

***In vitro* evaluation of antioxidant activity of *Leucas plukenetii* (Roth) Spreng**

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ABSTRACT

*The present study was carried out to evaluate the antioxidant activities of methanolic extract of *Leucas plukenetii* (Roth) Spreng whole plant in various systems. The free radical scavenging potential was studied by using different antioxidants models of screening using vitamin C (5mM) as standard. About 200, 400, 600 & 800 µg/ml methanolic extract inhibited the FeSO₄ induced lipid peroxidation in a dose dependent manner and showed IC₅₀ value 536 ± 1.26 µg/ml. The methanolic fraction at 800 µg/ml exhibited significant antioxidant activity in ferrous sulphate induced lipid peroxidation and Superoxide scavenging models with simultaneous improvement in hepatic glutathione (10.31 ± 0.2906 µg GSH/mg of wet tissue) and catalase levels (121.81 ± 0.6306 µM of H₂O₂ consumed /min/mg tissue) compared to standard group. The results suggest that the methanolic extract of *Leucas plukenetii* Smith. Whole plant plays an important role in the modulation of oxidative stress.*

Key Words: *Leucas plukenetii* Smith. Antioxidant activity, Lipid peroxidation, whole plant methanolic extract.

INTRODUCTION

Antioxidants are micronutrients that have gained importance in recent years due to their ability to neutralize free radicals or their actions [1]. Reactive oxygen species (ROS) including superoxide radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide are often generated as by products of biological reaction or from exogenous factors [1]. *In vivo*, some of these ROS play an important role in cell metabolism including energy production, phagocytosis and intercellular signaling [3]. However, these ROS produced by sunlight, ultraviolet light, ionizing radiation, chemical reactions and metabolic processes have a wide variety of pathological effects such as DNA damage, carcinogenesis and various degenerative disorders such as cardiovascular diseases, aging and neuro-degenerative diseases [4,5,6]. A potent broad spectrum scavenger of these species may serve as a possible preventive intervention for free radical mediated cellular damage and diseases [7]. Antioxidant based drugs and formulations for the prevention and treatment of complex diseases like Alzheimer's disease and cancer have appeared during last three decades [8]. Recent studies have shown that a number of plant products including polyphenols, terpenes and various plant extracts exerted an antioxidant action [9-12]. There is also a considerable amount of evidence revealing an association between individuals who have a diet rich in fresh fruits and vegetables and the decreased risk of cardiovascular diseases and certain forms of cancer [13,14]. There is currently immense interest in natural antioxidants and their role in human health and nutrition [15]. Considerable amount of data have been generated on antioxidant properties of food plants around the globe [16, 17]. However, a traditionally used medicinal plant awaits such screening. On the other hand, the medicinal properties of plants have also been investigated in the light of

recent scientific developments through out the world, due to their potent pharmacological activities, low toxicity and economic viability [18]. Several medicinal plants (Rasayana) have also been extensively used in the Indian traditional (Ayurveda) system of medicine for the treatment of number of diseases [18]. Some of these plants have shown potent antioxidant activity [17, 8]. However, majority of plants have not yet been screened for such activity. The whole plant is used for medicinal purpose specially leaves and flower buds. The plant is an erect or diffusely branched, annual aromatic herb with woody root stock. Stems are quadrangular, hairy. The leaves are linear or oblong, 2.5 to 7.5 centimeters long, and blunt-tipped, the margins being scalloped, and narrow elliptic, distantly serrated, emit scent. The whorls are large, terminal and auxiliary, often 2.5 centimeters in diameter, and crowded with flowers. Flowers are white bi-labiate with nectarines. The calyx is variable but always produces an upper lip and has short, triangular teeth. The corolla is small 2- lipped, upper lip short and hairy, lower lip twice as long as upper, 3- lobed; stamens 4, didynamous, enclosed within the upper lip; nutlets oblong, angled on inner face smooth. Flowering season is cold but fruit in hot season. These are common in open areas. Approximately 3500 species in 220 genera, distributed worldwide, but mostly in the Mediterranean region and SW Asia. China has 807 species in 96 genera [31]. About 100 species Africa, Asia, Australia, Pacific Islands, two naturalized in South America; eight species in China [19], India, Bangladesh, IndoChina boarder, Malaysia and Mauritius. It is found in most plains district in India up to the altitude of 3000 ft, as awed in cultivated field, waste lands and roadsides [20].

Leaves and flower buds are used as vegetable. It is considered medicinal for liver ailment, scorpion sting sinusitis, headache etc [21]. The leaves are very useful in chronic rheumatism. It is a good remedy for psoriasis and skin eruptions. It is erroneously reputed to be an antidote to snake venom. This is a home remedy for worms, fever and intestinal catarrh in children. It is antihistaminic, antipyretic, antiseptic, carminative, febrifuge and wormifuge. It is used in anorexia, cough, dyspepsia, fever, helminthic manifestation, jaundice, psoriasis, respiratory disorder and skin disorder [22]. Traditional medicine for coughs, colds, painful swellings and chronic skin eruptions [23].

Since polyphenolic and flavonoidal compounds present in the *Leucas plukenetii*, it was thought that it would be worthwhile to evaluate the plant for antioxidant activity. Lipids are one of the most susceptible target for free radicals. The oxidative destruction is known as lipid peroxidation and may induce many pathological events. So the purpose of the present study was to evaluate antioxidant potential of methanolic extract of *Leucas plukenetii* (Roth) Spreng plant.

MATERIALS AND METHODS

Plant material

The whole plant of *Leucas plukenetii* (Roth) Spreng was collected from Dhubri district of Assam in the month of August – September in the month of July, 2010 and was authenticated at Botanical survey of India, Gangtok, Sikkim (Specimen No-HPI/121/2010). A voucher specimen was deposited at the Department of Pharmacology, Himalayan Pharmacy Institute, Sikkim. The leaves of the plants were thoroughly washed in running water to remove the earthy material and/or adherent impurities and dried in shade. (Figure 1)



Figure 1: Photograph of *Leucas plukenetii* Plant and ripe fruit of *Leucas plukenetii*.

Preparation of extract

About 700 gm of the air-dried and powdered plant material was extracted by continuous hot percolation method in Soxhlet apparatus with methanol. The extract was concentrated by distilling off the solvent and evaporating to dryness on water bath. On removal of the methanol by evaporation, a sticky dark brown mass was obtained. The percentage yield was found to be 14.9 % w/w. phytochemical investigations showed the presence of alkaloids, glycosides, flavonoids and other phenolic compounds.

Antioxidant assays**1) Lipid peroxidation assay**

The extent of lipid peroxidation in goat liver homogenate was measured *In vitro* in terms of formation of thiobarbituric acid reactive substances (TBARS) by using standard method [24] with minor modifications [25] with the help of spectrophotometer.

Tissue sample preparation for Lipid peroxidation assay

Goat liver was collected from slaughter house; the liver lobes were washed with 0.9% Sodium Chloride solution. (To remove excess blood). The lobes were dried by blotting papers and were cut into small pieces with a heavy-duty blade. From that 1gm tissue were then homogenized with 10 ml of cold phosphate buffer (pH-7.4) to get 10% homogenate in glass-Teflon homogenizing tubes and filtered to get clear solution. The solution was centrifuged at 3000 r.p.m at 4° C for 10 min. The supernatant was diluted with phosphate buffer to obtain final concentration of protein equal to 8.0-15.0 mg/ml. This solution was taken for lipid peroxidation assay. [26]

Protein concentration was measured by using standard method of Lowry [27].

Assay procedure

Liver homogenates (3.0ml) were taken in six 10 ml test tubes. The first two test tubes were treated as control and standard where buffer and Vitamin C (5mM) were added. In the third to sixth test tubes different concentrations of extract were added. Lipid peroxidation was initiated by adding 100 µl of 15mM ferrous sulphate solution to 3.0 ml of liver homogenate [28]. After 30 min, 200µl of this reaction mixture was taken in a tube containing 3.0 ml of 10% trichloroacetic acid. After 10 min, tubes were centrifuged and supernatant was separated and mixed with 3.0 ml of 0.67% thiobarbituric acid in acetic acid. The mixture was heated in a water bath at 85°C for 30 minutes, followed by heating in boiling water bath to complete the reaction. The intensity of pink coloured complex formed was measured at 535 nm in a spectrophotometer. The percentage of inhibition of lipid peroxidation was calculated by comparing the results of the test with those of control as per the following formula:

$$\% \text{ Inhibition} = \frac{(\text{Control Absorbance} - \text{Test Absorbance})}{\text{Control Absorbance}} \times 100$$

The TBARS concentration was calculated by using the following formula and expressed as nM/mg of tissue [29].

$$\text{nM of TBARS/mg of tissue} = \frac{\text{OD} \times \text{Volume of homogenate} \times 100 \times 10^5}{(1.56 \times 10^5) \times \text{Volume of extract taken}}$$

Tissue sample preparation for Catalase, GSH, SOD assay

Liver tissue was collected from slaughter house, washed in normal saline and soaked in filter paper. 1gm tissue was then homogenized in 2.0ml M/15 phosphate buffer (pH-7.0) and centrifuged at 3000 r.p.m at 4° C for 1 hr. The supernatant collected was taken for the assay [30].

2) Catalase Assay (CAT)

Catalase activity was measured based on the ability of the enzyme to break down H₂O₂. 10 µl samples were taken in tube containing 3.0ml 30% (w/v) of H₂O₂ in phosphate buffer (M/15 phosphate buffer; pH-7.0). [31] Time required for 0.05 units change in absorbance was observed at 240 nm against blank containing the enzyme source in phosphate buffer free from H₂O₂. The absorbance was noted at 240 nm after the addition of enzyme; Δt was noted till absorbance was 0.45. If Δt was longer than 60 seconds, the procedure was repeated with more concentrated enzyme sample. Reading was taken at every 5 seconds interval. One unit catalase activity is the amount of enzyme that liberates half the peroxide oxygen from H₂O₂ solution of any concentration in 100 seconds at 25°C which is determined by CAT activity expression:

$$\text{Moles of H}_2\text{O}_2 \text{ consumed/min (units/mg of tissue)} = \frac{2.3}{\Delta t} \times \ln \left(\frac{E_{\text{initial}}}{E_{\text{final}}} \right) \times 1.63 \times 10^{-3}$$

Where E= optical density at 240nm,

Δt = time required for a decrease in the absorbance.

3) Reduced Glutathione Assay

Reduced glutathione (GSH) activity was assayed according to the method of Ellman. Reduced Glutathione in the liver homogenate was estimated spectrophotometrically by determination of 2-nitro 5-thiobenzoic acid (yellow colour) formed as a result of reduction of DTNB (Dithiobis-(2-nitrobenzoic acid) by GSH, expressed as $\mu\text{g}/\text{mg}$ of tissue. [32]

To 0.1 ml of different tissue samples, 2.4 ml of 0.02 M EDTA solution was added and kept on ice bath for 10 min. Then 2.0 ml of distilled water and 0.5 ml of 50 % w/v TCA were added. This mixture was kept on ice for 10-15 min and then centrifuged at 3000 r.p.m. for 15 min. To 1.0 ml of supernatant, 2.0 ml of Tris buffer (0.4M) was added. Then 0.05 ml of DTNB solution (Ellman's reagent; 0.01M DTNB in methanol) was added and vortexed thoroughly. OD was read (within 2-3 min after the addition of DTNB) at 412 nm in spectrophotometer against a reagent blank. Different concentrations (10-50 μg) of standard glutathione were taken and processed as above for standard curve. The amount of reduced glutathione was expressed as μg of GSH/mg of wet tissue.

4) Superoxide Dismutase (SOD) assay

Superoxide dismutase (SOD) activity was assayed according to the method of Marklund and Marklund. [33] The liver homogenates were prepared in Tris (ethylenediamine tetraacetic acid) buffer centrifuged for 40 min at 10000 r.p.m at 4°C, the supernatant was used for the enzyme assay. 2.8 ml Tris-EDTA and 100 μl Pyrogallol (2mM) were taken in the cuvette and scanned for 3 min at 420 nm wavelength. Then 2.8 ml Tris-EDTA buffer (pH -8.0), 100 μl Pyrogallol and 50 μl tissue homogenate were taken and scanned for 3 min at the same wavelength. One unit of SOD activity is the amount of the enzyme that inhibits the rate of auto oxidation of pyrogallol by 50% and was expressed as Units/mg protein/min. The enzyme unit can be calculated by using the following equations:

$$\text{Rate(R)} = \frac{\text{Final OD} - \text{Initial OD}}{3 \text{ min}}$$

$$\% \text{ of inhibition} = \frac{\text{Blank OD} - \text{R}}{\text{Blank OD}} \times 100$$

Enzyme unit (U) = (% of inhibition/50) x common dilution factor

[50% inhibition = 1 U]

RESULTS AND CONCLUSION

Anti-lipidperoxidation free radicals were scavenged in concentration like 200, 400, 600 and 800 $\mu\text{g}/\text{ml}$ were observed in 0.9466 ± 0.041 , 0.7402 ± 0.020 , 0.5372 ± 0.069 and 0.4114 ± 0.049 respectively. However, the extract showed encouraging response in IC50 values were given table.

The results presented in **Table-1** showed that the methanolic extract of *Leucas plukenetii* whole plant inhibited FeSO₄ induced lipid peroxidation in a dose dependent manner. The extract at 800 $\mu\text{g}/\text{ml}$ exhibited maximum inhibition ($61.07 \pm 2.34\%$) of lipid peroxidation nearly to the inhibition produced by Vit. C. The IC₅₀ value was found to be $480 \pm 1.87\mu\text{g}/\text{ml}$. The inhibition could be caused by the absence of ferryl-perferryl complex or by changing the ratio of Fe³⁺/Fe²⁺ or by reducing the rate of conversion of ferrous to ferric or by changing the iron itself or combination thereof [34] (**Figure 2**)

The H₂O₂ consumed /min/mg tissue in catalase assay on goat liver homogenate in different concentration like 200, 400, 600 and 800 $\mu\text{g}/\text{ml}$ were observed in 68.06 ± 0.9968 , 83.17 ± 0.9353 , 112.71 ± 0.8304 and 136.27 ± 0.4867 respectively.

Catalase is an enzymatic antioxidant widely distributed in all animal tissues including RBC and liver. Catalase decomposes hydrogen peroxide and helps protect the tissues from highly reactive hydroxyl radicals [35]. 800 $\mu\text{g}/\text{ml}$

methanolic extract shows higher rate of H₂O₂ consumption and provide the protection highly reactive hydroxyl radicals. (Table 2)(Figure 3).

In extract concentration like 200, 400, 600 and 800 µg/ml were observed in Reduced glutathione (µg of GSH/mg of wet tissue) concentration are 5.20 ± 0.2982 , 5.96 ± 0.5231 , 8.12 ± 0.3689 and 10.22 ± 0.2333 respectively.

Glutathione, a major non-protein thiol in living organisms, plays a central role in coordinating the body's antioxidant defense processes. Excessive peroxidation causes increased glutathione consumption. Reduced thiols have long been reported to be essential for recycling of antioxidants like vitamin E and vitamin C [36]. The tissue glutathione levels were significantly elevated in the 800µg/ml extract. Therefore, it clearly demonstrates that the extract at 800µg/ml have protective role against oxidative damage in the liver tissue. (Table 3) (Figure 4)

Superoxide free radicals were scavenged in concentration like 200, 400, 600 and 800 µg/ml were observed in percentage inhibition 34.18 ± 0.191 , 41.42 ± 0.407 , 50.88 ± 0.284 and 58.24 ± 0.749 respectively and amount of SOD are 0.68 ± 0.0034 , 0.83 ± 0.0103 , 1.02 ± 0.0033 and 1.17 ± 0.0136 in different concentration.

Superoxide anion is an oxygen-centered radical with selective reactivity. This species is produced by a number of enzyme systems in auto-oxidation reactions and by nonenzymatic electron transfers that univalently reduce molecular oxygen. It can also reduce certain iron complexes such as cytochrome [37-56]. The results indicate that of methanolic extract of *Leucas plukenetii* is an effective scavenger of superoxide anions and this may be due to the presence of multiple antioxidants with relatively high superoxide scavenging activity. (Table 3) (Figure 5).

Table 1. Effect of methanolic extract of *Leucas plukenetii* on ferrous sulphate induced lipid peroxidation on goat liver homogenate

Test tube no.	Treatment	% Inhibition	IC ₅₀ value and confidence interval (µg/ml)	TBARS (nM/mg of tissue)
1	Control	—	—	—
2	Vitamin C (5mM)	83.94 ± 0.92		0.2084 ± 0.043
Concentration of methanolic extract (µg/ml)				
3	200	28.37 ± 0.49	536 ± 1.26	0.9298 ± 0.075
4	400	41.68 ± 1.07		0.7570 ± 0.032
5	600	54.16 ± 1.87		0.5950 ± 0.019
6	800	66.62 ± 0.53		0.4332 ± 0.078

Values are expressed as Mean \pm SEM, n=3 in each group. The statistical analysis was performed by One-way ANOVA followed by post Dunnett's test, and the P value was found to be $P < 0.01$ when compared with control group.

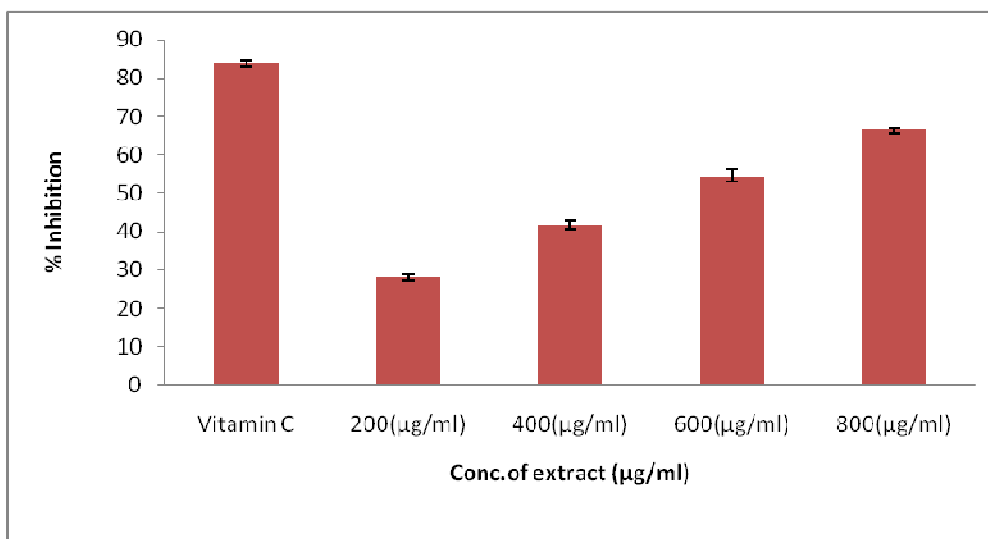
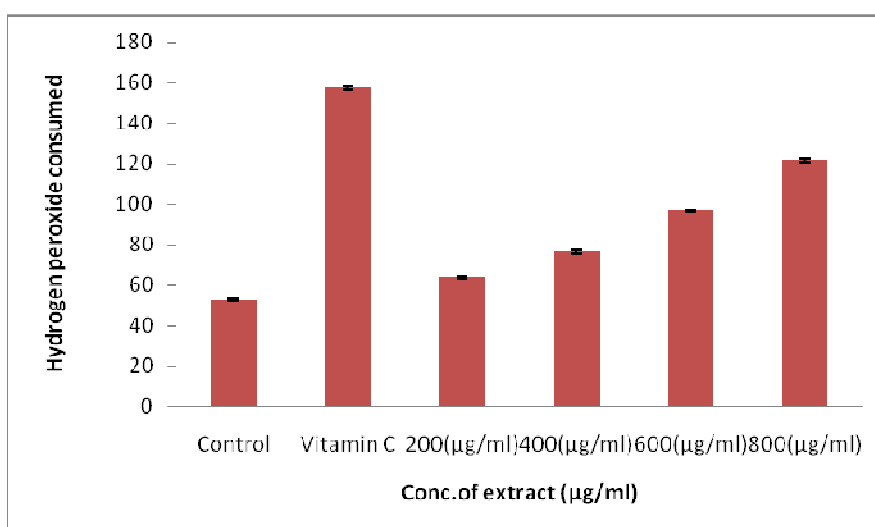


Figure 2: Percentage of inhibition of lipid peroxidation by different concentrations of methanolic extract of *Leucas plukenetii* where vitamin C was used as standard.

Table 2: Effect of methanolic extract of *Leucas plukenetii* on catalase assay on goat liver homogenate

Test tube no.	Treatment	Initial OD	Final OD	Time (min)	μM of H_2O_2 consumed /min/mg tissue
1	Control	0.7842 ± 0.0028	0.7613 ± 0.0025	0.30	52.81 ± 0.6988
2	Vitamin C (5mM)	0.8924 ± 0.0015	0.8378 ± 0.0013	0.30	157.89 ± 0.5357
Concentration of methanolic extract ($\mu\text{g/ml}$)					
3	200	0.8659 ± 0.0029	0.8441 ± 0.0029	0.30	63.78 ± 0.5774
4	400	0.8642 ± 0.0027	0.8278 ± 0.0025	0.30	76.74 ± 0.4073
5	600	0.8740 ± 0.0025	0.8278 ± 0.0022	0.30	96.92 ± 0.5290
6	800	0.8755 ± 0.0022	0.8338 ± 0.0023	0.30	121.81 ± 0.6306

Values are expressed as Mean \pm SEM, $n=3$ in each group. The statistical analysis was performed by One-way ANOVA followed by post Dunnett's test, and the P value was found to be $P<0.01$ when compared with control group.

**Figure 3: Amount of H_2O_2 consumed by different concentrations of methanolic extract of *Leucas plukenetii* where vitamin C was used as standard.****Table 3: Effect of methanolic extract of *Leucas plukenetii* on glutathione assay on goat liver homogenate**

Test tube no.	Treatment	Absorbance	Reduced glutathione (μg of GSH/mg of wet tissue)
1	Control	0.2367 ± 0.0249	4.12 ± 0.4096
2	Vitamin C (5mM)	0.9037 ± 0.0143	14.64 ± 0.2360
Concentration of methanolic extract ($\mu\text{g/ml}$)			
3	200	0.2640 ± 0.0138	4.14 ± 0.2287
4	400	0.3943 ± 0.0192	6.27 ± 0.3157
5	600	0.5230 ± 0.0197	8.39 ± 0.3243
6	800	0.6400 ± 0.0174	10.31 ± 0.2906

Values are expressed as Mean \pm SEM, $n=3$ in each group. The statistical analysis was performed by One-way ANOVA followed by post Dunnett's test and the P value was found to be $P<0.01$ for all, except test tube no. 6 where $P \leq 0.05$ when compared with control group.

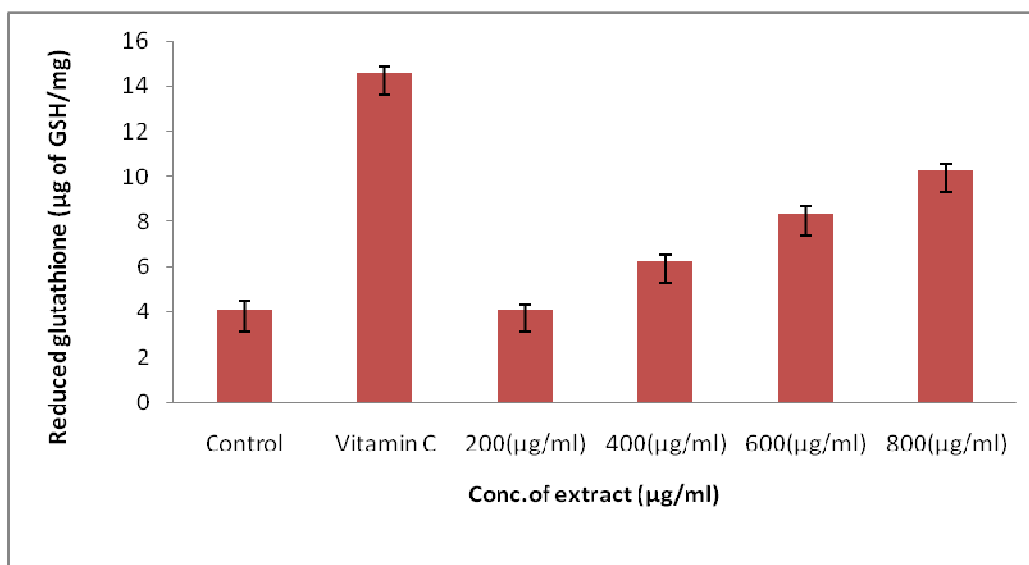


Figure 4: Amount of reduced glutathione formed for different concentrations of methanolic extract of *Leucas plukenetii* where vitamin C was used as standard.

Table 4: Effect of methanolic extract of *Leucas plukenetii* on superoxide dismutase assay on goat liver homogenate

Test tube no.	Treatment	Rate of absorbance change	% Inhibition	SOD (U/mg tissue wet)
1	Control	0.0453 ± 0.0055	29.15 ± 0.199	0.58 ± 0.0033
2	Vitamin C (5mM)	0.0753 ± 0.0068	61.79 ± 0.661	1.24 ± 0.0145
Concentration of methanol extract (µg/ml)				
3	200	0.1693 ± 0.0049	32.99 ± 0.521	0.66 ± 0.0116
4	400	0.1693 ± 0.0022	39.68 ± 0.533	0.79 ± 0.0087
5	600	0.1667 ± 0.0029	48.23 ± 0.414	0.97 ± 0.0067
6	800	0.1597 ± 0.0029	56.86 ± 0.554	1.14 ± 0.0100

Values are expressed as Mean ± SEM, n=3 in each group. The statistical analysis was performed by One-way ANOVA followed by post Dunnett's test, and the P value was found to be $P < 0.01$ when compared with control group.

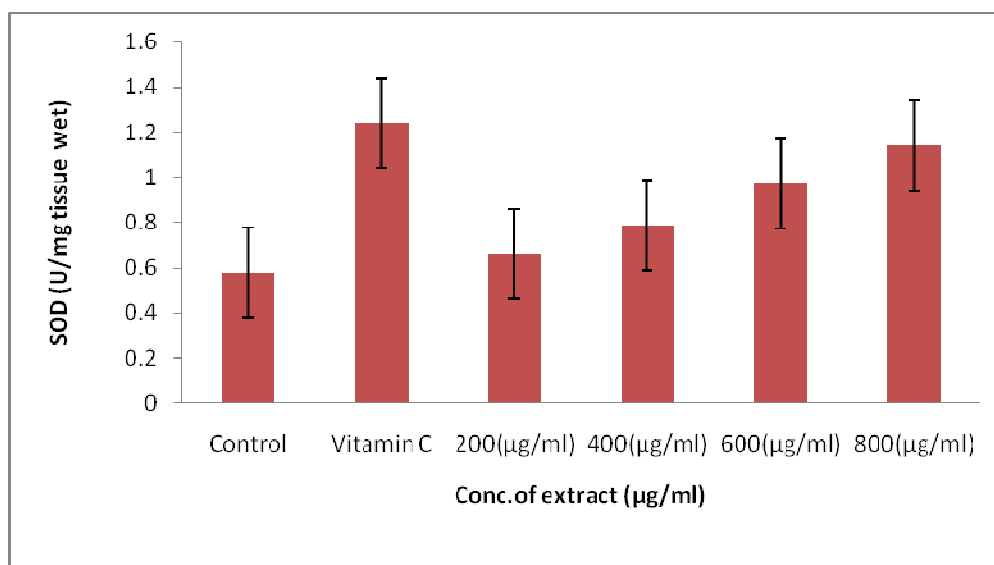


Figure 5: Amount of SOD for different concentrations of methanolic extract of *Leucas plukenetii* where vitamin C was used as standard.

CONCLUSION

The result of the present study showed that the methanolic extract of *Leucas plukenetii* plant, which contains phenolic and flavonoidal compounds, exhibited the great antioxidant activity. The high scavenging property of methanolic extract of *Leucas plukenetii* plant may be due to hydroxyl groups existing in the phenolic compounds' chemical structure that can provide the necessary component as a radical scavenger. Free radicals are often generated as byproducts of biological reactions or from exogenous factors. The involvements of free radicals in the pathogenesis of a large number of diseases are well documented. A potent scavenger of free radicals may serve as a possible preventative intervention for the diseases. Methanolic extract of *Leucas plukenetii* plant in this research exhibited antioxidant. The antioxidant potential may be attributed to the presence of polyphenolic compounds.

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