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Determination of *In Vitro* Antioxidant activity of *Passiflora Nepalensis* smith. Fruit extract

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ABSTRACT

The present study was carried out to evaluate the antioxidant activities of methanolic extract of *Passiflora nepalensis* Smith. (Passifloraceae) ripe fruits 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 510 ± 2.59µg/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 (10.22 ± 0.2333µg GSH/mg of wet tissue) and catalase levels (136.27 ± 0.4867µM of H_2O_2 consumed /min/mg tissue) compared to standard group. The results suggest that the methanolic extract of *Passiflora nepalensis* Smith. Fruits play an important role in the modulation of oxidative stress.

Key Words: *Passiflora nepalensis* Smith. Antioxidant activity, Lipid peroxidation, Ripe Fruit Extract.

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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 free radicals such as superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$), singlet oxygen ($^1O_2$) and non-free radical species such as hydrogen peroxide (H_2O_2) are various forms of activated oxygen and often generated by oxidation product of biological reactions or exogenous factors [2,3,4]. ROS have aroused significant interest among scientists in the past decade. Their broad range of effects in biological and medicinal systems has drawn on the attention of many experimental works [4,5]. In living organism, various ROS can form by different ways. Normal aerobic respiration stimulates polymorpho nuclear leukocytes and macrophages, and peroxisomes appear to be the main endogenous sources of most of the oxidants produced by cells. Exogenous sources of ROS include tobacco smoke, certain pollutants, organic solvents, and pesticides [6, 7, 8, 9, 10]. ROS can cause lipid peroxidation in foods, which leads to the deterioration of the food [11, 12]. In addition, it is well known that ROS induce some oxidative damage to biomolecules like lipids, nucleic acids, proteins, amines, deoxyribonucleic acid and carbohydrates. Its damage causes ageing, cancer, and other many diseases. As a result of this, ROS have been implicated in more than 100 diseases, including malaria, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes, and cancer[14] ROS are continuously produced during normal physiologic events, and removed by antioxidant defense mechanisms. There is a balance between generation of ROS and antioxidant system in organisms. In pathological condition, ROS are overproduced and result in lipid peroxidation and oxidative stress. The imbalance between ROS and antioxidant defense mechanisms leads to oxidative modification in cellular membrane or intracellular molecules. Various endogenous antioxidant defense mechanisms play an important role in the elimination of ROS and lipid peroxides, and therefore, protect the cells against toxic effects of ROS and lipid peroxides [15, 16].

Passiflora nepalensis Smith belonging to the family Passifloraceae is commonly known as Passion fruit (English), Krishna Fal (Hindi), Pansara (Bengali) and Garindai (Nepali). Passion fruit is grown in most tropical and subtropical parts of the world, passion fruit are particularly important commercially in Australia, Hawaii, South Africa and Brazil. In India it is widely distributed in Sikkim, Himalayan region, at about 4000-6000 ft altitude. It is also found run wild in parts of Assam and Bengal and in areas near Ootacamund, Kodaikanal and Yercaud. Alkaloids, phenols, glycosyl flavonoids and cyanogenic compounds are known in the genus.[17-24].

Ripe fruits are eaten raw. Juice is highly acidic and is preserved for use in blends with less acidic fruit juices and in the preparation of squashes, cordials, syrups, carbonated beverages, jellies etc. Also used in flavouring candy, ice-creams, cake fillings and frostings. Peels are used for recovery of pectin as stock-feed. Seeds yield semi-drying oil, suitable for use in paints and varnishes. *Passiflora edulis* has been used intraditional medicine as a sedative and to treat or prevent central disorders such as anxiety and insomnia [25, 26, 27].

Since polyphenolic compound present in the Passiflora nepalensis, 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 *Passiflora nepalensis* Smith. (Passifloraceae) ripe fruits.

MATERIAL AND METHODS

Plant material

The ripe fruits of *Passiflora nepalensis* Smith were collected from local areas of Sikkim Himalayan region at a high altitude, in the month of July, 2010 and were 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 *P. nepalensis* Plant and ripe fruit of *P.nepalensis* Wall.

Preparation of extract

About 700 gm of the air-dried and powdered whole 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 [28] with minor modifications[29] with the help of spectrophotometer.

Tissue sample preparation for Lipid peroxidation assay[30]

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.

Protein concentration was measured by using standard method of Lowry [31].

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[32]. 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 85^0C 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:

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

The TBARS concentration was calculated by using the following formula and expressed as nM/mg of tissue[33].

$$\text{nM of TBARS/mg of tissue} = \frac{OD \times Volume\ \ of\ homogenate\ \times 100 \times 10^3}{(1.56 \times 10^5) \times Volume\ \ of\ extract\ \ taken}$$

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[34].

2) Catalase Assay (CAT)

Catalase activity was measured based on the ability of the enzyme to break down H_2O_2. 10 µl samples were taken in tube containing 3.0ml 30% (w/v) of H_2O_2 in phosphate buffer (M/15 phosphate buffer; pH-7.0). 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 H_2O_2[35]. 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 H_2O_2 solution of any concentration in 100 seconds at 25^0C which is determined by CAT activity expression:

$$\text{Moles of } H_2O_2 \text{ consumed/min (units/mg of tissue)} = \frac{2.3}{\Delta t} \times \ln\left(\frac{E_{initial}}{E_{final}}\right) \times 1.63 \times 10^{-3}$$

Where E= optical density at 240nm,

$\Delta\ t$ = 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 µg/mg of tissue[36].

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, 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 (10-50µg) 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. 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[37]. 2.8 ml Tris-EDTA and 100µl 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), 100µl Pyrogallol and 50µl 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:

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

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

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

[50% inhibition = 1 U]

RESULTS AND DISCUSSION

Anti-lipid peroxidation free radicals were scavenged in concentration like 200, 400, 600 and 800 µg/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 *Passiflora nepalensis* ripe fruits inhibited FeSO4 induced lipid peroxidation in a dose dependent manner. The extract at 800µg/ml exhibited maximum inhibition (61.07 ±2.34%) of lipid peroxidation nearly to the inhibition produced by Vit. C. the IC50 value was found to be 480 ± 1.87µg/ml. The inhibition could be caused by the absence of ferryl-perferryl complex or by changing the ratio of Fe^{3+}/Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or by changing the iron itself or combination thereof[38].(Figure 2)

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[39]. 800µg/ml methanolic extract shows higher rate of H_2O_2 consumption and provide the protection highly reactive hydroxyl radicals.(Table 2)(Figure 3).

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[40]. 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)

Table 1: Effect of methanolic extract of *Passiflora nepalensis* fruit 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)	84.35 ± 2.41		0.2031 ± 0.065
	Conc. of methanol extract (µg/ml)			
3	200	27.07 ± 0.31	510 ± 2.59	0.9466 ± 0.041
4	400	42.97 ± 1.63		0.7402 ± 0.020
5	600	58.61 ± 0.87		0.5372 ± 0.069
6	800	68.30 ± 1.18		0.4114 ± 0.049

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.

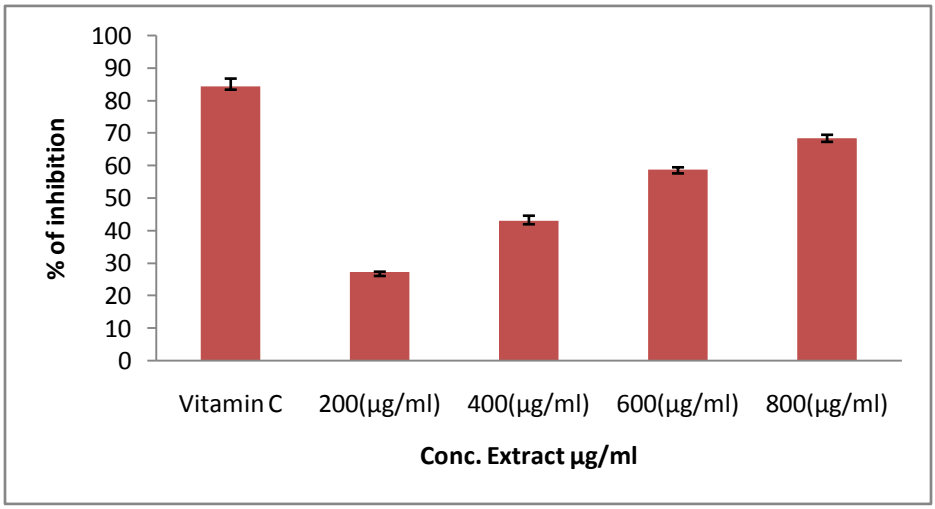

Figure 2: Percentage of inhibition of lipid peroxidation by different concentrations of methanolic extract of *Passiflora nepalensis* fruit where vitamin C was used as standard.

The H_2O_2 consumed /min/mg tissue in catalase assay on goat liver homogenate in different concentration like 200, 400, 600 and 800 µg/ml were observed in 68.06 ± 0.9968, 83.17 ± 0.9353, 112.71 ± 0.8304 and 136.27 ± 0.4867 respectively.

Table 2: Effect of methanolic extract of *Passiflora nepalensis* 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	0.7659 ± 0.0025	0.7433 ± 0.0019	0.30	53.25 ± 0.6056
2	Vitamin C(5mM) Conc. of methanol extract (µg/ml)	0.8934 ± 0.0018	0.8368 ± 0.0014	0.30	163.22 ± 0.9615
3	200	0.8756 ± 0.0019	0.8521 ± 0.0021	0.30	68.06 ± 0.9968
4	400	0.8747 ± 0.0026	0.8297 ± 0.0066	0.30	83.17 ± 0.9353
5	600	0.8827 ± 0.0023	0.8286 ± 0.0018	0.30	112.71 ± 0.8304
6	800	0.8836 ± 0.0025	0.8367 ± 0.0023	0.30	136.27 ± 0.4867

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.

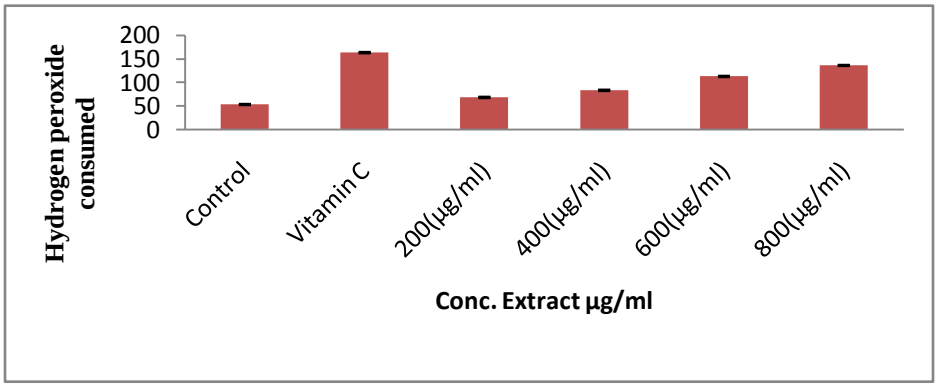

Figure 3: Amount of H_2O_2 consumed by different concentrations of methanolic extract of *Passiflora nepalensis* where vitamin C was used as standard.

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.

Table 3: Effect of methanolic extract of *Passiflora nepalensis* fruit on glutathione assay on goat liver homogenate.

Test tube no.	Treatment	Absorbance	Reduced glutathione (µg of GSH/mg of wet tissue)
1	Control	0.3253 ± 0.0133	5.06 ± 0.2198
2	Vitamin C (5mM) Concentration of methanolic extract (µg/ml)	0.9093 ± 0.0290	14.74 ± 0.4788
3	200	0.3287 ± 0.0181	5.20 ± 0.2982
4	400	0.3747 ± 0.0318	5.96 ± 0.5231
5	600	0.5063 ± 0.0224	8.12 ± 0.3689
6	800	0.6340 ± 0.0140	10.22 ± 0.2333

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 for all, except test tube no. 6 where *P* < 0.05 when compared with control group.

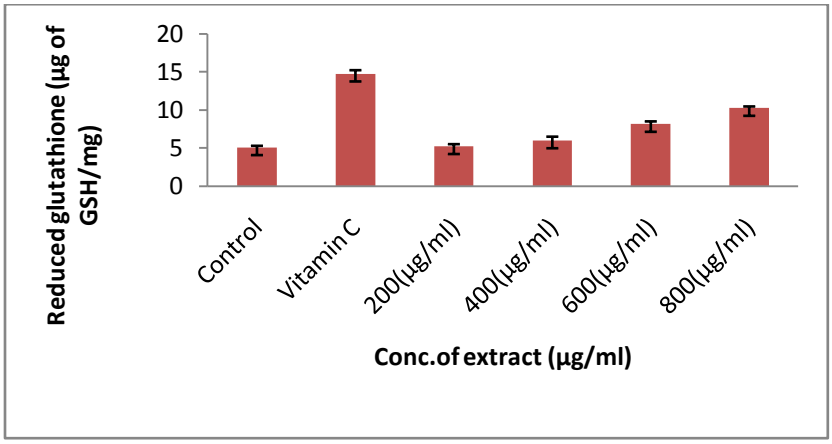

Figure 4: Amount of reduced glutathione formed for different concentrations of methanolic extract of *Passiflora nepalensis* fruit where vitamin C was used as standard.

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[41]. The results indicate that of methanolic extract of *Passiflora nepalensis*) ripe fruits 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 4: Effect of methanolic extract of *Passiflora nepalensis fruit* on superoxide dismutase assay on goat liver homogenate.

Test tube no.	Treatment	Rate of absorbance change	% Inhibition	SOD(U/mg tissue wet)
1	Control	0.0486 ± 0.0063	28.09 ± 0.133	0.56 ± 0.0034
2	Vitamin C (5mM)	0.0770 ± 0.0032	62.82 ± 0.416	1.26 ± 0.0097
	Concentration of methanolic extract (µg/ml)			
3	200	0.1757 ± 0.0073	34.18 ± 0.191	0.68 ± 0.0034
4	400	0.1697 ± 0.0015	41.42 ± 0.407	0.83 ± 0.0103
5	600	0.1667 ± 0.0066	50.88 ± 0.284	1.02 ± 0.0033
6	800	0.1617 ± 0.0012	58.24 ± 0.749	1.17 ± 0.0136

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.

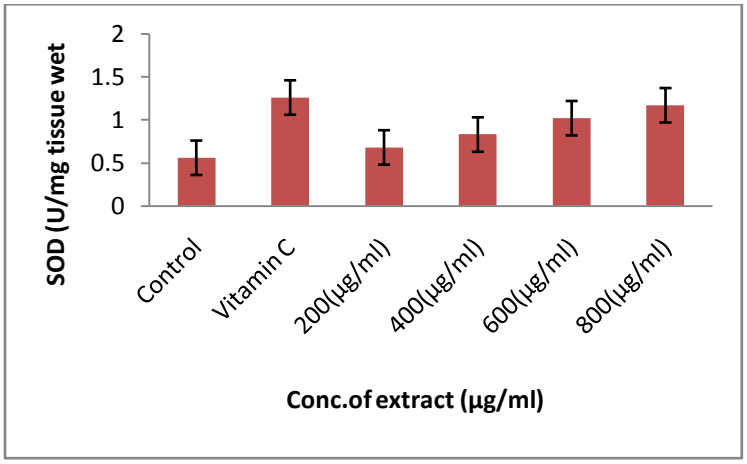

Figure 5: Amount of SOD for different concentrations of methanolic extract of *Passiflora nepalensis* where vitamin C was used as standard.

CONCLUSION

The result of the present study showed that the methanolic extract of *Passiflora nepalensis* ripe fruits, which contain phenolic compounds, exhibited the great antioxidant activity. The high scavenging property of methanolic extract of *Passiflora nepalensis* ripe fruits 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 *Passiflora nepalensis* ripe fruits in this research exhibited antioxidant. The antioxidant potential may be attributed to the presence of polyphenolic compounds.

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