENTIAL OF ROOTS OF RED SAGE

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ABSTRACT

Objective: The objective of the present work is to study the *in-vitro* anti-oxidant activities of petroleum ether, ethyl acetate and methanolic extracts of *Salvia splendens* roots.

Methods: The extracts were studied using 1,1-diphenyl-2-picryl hydrazyl, hydrogen peroxide (H_2O_2), total phenolic content (TPC) and total flavonoid content (TFC). The TPC and TFC were estimated taking gallic acid and rutin calibration curve respectably.

Results: All the extracts possess *in-vitro* anti-oxidant activities. However, the order of possessing activities were methanolic > ethyl acetate > petroleum ether extracts of *S. splendens* roots. The TPC and TFC was highest in methanolic extract.

Conclusion: It can be concluded that *S. splendens* roots extracts possess anti-oxidant activities. The methanolic extract of *S. splendens* roots possess highest anti-oxidant activity *in-vitro*.

Keywords: *Salvia splendens*, *In-vitro* anti-oxidant, 1,1-diphenyl-2-picryl hydrazyl, Ferric reducing power activity, Hydrogen peroxide scavanging, Total phenolic content, Total flavonoid content

INTRODUCTION

Oxidative damage to cellular biomolecules such as lipids, proteins and DNA is thought to play a crucial role in the incidence of several chronic diseases [1-5]. Flavonoids are a group of polyphenolic compounds found abundantly in the plant kingdom. Interest in the possible health benefits of flavonoids and other polyphenolic compounds has increased in recent years owing to their potent antioxidant and free-radical scavenging activities [6-12].

The effects of free radicals on human beings are closely related to toxicity, disease and aging [1] Most living species have an efficient defense system to protect themselves against the oxidative stress induced by reactive oxygen species [2]. Recent investigations have shown that the antioxidant properties of plants could be correlated with oxidative stress defense and different human diseases including cancer, atherosclerosis and the aging process [3-5]. The antioxidants can interfere with the oxidation process by reacting with free radicals, chelating free catalytic metals and also by acting as oxygen scavengers.

Salvia splendens of family Lamiaceae/Labiatae (Mint family) is commonly known as Scarlet sage [13-15]. It also reported the activity like analgesic and anti-inflammatory of roots, anti-ulcerative activity, antimicrobial activity, laxative activity, anti-oxidant, hepatoprotective and anti-hyperlipidemic activity have also been reported [13-19].

Hence, the present investigation was conducted to study *in-vitro* antioxidant activities of various roots extracts so as to make researcher to route for other pharmacological activities.

METHODS

Plant material, authentication and extraction procedures

S. splendens plant were collected from Bhopal (Madhya Pradesh) and Hazaribag, (Jharkhand) and was authenticated by Dr. V.P. Prasad, Scientist-C, Botanical Survey of India, Government of India, Howrah, (West Bengal). The specimen no. PY/JVD 1026/2011 had been submitted to Faculty of Pharmaceutical sciences, Jyoti Vidhyapeeth Women's University, Jaipur (Rajasthan). The air-dried roots were made into coarse powder and extracted with methanol, ethyl acetate and petroleum ether and percentage yield were calculated. The dried roots were extracted with Hot continuous soxhlet apparatus for 72 hrs with three different solvents i.e. methanol, ethyl acetate and petroleum ether and concentrated to dryness under reduced temperature.

Preliminary phytochemical analysis

The various extracts of *S. splendens* were tested for different phytoconsituents such as alkaloids, glycosides, saponinins, tannins, terpinoids, phenolic compounds, protein, carbohydrates using standard procedures [20].

In-vitro anti-oxidant activity

1,1-diphenyl-2-picryhydrazyl (DPPH) radical scavenging activity

The DPPH assay of *S. splendens* extract was determined by the method as reported by Patil *et al*. (2009).

The procedure involved UV-spectrophotometric determination. Three solutions i.e. standard, test and control were prepared.

Preparation of standard ascorbic acid solutions

Different solutions (1-10 µg/ml) of the ascorbic acid were prepared in methanol. 1.5 ml of each solution of ascorbic acid were mixed with 1.5 ml of 200 µM DPPH solution and incubated for 30 minutes at room temperature in dark. Absorbance of each solution was taken after 30 minutes against methanol (as blank) at 517 nm [21,22].

Preparation of test solutions

Different solutions of the *S. splendens* extract were prepared in methanol to give concentrations (10-100 µg/ml). 1.5 ml of each solution of *S. splendens* extract was mixed with 200 µM DPPH solution and incubated for 30 minutes at room temperature in dark. Absorbance of each solution of *S. splendens* extract was taken after 30 minutes against methanol (as blank) at 517 nm [21,22].

Preparation of control solution

For control, 1.5 ml of methanol was mixed with 200 µM DPPH solution and incubated for 30 minutes at room temperature in dark. Absorbance of the control was taken after 30 minutes against methanol (as blank) at 517 nm.

Percentage inhibition was calculated using equation (1), while IC_{50} values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm. The data were presented as mean values ± standard deviation (n=3) [21,22].

$$I\% = \frac{Ac - (At - Ab)}{Ac} \times 100$$

Where Equation (1)

I% = Percentage inhibition

Ac = Absorbance of control (methanol and 200 µM DPPH solution)

At = Absorbance of ascorbic acid/plant extract with 200 µM DPPH solution after 30 minutes.

Ab = Absorbance of ascorbic acid/plant extract without 200 µM DPPH solution.

Ferric reducing power activity

This method is based on the principle of increase in the absorbance of the reaction mixture. Increase in the absorbance indicates increase in the antioxidant activity. Substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium Fe^{2+}, which then react with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. Increase in absorbance of the reaction mixture indicates the reducing power of the Samples [23].

Antioxidant= Potassium ferricyanide + Ferric chloride→Potassium ferrocyanide + Ferrous chloride

Preparation of standard ascorbic acid solutions

Different concentrations of the ascorbic acid were prepared in distilled water to give solutions of concentration (20-100 µg/ml). 1ml of each concentration of ascorbic acid solutions were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide solution. The reaction mixtures were incubated for 20 minutes at 50°C. Afterwards 2.5 ml of 10% trichloroacetic acid solution was added and centrifuged at 560 × g for 10 minutes. After separation, 2.5 ml of upper layer of each solution were mixed with 2.5 ml of distilled water and 1 ml of 0.1% ferric chloride (freshly prepared solution). Absorbance was recorded for each solution of ascorbic acid against (0.2 M, pH 6.6) phosphate buffer (used as blank) at 700 nm [23].

Preparation of test solutions

Different solutions of extract were prepared in distilled water to give various concentrations (20-100 µg/ml). 1 ml of each solution of plant part extract was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide solution. The reaction mixtures were incubated for 20 min at 50°C. Afterwards 2.5 ml of 10% trichloroacetic acid solution was added and centrifuged at 560 × g for 10 minutes. After separation, 2.5 ml of upper layer of each solution were mixed with 2.5 ml of distilled water and 1 ml of 0.1% ferric chloride (freshly prepared solution). Absorbance was recorded for each solution of plant part extract against (0.2 M, pH 6.6) phosphate buffer (used as blank) at 700 nm.

The absorbance versus concentration curve for ascorbic acid and extract was plotted. The 'Y' and 'R^2' values obtained in both curve and the cases were comparatively studied to determine the reducing power of extract [23].

Hydrogen peroxide (H_2O_2) scavenging activity

H_2O_2 is a biologically important oxidant because of its ability to generate the hydroxyl radical (•OH) which is extremely potent. The ability of the •OH to remove or add hydrogen molecules to unsaturated hydrogen bonds of organic lipids makes it potentially one of the most reactive oxidants in biological systems. It's very short half-life (1×10^{-9} at 37°C), however, restricts its diffusion capability and potency.

The ability of the *S. splendens* extract to scavenge H_2O_2 was determined according to the method reported by Neha Panday *et al.* (2011).

The procedure involved UV-spectrophotometric determination of H_2O_2 radical scavenging. Three solutions i.e. standard, test and control were prepared.

Preparation of standard ascorbic acid solutions

Different concentrations (10-100 µg/ml) of the ascorbic acid were prepared in distilled water. 1ml of each solution of ascorbic acid was mixed with 2 ml of 0.1 M phosphate buffer solution and 600 µl of 100 mM H_2O_2 solution. After 10 minutes absorbance of different concentration of ascorbic acid solutions was taken at 230nm [24].

Preparation of test solutions

Various concentrations (10-100 µg/ml) of the *S. splendens* aq. extract were prepared in distilled water. 1 ml of each solution of aq. extract was mixed with 2 ml of 0.1 M phosphate buffer solution and 600 µl of 100 mM H_2O_2 solution. After 10 minutes (approximately) absorbance of different concentration of *S. splendens* extract solutions were taken at 230 nm [24].

Preparation of control solution

For control 2 ml of 0.1 M phosphate buffer solution was mixed with 600 µl of 100 mM H_2O_2 solution. After 10 minutes absorbance of control was taken at 230 nm.

The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples using Equation 1. IC_{50} values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm [24].

$$I\% = \frac{Ac - (At - Ab)}{Ac} \times 100$$

Where,

I% = Percentage inhibition.

Ac = Absorbance of control (0.1 M phosphate buffer solution and H_2O_2).

At = Absorbance of ascorbic acid/plant extract with H_2O_2 after 10 minutes.

Ab = Absorbance of ascorbic acid/plant extract without H_2O_2.

Estimation of total phenolic content (TPR)

The amount of total TPC in extracts was determined with the Folin–Ciocalteu reagent. Gallic acid (GA) was used as a standard and the total phenolic were expressed as mg/g GA equivalent (GAE). Concentration of 0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml of GA were prepared in methanol. Concentration of 0.1 and 1 mg/ml of plant extract were also prepared in methanol and 0.5 ml of each sample were introduced in to test and mixed with 2.5 ml of a 10 fold dilute Folin–Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The tubes were covered with parafilm and allowed to stand for 30 minutes at room temperature before the absorbance was at read at 760 nm spectrometrically. All determination was performed in triplicate. The Folin–Ciocalteu reagent is sensitive to reducing compounds including polyphenols. They produce a blue color upon reaction. This blue color was measured spectrophotometrically.

Line of regression from GA was used for estimation of unknown phenol content. From standard curve of GA line of regression was found to be

$y = 0.005x + 0.065$ and $R^2 = 0.976$

Thus the goodness of fit was found to be good for selected standard curve. By putting the absorbance of test sample (y = absorbance) in line of regression of above mentioned GA [25].

Total flavonoids determination

Total flavonoids were measured by a colorimetric assay according to Dewanto *et al*. An aliquot of diluted sample or standard solution of rutin was added to a 75 µl of $NaNO_2$ solution, and mixed for 6 minutes, before adding 0.15 mL $AlCl_3$ (100 g/L). After 5 minutes, 0.5 mL of NaOH was added. The final volume was adjusted to 2.5 ml with distilled water and thoroughly mixed. Absorbance of the mixture was determined at 510 nm against the same mixture, without the sample, as a blank. Total flavonoid content was expressed as mg rutin/g dry weight (mg rutine/g DW), through the calibration curve of rutin. All samples were analyzed in three replications.

Line of regression from rutin was used for estimation of unknown flavonoid content. From standard curve of rutin, line of regression was found to be

$y = 0.001x - 0.118$ and $R^2 = 0.985$

Thus the goodness of fit was found to be good for selected standard curve. By putting the absorbance of test sample (y = absorbance) in line of regression of above mentioned rutin [26-28].

RESULTS

The successive solvent extraction was done using petroleum ether, ethyl acetate and methanol using standard procedure. The behavior of various extracts like texture and colour and extractive yield were calculated.

DPPH free radical scavenging activity

The DPPH radical scavenging activity of petroleum ether extract of *S. splendens* (PEESS), ethyl acetate extract of *S. splendens* (EAESS) and methanol extract of *S. splendens* (MESS) roots were detected and compared with ascorbic acid. The percentage inhibition (% inhibition) at various concentration (10-100 µg/ml) of PEESS, EAESS and MESS as well as standard ascorbic acid (1-10 µg/ml) were calculated and plotted in Fig. 1. The IC_{50} values of ascorbic acid were 25.07 µg/ml), PEESS (126.25 µg/ml), EAESS (70.88 µg/ml) and MESS (67.61 µg/ml).

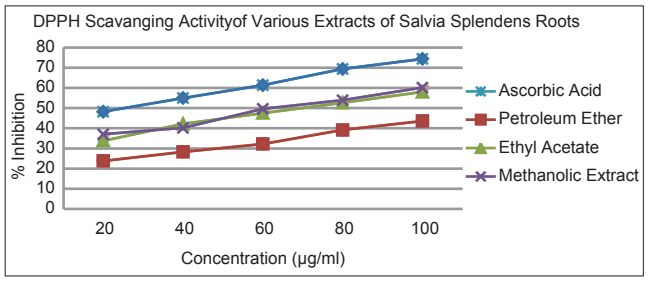

Fig. 1: 1,1-diphenyl-2-picryhydrazyl scavanging activity of different *Salvia splendens* roots extracts

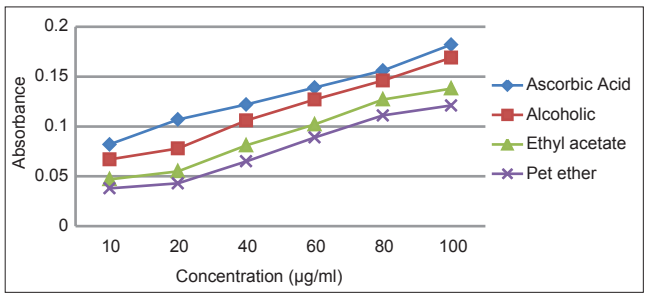

Fig. 2: Reducing power activity of different *Salvia splendens* roots extracts

Ferric reducing power activity

The reductive capabilities of PEESS, EAESS and MESS roots were detected and compared with ascorbic acid. The mean absorbance at various concentration (20-100 µg/ml) of PEESS, EAESS and MESS as well as standard Ascorbic acid (20-100 µg/ml) were calculated and plotted in Fig. 2. The reductive capabilities were found to increase with increasing of concentration in various extract as well as standard ascorbic acid.

H_2O_2 scavenging activity

The H_2O_2 scavenging activity of PEESS, EAESS and MESS roots were detected and compared with ascorbic acid. The percentage inhibition (% inhibition) at various concentration (10-100 µg/ml) of PEESS, EAESS and MESS as well as standard Ascorbic acid (10-100 µg/ml) were calculated and plotted in Fig. 3. The IC_{50} values are calculated from graph and were found ascorbic acid (36.27 µg/ml), PEESS (145.13 µg/ml), EAESS (95.57 µg/ml) and MESS (64.11 µg/ml).

Total phenolic contents (TPC)

The TPC in PEESS, EAESS and MESS roots were estimated using standard GAE of phenols. The various concentration of GA (10-50 µg/ml) calibration curve was plotted and the results were given in Table 1 and in Fig. 4. The TPC for PEESS, EAESS and MESS were obtained for 1 mg/ml of extracts from TPC calibration of GA and the result are given in Table 2. The phenolic compounds are absent in the petroleum ether. The TPC for EAESS and MESS were calculated using standard calibration curve ($y = 0.007x + 0.056$, $R^2 = 0.995$) and found to have 202.06±0.611and 213.0±0.721 mg/g equivalent of GA respectably.

Total flavanoid content (TFC)

The TFC in PEESS, EAESS and MESS roots were estimated using standard rutin equivalent of phenols. The various concentration of rutin (25-100 µg/ml) calibration curve was plotted and the results were given in Table 3 and in Fig. 5. The TFC for PEESS, EAESS and MESS

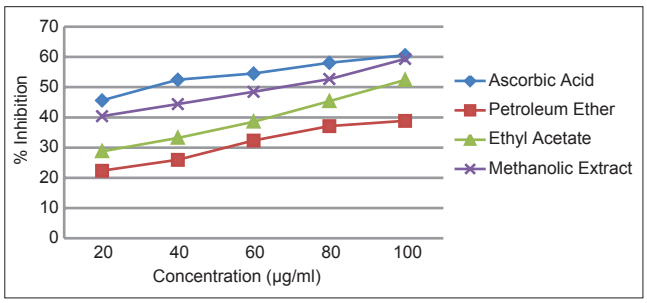

Fig. 3: Hydrogen peroxide scavenging activity of different *Salvia splendens* roots extracts

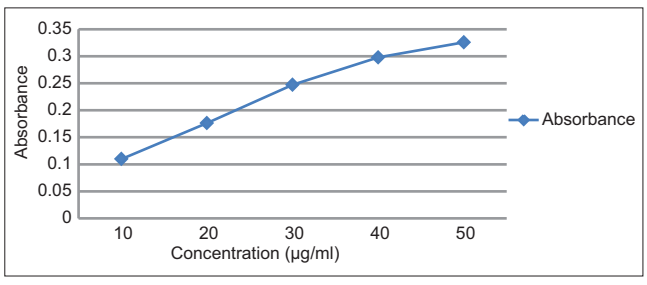

Fig. 4: Standard gallic acid curve for total phenolic content

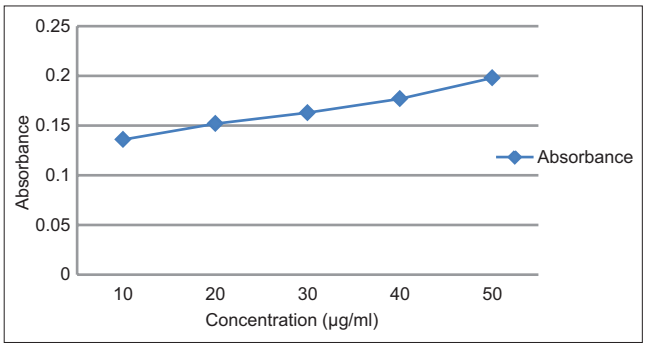

Fig. 5: Standard curve for rutin for total flavanoid content

Table 1: TPC of calibration of standard curve of GA

Serial no.	Concentration (ug/ml)	Absorbance
1	10	0.1098
2	20	0.1763
3	30	0.2471
4	40	0.2979
5	50	0.3258

TPC: Total phenolic content, GA: Gallic acid

Table 2: TPC of different extracts of *S. splendens*

Extracts	Concentration (mg/ml)	TPC (mg/g GAE)
EAESS	1 mg/ml	202.06±0.611
MESS	1 mg/ml	213.0±0.721

Values are in mean±SD for three readings, TPC: Total phenolic content, *S. splendens: Salvia splendens*, SD: Standard deviation, MESS: Methanol extract of *Salvia splendens*, EAESS: Ethyl acetate extract of *Salvia splendens*, GAE: Gallic acid equivalent

Table 3: TFC of calibration of standard curve of rutin

Serial no.	Concentration (µg/ml)	Absorbance
1	10	0.136
2	20	0.152
3	30	0.163
4	40	0.177
5	50	0.198

TFC: Total flavanoid content

Table 4: TFC of different extracts of *S. splendens*

Extracts	Concentration (mg/ml)	TFC in mg/g equivalent of rutin
EAESS	1 mg/ml	121.66±3.055
MESS	1 mg/ml	148.66±2.516

TFC: Total flavanoid content, values are in mean±SD for three readings, SD: Standard deviation, *S. splendens: Salvia splendens*, MESS: Methanol extract of *Salvia splendens*, EAESS: Ethyl acetate extract of *Salvia splendens*

were obtained for 1000 µg/ml of extracts from TFC calibration of rutin and the result are given in Table 4. The phenolic compounds are absent in the petroleum ether. The TFC for EAESS and MESS were calculated using standard calibration curve (y = 0.001x+ 0.120, R^2 = 0.998) and found to have 92.33±3.055, and 115.33±1.154 mg/g equivalent of rutin respectably.

DISCUSSION

The *S. splendens* roots were made coarse powder and extracted with using petroleum ether, ethyl acetate and methanol as solvent using standard procedure.

The various extracts of *S. splendens* roots were tested for different phytoconsituents like alkaloids, glycosides, saponinins, tannins, terpinoids, reducing sugars, phenolic compounds, flavonoids, protein, carbohydrates and volatile oils. The phenolic and flavonoids are widely distributed secondary metabolites in plants having anti-oxidant activity and have wide range of biological activities as anti-apoptosis, anti-aging, anti-carcinogen, anti-inflammation, anti-atherosclerosis, cardiovascular protection and improvement of endothelial function, as well as inhibition of angiogenesis and cell proliferation activities [29,30]. Recent studies have shown that many dietary polyphenolic constituents derived from plants are more effective antioxidants *in-vitro* than vitamins E or C, and thus might contribute significantly to the protective effects *in vivo* [31].

In-vitro antioxidant studies are widely carried to screen various plant containing phenolic and flavonoids constituents. Plant derived antioxidant compounds, flavonoids and phenolics have received considerable attention because of their physiological effect like antioxidant, anti-inflammatory, antitumor activities and low toxicity compared with those of synthetic phenolics antioxidant such as butylated hydroxyanisole, butylated hydroxytoluene and Propyl gallate [32,33].

DPPH is a purple colored stable free radical; when reduced it becomes the yellow-colored diphenyl-picryl hydrazine. DPPH radicals react with suitable reducing agents and then electrons become paired-off and the solution loses colour stoichimetrically with the number of electrons taken up [34]. Such reactivity has been widely used to test the ability of compounds/plant extracts to act as free radical scavengers [35]. In this present study, the DPPH radical scavenging activity of MESS, PEESS and EAESS roots were detected and compared with ascorbic acid. The IC_{50} values for DPPH assay of for methanolic extract was found maximum, followed by ethyl acetate extract and for petroleum ether extract was minimum. Though the extracts showed good DPPH scavenging activity, but it was less effective than standard ascorbic acid. The difference of activity is due to presence of phenolic components in different extracts. Thus, choosing the appropriate solvent is one of the most important factors for obtaining extracts with a high content of bioactive compounds and antioxidant activity [35].

In ferric reducing antioxidant power assay, a yellow color of the test solution changes to various shades of green and blue is depending on the reducing power of each compound. The presence of radicals (i.e. antioxidant) causes the conversion of the Fe^{3+}/ferricyanide complex used in this method to the ferrous form. Therefore by measuring the formation of pearls prussian blue spectroscopically, the Fe^{2+} concentration can be monitored; a higher absorbance indicates a higher reducing power. The reductive capabilities of PEESS, EAESS and MESS roots were detected and compared with ascorbic acid. The methanolic extract showed highest reducing power, followed by ethyl acetate extracts and petroleum ether extracts respectively. The increased reducing power in the extracts indicated that some components in the extract were electron donors that could react with the free radicals to convert them into more stable products to terminate radical chain reaction. Antioxidants are strong reducing agents and this is principally based on the redox properties of their hydroxyl groups and the structural relationships between different parts of their chemical structure [36,37].

H_2O_2, a biologically relevant, non-radical oxidizing species, may be formed in tissues through oxidative processes. H_2O_2 which in turn generate •OH resulting in initiation and propagation of lipid peroxidation [37]. The H_2O_2 scavenging activity of PEESS, EAESS and MESS roots were detected and compared with ascorbic acid. The IC_{50} values for H_2O_2 scavenging activity of for methanolic extract was found maximum followed by ethyl acetate extract and for petroleum ether extract was minimum. Though the extracts showed good H_2O_2

scavenging activity but it was less effective than standard ascorbic acid. The ability of the extracts to quench OH_ seems to be directly related to the prevention of the lipid peroxidation and appears to be moderate scavenger of active oxygen species, thus reducing rate of chain reaction [38].

The TPC in PEESS, EAESS and MESS roots were estimated using standard GAE of phenols. The phenolic compounds are absent in the petroleum ether. The TPC for EAESS and MESS were found to have 202.06 and 213.00 mg/g equivalent of GA respectably. The methanolic extract was found to have maximum phenolic components and which may be one the reason of its to possess maximum antioxidant activity then other two extracts [39].

But in TFC, it was found methanolic extract to possess maximum 148.66 mg/g equivalent of rutin then other ethyl acetate (121.66 mg/g Eq). Flavonoids play some important pharmacological roles against diseases, such as cardiovascular disease, cancer, inflammation and allergy and other oxidative stress related diseases [39].

From, above discussion, it was clear that the most powerful anti-oxidant extract is MESS roots.

CONCLUSION

It can be concluded that *S. splendens* roots extracts possess anti-oxidant activities and the potency of anti-oxidant activities depends on the type of extract. The MESS roots possess highest anti-oxidant activity *in-vitro*. This anti-oxidant power depends on total phenolic and flavonoid contents on particular extract.

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