Title: Evaluation of Free Radical Scavenging Activity of *Calpurnia aurea* Root Extract by Using Methanol Solvent for DPPH Selectively

Dula Daksa Ejeta

Keywords: Absorbance, Free radical scavenging*;* DPPH; *Calpurnia aurea, In vitro,* % of Inhibition*,* Qualitative Analysis, Quantitative Analysis, Radical Scavenging, Spectrophotometric Method, TLC Method

Abstract

In Ethiopia, any part of *Calpurnia aurea* is used for the treatment of different ailments. Hence; the researcher was interested to evaluate the *in vitro* scavenging potential of the four root extracts of *Calpurnia aurea* by using methanol as DPPH solvent selectively and Ascorbic acid as a standard reference. The methanol and ethanol extracts of the root part of *Calpurnia aurea* have shown better free radical scavenging activity at 100 µg/mL 67.3% for and 57.8% for ethanol when compared to the standard reference (ascorbic acid, 92.5%) and other extracts (chloroform and n-hexane i.e. 25.1% and 15.8%, respectively) at the same concentration. n-hexane root extract comparatively showed very weak activity. Therefore, it is evident from this study that free radical scavenging potential of the plant species adds its value to be quantified for application in pharmaceutical industry.

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INTRODUCTION

Free radical and reactive oxygen species (ROS) are basically the main cause of several disorders in humans that are generated as an imbalance between formation and neutralization of perooxidants resulting in oxidative stress. They cause oxidative damage to lipids, proteins and DNA, eventually leading to many chronic diseases, such as cancer, diabetes, aging, and other degenerative diseases in humans along with lipid peroxidation. To protect the adverse effects of free radicals, human cells generate enzymes such superoxide dismutase (SOD) and catalase or compounds such as ascorbic acid, tocopherol, and glutathione [1].

Plants are rich in free radical scavengings; so much attention has been directed towards the development of ethnomedicines as they contain phenols, flavonoids, alkaloids, tannins, vitamins, terpenoids, and many more phytochemicals responsible for different pharmacological activities. Current research has proved that ingestion of natural free radical scavengings has been associated with reduced risk of cancer and many chronic diseases [1; 2].

Calpurnia aurea is a genus of Flowering Plants within the family of *fabaceae*. The genus comprises shrubs or small trees in or along the margin of forests in many parts of Ethiopia and widely distributed in Africa from

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Cape Province to Eritrea and which also occurs in Southern India [3]. Literature survey brings to light that, all parts of the plant species has been used for different human and animal disease [4]. In native countries like Ethiopia, traditionally, the leave and powdered roots of *Calpurnia aurea* is used for the treatment of syphilis, malaria, rabies, diabetes, lung TB, hypertension, diarrhoea, leishmaniasis, trachoma, elephantiasis, fungal diseases, different swellings, stomach-ache, abscesses, bowel, bladder disorders, to destroy maggots, to destroy lice, to relieve itches, used as a fish-poison or as a cure for dysentery, exhibit activity against amoebiasis and giardiasis, cough and snake bite [5]. Plant products have been part of phytomedicines since time immemorial. These can be derived from any part of the plant like stem bark, leaves, flowers, seeds and root i.e., any part of the plant may contain active components. Knowledge of the chemical constituents of plants is desirable because such information will be of value for the synthesis of complex chemical substances [6].

Thus, here in this study, qualitative and quantitative free radical scavenging activities analysis of the root extracts of *Calpurnia aurea* were assessed by using Spectrophotometric and Thin Layer Chromatography (TLC) methods. I hope that the findings from this work may add to the overall value of the medicinal potential of the plant species.

Materials and Methods

The plant sample of the study was collected from the compound of Nekemte College of Teacher Education (East Wollega Zone, Nekemte, Ethiopia) at the end of September 2017 and the identity of the plant was confirmed by botanical scholars from Wollega University Biological Science Department with the reference of National Museums of Ethiopia Herbarium. Appropriate voucher specimen designated was deposited at the School of Botanical science, Wollega University.

Extraction

The whole root part was washed in tap water and cut in to small bits to facilitate drying. After weighing the wet sample, it was hot-air oven-dried below 55°C for 14 h until it came to constant weight. Then the preliminary quantitative moisture difference was calculated and the complete dry sample was powdered to suitable size first the root bark and the inner part separately, and then homogenized. The powdered sample was stored in clean glassware container. The prepared powder weighed (400 g) and then macerated by using four organic solvents hexane (99%), chloroform (99.9%), ethanol (97%) and methanol (99.8%) according to their increasing polarity index for 72 hours with mechanical shaking within 4 hours interval in average and it was filtered through Whatman No.340 filter paper and the filtrate was dried using Rotatory evaporator.

The *In vitro* Free Radical Scavenging Activity Assay

The quantitative and qualitative analysis of *in vitro* free radical scavenging activity was done to evaluate the free radical scavenging potential of the *Calpurnia aurea* root extract by using DPPH scavenging assay method and methanol as solvent selectively. The *in vitro* free radical scavenging potential of *Calpurnia aurea* root extract was measured using DPPH according to Blois (1958) [7].

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The DPPH radical (Hi-media) is stable due to the delocalization of a spare electron over the molecule, thus preventing dimer formation. This radical is used in the DPPH radical scavenging capacity assay to quantify the ability of free radical scavenging potential to quench the DPPH radical. The dark purple colour of DPPH will be lost when it is reduced to its non-radical form by the scavenging arrays becomes colourless. DPPH radicals are widely used in the model system to investigate the scavenging activities of several natural compounds. When the DPPH radical is scavenged, the colour of the reaction mixture changes from purple to yellow with decreasing of absorbance at wavelength of 517 nm.

Using Spectrophotometric Method

The DPPH radical-scavenging activity of the test extracts was examined according to the method of Blois (1958) with some modifications. 1 mL of different concentrations, 10 μg/mL, 20 μg/mL, 40 μg/mL, 60 μg/mL, 80 μg/mL, and 100 μg/mL, of each extract and standard were taken in different test tubes at an equal volume and to these solutions, 3 mL of methanol solution of DPPH (0.1 mM) was added and the mixture was allowed to react at 27°C in the dark within 30 minutes of incubation. Vitamin C (ascorbic acid) was used as standard controls. Three replicates were made for each test sample. After 20 minutes, the absorbance (A) was measured at 517 nm and converted into the percentage free radical scavenging activity and interpreted in terms of % scavenged. IC_{50} value (concentration of sample where absorbance of DPPH decreases 50% with respect to absorbance of the control) of extracts were determined. The higher the free radical scavenging activity, the lower IC_{50} value [2; 6].

Using Thin Layer Chromatography (TLC) Method

The *in-vitro* free radical scavenging activity of Calpurnia *aurea* root extract was measured using DPPH according to Blois (1958). The experiment was conducted in two ways: solvent fractionation and reagent spraying.

Thin-layer chromatography was carried out on all the fractions using TLC pre-coated plates (silica gel $60F_{254}$) by using one way ascending technique. The plates were activated in an oven at 100^{0}C for 20 min to drive off the water molecule that bond to the polar site on the plate. And then the plates were cut with surgical blade and marked with pencil about 1cm from the bottom of the plate. The extracts to be analysed were diluted with respective solvents (n-hexane, chloroform, ethanol and methanol) and then spotted with help of capillary tube just 1 cm above its bottom and allowed to dry. The plates were developed in a chromatographic tank or chamber using the different solvent systems. Solvents were analysed as its order of increasing polarity. After several trials, the best solvent system was selected which showed good positive result upon colour detection. For each test, the plates were dried and visualized under normal day light after spraying with DPPH in methanol solution reagent and approved by using Uv lamp [8].

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According to Blois (1958), the developed bands were sprayed with spraying reagents by using 2.5µL micropipette of the prepared sample solution as stated above and observed in daylight and by using Uv lamp after being dried. For application of simple diffusion of 0.1 mM DPPH methanol solutions on the dried plate carrying sample spot, *in vitro* free radical scavenging properties of these various concentrations of four different solvents extract of *Calpurnia aurea* root part (100, 200, 300 mg/ 100 mL for each) were tested. The spot exhibiting radical scavenging activity of the free radical scavenging against the colour of the background was observed and related in both cases. The experiment was performed in triplet.

Results

A. Some Physical Properties and Fractional Extraction of the Plant Extracts

The root part of *Calpurnia aurea* plant was successively extracted with n-hexane, chloroform, ethanol and methanol by using maceration. These extracts after removal of the solvents were used for the scavenging activity tests.

The dried n-hexane, chloroform, and ethanol extracts have waxy gel appearance whereas that of methanol has semi-solid liquid appearance. The four solvent extracts were reddish-brown coloured even though the deepness was slightly showing difference.

The yield from different solvent extracts is presented in Table 1 below.

Table 1: Extracted sample weight and Percentage yields of the crude extracts of *Calpurnia aurea* root

S/N	Solvent	Weight (g) of the crude extract	Percentage of the extract
1	n-hexane	10.10	1.48
2	Chloroform	15.78	2.32
3	Ethanol	38.92	5.72
4	Methanol	105.70	15.54

The ability of a solvent to extract the bioactive compounds from plant is determined by calculating the percentage yield of extraction [9]. The percentage yields of plant extraction are mainly depending on the solvent used in the extraction. Different polarity index of solvents give different percentages yield and extract different phytochemical compounds. Nur Syukriah (2014) reported that polar solvent usually has the higher extraction yield compared to non- polar solvent [10]. This is because the polar compounds such as polyphenols are highly extracted in polar solvent compared with non-polar solvent [11]. This indicates that the plant usually contain more polar compounds as these compounds will be dissolved in similar polarity of solvents which apply to the “like dissolves like” principle [12]. The methanol extract obtained the highest percentage yield of extraction, 15.54%. Meanwhile, the hexane extract obtained the lowest percentage yield of extraction, 0.74%.

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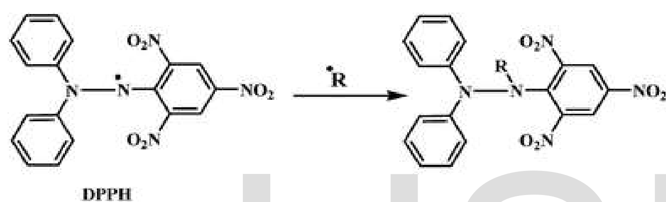
B. *In vitro* Free radical scavenging Activity by Using Spectrophotometric Method

Assay of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

Evaluation of the antioxidant activity of any compound can be carried out either by *in vitro* or *in vivo* models. DPPH is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule.

Due to its odd electron, the methanolic solution of DPPH shows a strong absorption band at 517 nm. As shown in the Scheme 1, the DPPH radical reacts with suitable reducing agent producing new bond, thus changing the color of solution. The solution loses color with the increase in the concentration of antioxidant as the electrons taken up by DPPH radical from the antioxidant. Such reactivity has been used to test the ability of compounds/plant extracts to act as free radical scavengers. Reduction of the DPPH radicals can be monitored spectrophotometrically by the decrease in absorbance at 517 nm [1].



Scheme 1 Reaction of DPPH radical with other radicals

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Methanol and ethanol extracts of the root sample of *Calpurnia aurea* have shown better free radical scavenging activity when compared to the standard reference (ascorbic acid, which was 92.5%) and other extracts tested. The methanol root extract has shown 67.3% of free radical scavenging activity at 100 µg/mL as compared to others (chloroform and n-hexane i.e. 25.1% and 15.8% respectively) solvents root extract whereas, the ethanol extract has shown 57.8%. n-Hexane root extract showed comparatively very weak. All the free radical scavenging activities in different concentrations are shown as absorbance and %inhibition in a bar and line graphs in Figure 1 and 2 respectively.

From the free radical scavenging activity, the IC_{50} values (efficient concentration value i.e. concentration of the substrate that causes 50 % loss of the DPPH activity, color, [2] of test and standard samples were determined. The concentration that causes a decrease in the initial DPPH concentration by 50% was calculated from the kinetic curve [13].

The standard i.e. ascorbic acid shows 16.33 µg/mL IC_{50} value. In the various extracts the methanol root showed 65.64 IC_{50} value which is less in all extracts and next to this ethanol has 80.20 µg/mL. The other two extracts i.e. hexane and chloroform showed very high IC_{50} value which was 131.37 and 127.41 µg/mL. The experimental analysis of all extracts showed that for the entire examined root extracts rank order in terms of % free radical scavenging activity efficiency was always: methanol > ethanol > chloroform > hexane. But it was also observed

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that all the sample extracts have lesser activity than that of standard ascorbic acid which was found IC_{50} value 16.33 µg/mL in the methanol solution (shown by figure 3)

Table 2: Absorbance, %inhibition and EC_{50} by DPPH for Samples of *Calpurnia aurea* Root Extract of Four Different Solvents and Standard Reference (Ascorbic Acid)

Conc.	Hexane Extract			Chloroform Extract			Ethanol Extract			Methanol Extract			Ascorbic Acid		
(µg/mL)	A	%I	EC_{50} (µg/mL)	A	%I	EC_{50} (µg/mL)	A	%I	EC_{50} (µg/mL)	A	%I	EC_{50} (µg/mL)	A	%I	EC_{50} (µg/mL)
10	1.736	3.56	**131.37**	1.685	6.39	**127.41**	1.377	23.50	**80.2**	1.088	39.56	**65.64**	0.367	79.61	**16.33**
20	1.661	7.72		1.671	7.17		1.117	37.94		0.987	45.17		0.190	89.44	
40	1.639	8.94		1.633	9.28		1.050	41.67		0.875	51.39		0.187	89.61	
60	1.616	10.22		1.583	12.06		0.885	50.83		0.677	62.39		0.167	90.72	
80	1.582	12.11		1.534	14.78		0.762	57.67		0.655	63.61		0.164	90.89	
100	1.515	15.83		1.349	25.06		0.759	57.83		0.589	67.28		0.135	92.50	

Percentage of scavenging activity was calculated by using [14; 15]:

$$\% \text{ DPPH Scavenged} = \left[1 - \left(\frac{A_{Sample} - A_{Blank}}{A_{Control}}\right) x100\right]$$

Where A_{Sample} is the absorbance of the sample extract and $A_{Control}$ is the absorbance of the control in which ascorbic acid was used and its blank absorbance was 1.8.

Lower absorbance of the reaction mixture indicates higher free radical scavenging activity.

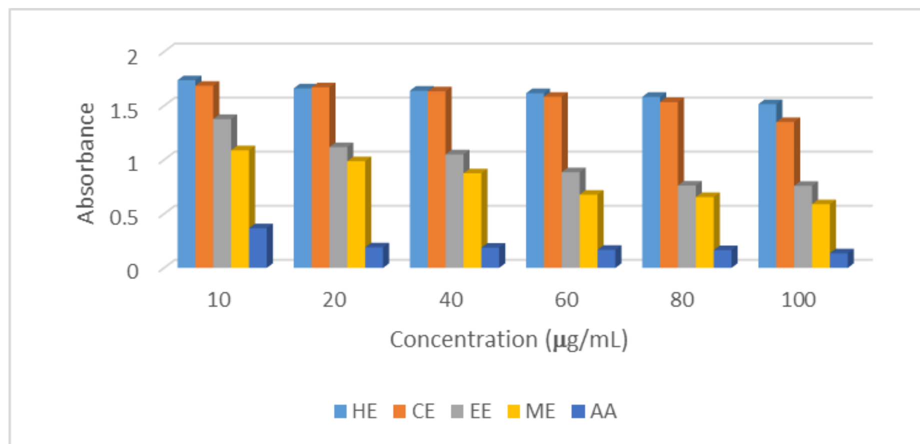

Figure 1: Bar graph plot of absorbance versus concentration of standard and different fractions of *Calpurnia aurea* root extract (HE-Hexane extract; CE-Chloroform extract; EE-Ethanol extract; ME-Methanol extract)

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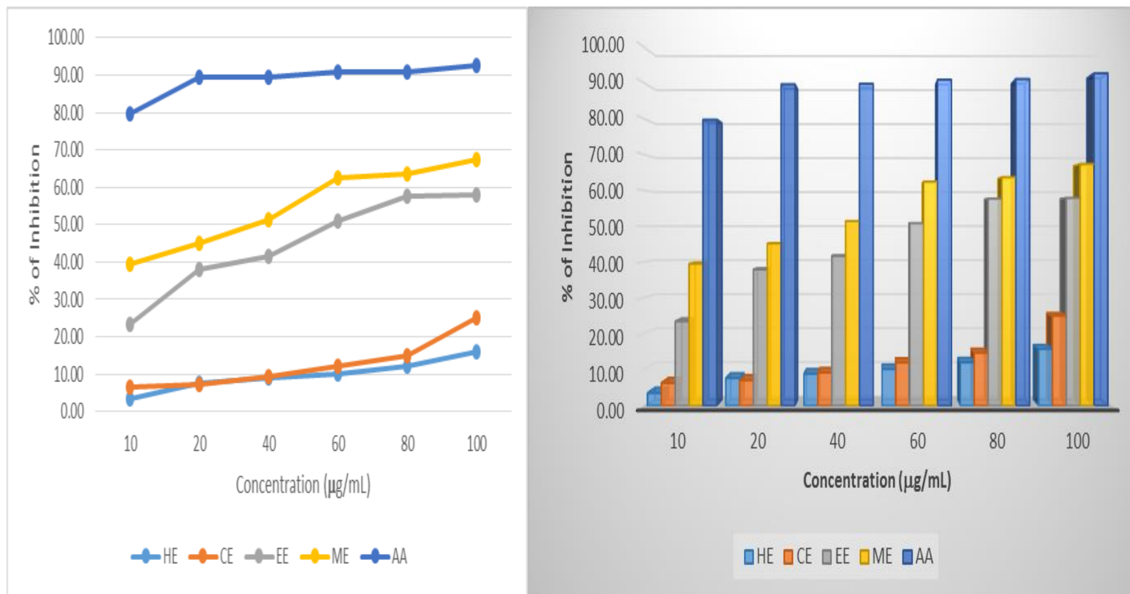

Figure 2: Line graph and bar graph plot of % of inhibition versus concentration of standard and different fractions of *Calpurnia aurea* root extract (HE-Hexane extract; CE-Chloroform extract; EE-Ethanol extract; ME-Methanol extract)

The percentage inhibition in *Calpurnia aurea* root extract and standard Ascorbic acid Vs concentration showed that the free radical scavenging activities of 99.8% methanol and 97% ethanol extracts of *Calpurnia aurea* root and the standard Ascorbic acid was found to be positively correlated with the % inhibition as determined from their corresponding regression curves. This comparison suggested that the solvent extracts are relatively potential free radical scavenging agents and compatible with the commercial free radical scavenging agent.

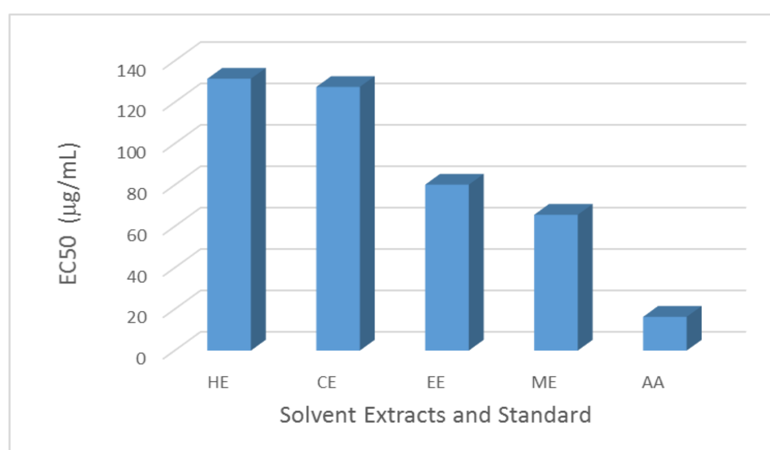

Figure 3: EC_{50} by DPPH for Samples of *Calpurnia aurea* Root Extract of Four Different Solvents (HE-Hexane extract; CE-Chloroform extract; EE-Ethanol extract; ME-Methanol extract) and Standard Reference (Ascorbic Acid)

In vitro Free radical scavenging Activity by Using TLC Method

The free radical scavenging capacities of the root of *Calpurnia aurea* plant extracts as measured by the DPPH method qualitatively were run through different solvent systems by using thin layer chromatography (TLC) using aluminum-backed TLC plates (Merck, silica gel 60 F_{254}). The TLC plates were developed with one of the three eluent systems developed via repeated separation experiments i.e.: ethyl acetate/methanol/water (40:5.4:5): [EMW] (polar/neutral); chloroform/ethyl acetate/formic acid (5:4:1): [CEF] (intermediate polarity/acidic); benzene/ethanol/ammonium hydroxide (90:10:1): [BEA] (non-polar/basic) [16]. Development of the chromatograms was in a closed tank in which the atmosphere had been saturated with the eluent vapor by lining the tank with filter paper wetted with the eluent.

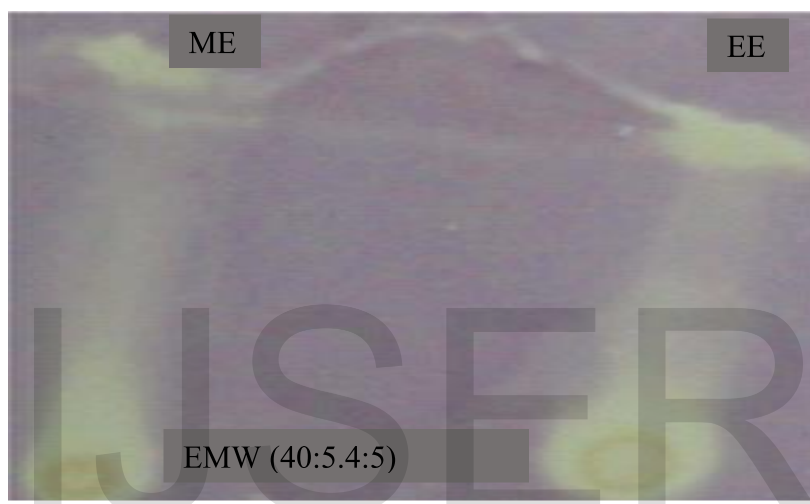

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Figure 4: Chromatograms of free radical scavenging activity sprayed with DPPH in different solvent system: EE-Ethanol extract; ME-Methanol extract; E-Ethyl acetate; M-Methanol; E-Ethanol.

The plant extracts which were run under the EMW (40:5.4:5) solvent system showed better activity by methanol and ethanol extracts (figure 4) but chloroform and n-hexane extracts showed no or almost negligible activity. This is related to the science of polarity of the components of the compounds present in the extractants. On the other hand, the direct diffusion that was applied based on three different respective concentrations of the sample extracts (100, 200, 300 mg/ 100 mL) showed supporting results. The diameter of the diffused spots of methanol and ethanol extracts showed significant evidence and upon increasing in concentration of *Calpurnia aurea* root extracts of these solvent extracts increased the scavenging property, zone of scavenged inhibition directly proportional to the concentration of *Calpurnia aurea* root extract in strong oxidizing agent (free radical) DPPH methanol solution.

Discussion

Gorinstein *et al*. (2007) has reported that the high free radical scavenging activity of plant extracts were due to the presence of high phenolic, tannins and flavonoids compounds which are polar compounds in the hydroalcoholic or alcoholic extracts [17]. In addition, Guha (2011) has conducted a study that showed that the polar solvent extract (lower molecular weight alcohols and aqueous) possessed a higher free radical scavenging activity when compared to non- polar (e.g. hexane) or low polar (e.g. chloroform) extracts over 56 different type of plants [18]. There have been also many studies reported that the other parts of the plant species showed significant DPPH radical scavenging activity and possessed high free radical scavenging activity. As an instance, the same part of the plant species has shown significant antioxidant activity when evaluated by using DPPH ethanol solution [19]. The stems and leaves of *Calpurnia aurea* showed significant activity where the leaves showing higher potential when evaluated by using DPPH and FRAP standard methods [20]. The extract showed significant activities in all free radical scavenging assays compared to the reference free radical scavenging ascorbic acid in a dose dependent manner [21; 22].

Here in the present study, methanol and ethanol extracts of the root part of *Calpurnia aurea* have relatively shown better free radical scavenging activity at 100 µg/mL when compared to the standard reference (ascorbic acid, which was 92.5%) and other extracts tested. The methanol root extract has shown 67.3% of free radical scavenging activity as compared to others (ethanol, chloroform, and n-hexane i.e. 57.83%, 25.1% and 15.83%, respectively) at the same concentration. Here, n-hexane root extract showed very weak activity.

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Conclusion

According to this study, the methanol extract of *Calpurnia aurea* root gave the highest percentage yield of extraction and followed by ethanol in contrast to n-hexane extract indicating that the root extract contained more polar compounds than non-polar compounds.

The DPPH assay showed that methanol extract gave the highest free radical scavenging activity and ethanol extract also can be a potential free radical scavenging agent as it was able to scavenge high percentages of free radical when compared to the other extracts. The qualitative thin layer chromatography also revealed that the *Calpurnia aurea* root extract contained several polar and slightly non polar compounds as they did show higher positive results for all solvents extract (except hexane extract) showing yellowish colour spots on purple background upon spraying and diffusing DPPH methanol solution on developed chromatogram. The methanol extract showed significant free radical scavenging activity in comparative to standard ascorbic acid means that in future this plant may found the main source for drug supplement as an free radical scavenging since the free radical scavenging activity has potential in the treatment of cancer, cardiovascular disease, neural disorders, Alzheimer's disease, mild cognitive impairment, Parkinson's disease, alcohol induced liver disease, ulcerative

colitis, aging and atherosclerosis. The exploitation of these pharmacological properties involves further investigation of these active ingredients by implementation techniques of extraction, purification, separation, crystallization, characterisation and identification.

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