

**FREE RADICAL SCAVENGING AND ANTIOXIDANT POTENTIAL ACTIVITY OF CASSAVA PLANTS**

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*Received: 22 November 2013, Revised and Accepted: 20 December 2013***ABSTRACT**

To isolate and quantify the protein and reducing sugar content of the cassava roots together with the antioxidant potential assays, which also included the inhibitory values on free radicals. The cassava roots were pulverized and extracted with both aqueous and organic extraction phases. The total protein content and reducing sugars and the flavonoid contents were estimated as per the protocol for all the four extract fractions. In addition, the free radical scavenging activity and SOD assay was done on all the four extracts. The total polyphenol content and the antioxidant potential assay was also estimated and to correlate the positive relation between the polyphenol content and the antioxidant power. The aqueous extract showed more content of reducing sugars and total protein content and flavonoids. The free radical scavenging activity was also more for the aqueous extract. In contrary the SOD activity was more towards the hexane fraction than the other three extraction phases. There is also positive correlation observed between the polyphenol content and the antioxidant power of the extracts. The cassava, a staple food though lacks the vitamin C content and is responsible for malnutrition among the consumers, it still has a good load of antioxidants which can make the plant a natural protective towards stress related diseases.

**Keywords:** Cassava, DPPH, SOD, Antioxidant potential activity.

**INTRODUCTION**

Cassava is a cheap and the most staple food for many of the developing countries. But the main drawback of this valuable crop is it has little protein content. Cassava (*Manihot esculenta* Crantz) is the most valuable starchy food crop. Many of the scientists all over the world are into the research on cassava to transform this crop into a disease resistant and to improve the nutritional qualities.

Cassava research has also been extended to produce transgenic cassava were they are raised to gain resistance to cassava mosaic disease (CMD) and also to cassava bacterial blight (CBB)[1]. Transgenic cassava crops are being raised to increase the content of several vitamins which adds nutritional as well as commercial value in the market. According to the survey in terms of world production in 2011, Cassava ranks among the top five crops like maize, rice, wheat and potato. Cassava is the most valuable crop and it is likely that it almost serves more than 600 million people all over the world. This transformation of crop is at a very high demand keeping in view of its highly susceptible nature to abiotic stresses and several diseases which include bacterial, viral and fungal diseases. Though cassava do contains about 85% of starch, but lacks any storage protein. It also has very low level of essential amino acids like lysine, leucine, methionine and cysteine[2].

As such this staple crop is neglected the most due to its drawback in supplementing the essential nutritional qualities. And most of the parts where it is the staple crop, it is bringing about malnutrition among the people[3]. This crop is staple and is mostly cultivated in the tropics. Most of the diseases correlate to the oxidative stress. Even diseases like atherosclerosis and cancer have always shown positive correlation to the stress. This stress is mostly caused due to the reactive oxygen species(ROS). Some examples of ROS are perhydroxy radical ( $\text{HO}\cdot$ ) and hydroxyl radical ( $\text{HO}\cdot$ ) and superoxide anion ( $\text{O}_2^{\cdot-}$ ).

ROS mainly acts on the membrane lipids causing damage to them via lipid peroxidation. This oxidatative damage is very fatal to the membrane biology as such production of antioxidants within the body is a must. Supplementing the body with the natural antioxidants is also a method of boosting up the immune system and protecting the body from the ROS[4].

To fight with the naturally produced ROS, antioxidants defense systems have been coevolved to counteract the damage. Almost all

the living species are gifted with protective defense systems to protect from ROS[5]. Recent studies have also proved that the antioxidant properties of plants could be supplemented into the systems to correlate with oxidative stress defense. Moreover these antioxidants produced by the plants can reduce different human diseases and can also stop the aging process[6]. Keeping in view of this, many of the plants have been screened for their antioxidant activities all over the world.

Recent studies showed that finding chemotherapeutic agents have made the world focus on natural products. Many of the plants have shown to contain bioactive compounds which are proved to be good in antioxidant properties[7]. Moreover some studies proved that there is a reported positive correlation between antioxidant activities of plants and their anti-proliferative effects. This proves of the compounds to be useful in inhibiting proliferative capacity of the cell[8].

As stated antioxidants neutralize most of the ROS relesad in the living systems. The antioxidants are also available in a wide variety of food sources. Artificially added antioxidants in food storage and conservation are costly at the same time cause deleterious effects on human health. The toxicological studies showed that they can provoke harmful effects in human and animals. Therefore, the need to search for natural occurring antioxidants is very much necessary. Moreover these natural antioxidant supplements are cheap and readily available[9,10,11].

In this study, we tried to estimate the protein and reducing sugar content of the cassava roots together with the antioxidant potential assays, which also included the inhibitory values on free radicals.

**Materials and methods****Plant material and extract preparation**

The roots of cassava was extracted with both aqueous and organic solvents as described elsewhere[12]. The roots of cassava were cleaned of any dirt and washed several times with water. The roots are sun dried and then finely pulverized into powder. The samples are kept at  $-20^\circ\text{C}$  until further use. About 25g of dried powder of *Cassava* root was extracted two times in 125ml of ethyl alcohol-water (4:1v/v) at room temperature over night with shaking. The

solution was then filtered through whatmann No.1 filter paper in a Buchner funnel and partitioned against Hexane (1:2 v/v) to obtain two fractions. H<sub>2</sub>O was added to the ethyl alcohol residue (1:1.5 v/v) and the mixture was washed with Ethyl acetate (1:2, v/v); the resulting two fractions were separated and the organic extraction was dried by addition of Na<sub>2</sub>SO<sub>4</sub>. The three fractions were evaporated in water bath to near dryness. The extract is then freeze dried for about 48 hours to achieve complete dryness.

#### Analysis of flavonoid content

Flavonoid content of the extracts was quantified using the aluminium chloride assay method. The C-4 keto group and the hydroxyl group of either the C-3 or C-5 of flavonoids reacts with the aluminium chloride to form an acid-stable complex[13]. In brief 500 µl of plant extract was dissolved in 1.5 ml of ethanol (95%) and 0.1 ml of 10% aluminium chloride. To this 0.1 ml of 1M sodium acetate are added. The volume is made up to 5ml with distilled water. Absorbance of the yellow-green complex was measured at 415 nm after 30 min. Quercetin was used as standard. Flavonoid content of the plant extracts was expressed as mg quercetin equivalents per gram dried weight (mg QE/g dried weight). All the experiments were carried out in triplicates.

#### Analysis of phenolic content

The extracts collected were then analyzed for the total phenol content. The phenolic content was assayed using the Folin-Ciocalteu assay[14]. Phenolic compounds, at basic pH, reduces the phosphomolybdic and phosphotungstic acid reagent, forming a blue complex. This blue colour complex can be measured at a wavelength of 765 nm. In brief, 0.5ml of extract was mixed with 0.5 ml of Folin-Ciocalteu reagent and incubated for 5 min at room temperature. This was followed by the addition of 0.5 ml sodium carbonate. The mixture was incubated in the dark at room temperature for about 2 hours. After the blue colour was developed, absorbance was taken at 765 nm. Gallic acid was used as standard. The concentration of phenolic content was expressed as milligram gallic acid equivalents per gram of dried weight (mg GAE/g dried weight). All the experiments were carried in triplicates.

#### Reducing Sugar Estimation

The reducing sugars are estimated according the protocol described elsewhere[15]. For estimation of reducing sugar, 1gm of the dried cassava powder is extracted using 1 ml of hot 80% ethanol. Ethanol is evaporated on hot water bath. The sugars are then dissolved in 1 ml of distilled water. The working standard glucose (250mg/100ml) solution (0.2, 0.4, 0.6, 0.8, and 1 ml) is taken separately into a series of test tubes. Volume of each sample is made up to 2 ml using distilled water. The distilled water (0.2 ml) is taken in separate tubes to serve as blank. Nelson's reagent (1 ml) is added to each tube and the tubes are placed in boiling water bath for 20 min and then cooled. Arsenomolybdic acid reagent (1 ml) is added to all tubes. The volume in each tube is made up to 10 ml with distilled water and observations are recorded at 540 nm after 10 min. The concentration of reducing sugar is expressed in terms of µg/µl.

#### Protein Estimation

1gm of the dried cassava powder is weighed and ground with 2 ml of 20% TCA. Ground samples are centrifuged at 10,000 rpm for 15 minutes. The Supernatant is discarded and 2 ml of 20% TCA is added to the pellet and mixed. The suspension is then centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant is then discarded and 1.2 ml of 1N NaOH is added to the pellet. The mixture is then boiled for 1-2 hours till the formation of a brown precipitate is appeared. The contents are then centrifuged at 10,000rpm for 5min. The supernatant is collected in a fresh centrifuge tube and used for protein estimation.

Working standard solution (0.02, 0.04, 0.06, 0.08, and 0.1 ml) of BSA is taken separately into a series of test tubes. Volume of samples is made up to 1.2 ml using distilled water. Distilled water to serve as blank. Lowry's mix (1.2 ml) is added to each tube. The tubes are then incubated for 10 min at room temperature. The Folin reagent (0.6ml) is added to all tubes. The tubes are incubated for 30 min at

room temperature. Light greenish color developed. The absorbance at 660nm is observed in a UV-visible spectrophotometer (Shimadzu, 1800). The unit of protein estimation is expressed in µg/µl. The method followed was described elsewhere[16].

#### DPPH radical scavenging activity

This assay was used to evaluate the radical scavenging activity of antioxidants in the plant extracts against a chemically-synthesised radical, 2,2-diphenyl-1-picryl-hydrazyl (DPPH)[17]. In this assay, 100µl of the extract (0-400 µg/ml) was added to 600 µl of DPPH reagent (100 µM), mixed thoroughly and incubated in the dark at room temperature for 20 min.

The decrease in absorbance was measured at 517 nm. The experiment was carried out in triplicate using ascorbate as standard. Quercetin were used as positive controls. The DPPH radical scavenging activity was calculated using the following equation: %of inhibition =  $\frac{\text{Abs}[\text{blank}] - \text{Abs}[\text{sample}]}{\text{Abs}[\text{blank}]} \times 100$ .

#### SOD assay

Superoxide dismutase (SOD) was assayed according to the technique[18]. The enzyme SOD is extracted using extraction buffer. 1 gm of dried powder of cassava Leaves are homogenized with extraction buffer (100mM potassium phosphate (pH 7.5), 2mM EDTA at 4°C). The homogenate is filtered through four layers of cheese cloth and centrifuged at 15,000 rpm for 20min. The supernatant obtained is used for SOD assay. To 0.75ml of sample equal amount of water is added and mixed properly. To the suspension 1ml of 0.01M Tris-Cl (pH 8.5) and 0.5ml of pyrogallol are added and mixed properly. For blank, 1.5ml of water is used as blank together with 1ml of 0.01M Tris-Cl (pH 8.5) and 0.5ml of pyrogallol. The contents are mixed properly. Incubate the contents at 37°C for 20 minutes. Absorbance at 420nm are recorded at 0 and 20minute time intervals. The rate of increase on absorbance units (A) per minute for the blank and for the test sample are calculated by the equation  $(A_{420\text{nm}}/\text{min} = \text{O.D at last interval} - \text{O.D at first interval}/\text{time in min})$ . The % inhibition is given by the formula  $(A_{420\text{nm}}/\text{min}[\text{blank}] - A_{420\text{nm}}/\text{min}[\text{test}] / A_{420\text{nm}}/\text{min}[\text{blank}] \times 100$ .

#### Antioxidant potential assays

The antioxidant power of the extracts has been assessed with the phosphomolybdenum reduction assay[19]. The reagent solution contained ammonium molybdate (4mM), sodium phosphate (28mM) and sulphuric acid (600mM) mixed with the extracts diluted in Methyl alcohol at the concentrations of 10mg/ml. The extracts were incubated for 60 min at 37°C or 90°C and the absorbance of the green phosphomolybdenum complex was measured at 695nm. The appropriate solutions of ascorbic acid have been used. The reducing capacity of the extracts has been expressed as the ascorbic acid equivalent. The antioxidant activities of the extracts were expressed as ascorbic acid equivalents by reference to the ascorbic acid standard calibration curve at 37° C and 90° C. The calibration equation for ascorbic acid at 37° C was  $y = 1.83x + 0.136$  ( $R^2 = 0.995$ ), and the calibration equation for ascorbic acid at 90° C was  $y = 1.331x - 0.118$  ( $R^2 = 0.997$ ), where x is the ascorbic acid concentration in mg/L and y is the absorbance reading at 765 nm.

#### RESULTS AND DISCUSSION

The dried *Cassava* powder obtained from the roots was analyzed for different biochemical parameters like phenolic content, flavonoid content, reducing sugar, protein estimation, and antioxidant enzyme assay for superoxide dismutase (SOD) and DPPH free radical scavenging activity. The reducing sugar content is estimated as per the method described elsewhere<sup>15</sup>. The total soluble proteins are estimated as per the method suggested elsewhere<sup>16</sup>. Antioxidant enzyme activity is done for the enzyme superoxide dismutase (SOD) as described elsewhere [20,21, 22].

#### Analysis of flavonoid content

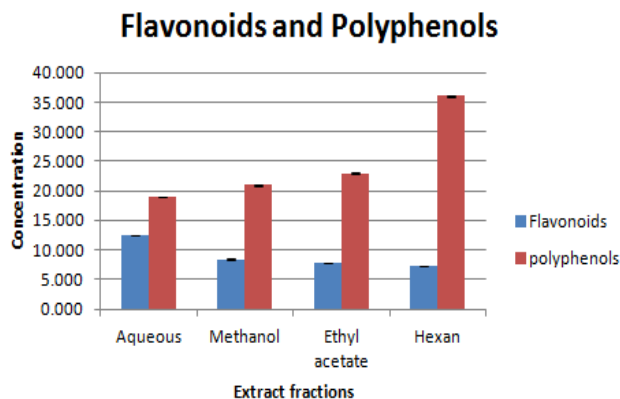
Flavonoid content of the plant extracts was expressed as mg quercetin equivalents per gram dried weight (mg QE/g dried weight). The aqueous extract was found to show more flavonoid content than the organic extracts. The concentration of the

flavonoids found for the aqueous extraction was found to be 127.093 $\mu\text{g}/\text{ml}$  of quercetin equivalents (Table 1). The calibration equation for Gallic acid was  $y = 0.0015x - 0.002$  ( $R^2 = 0.9908$ ), where  $x$  is the quercetin concentration in  $\text{mg}/\text{L}$  and  $y$  is the absorbance reading at 415nm. All the experiments were carried out in triplicates. The concentration of the quercetin used was 0.5 $\text{mg}/\text{ml}$ . The flavonoid content was found to be more (12.45  $\mu\text{g}/\text{ml}$ ) for the aqueous extraction phase than the organic phase (Table 1, Fig 1).

#### Estimation of polyphenols

The absorbance of the blue colour that developed was read at 765 nm using a spectrophotometer. The concentration of total phenolic content was expressed as Gallic acid equivalents by reference to the Gallic acid standard calibration curve. The calibration equation for Gallic acid was  $y = 1.0703x - 0.002$  ( $R^2 = 0.9906$ ), where  $x$  is the Gallic acid concentration in  $\text{mg}/\text{L}$  and  $y$  is the absorbance reading at 765 nm. The concentration of total phenolic compounds in the extract was determined by using the formula:  $T = CV/M$ ; Where, T= Total phenolic content  $\text{mg}/\text{gm}$  of plant extract in GAE, C= Concentration of Gallic acid from the calibration curve, V= volume of the extract in ml, M= wt of the pure plant methanol extract.

The results of Folin -Ciocalteu total phenols photometric assay are reported in table 1 and figure 1. Hexane fraction was shown to contain more polyphenols (36%), than the other three extracts.



**Fig1: Graph showing the values of flavonoids and total polyphenols of the extracts of cassava roots. The flavonoids are expressed in  $\mu\text{g}/\text{ml}$  and the total polyphenols are expressed in percentage. All the values are average of triplicates.**

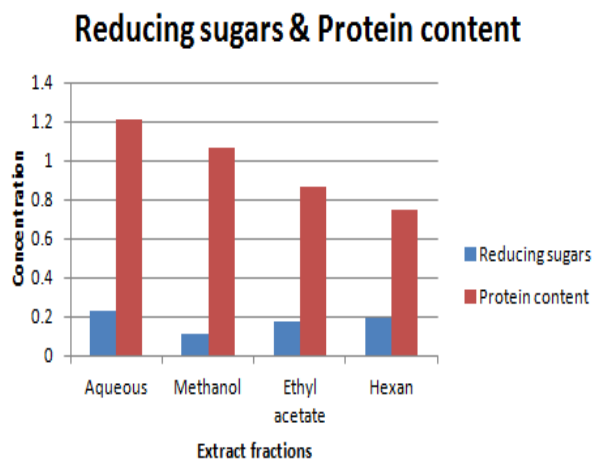
#### Estimation of reducing sugars

The reducing sugars are estimated according the protocol described elsewhere<sup>15</sup>. The working standard glucose (250 $\text{mg}/100\text{ml}$ ) solution (0.2, 0.4, 0.6, 0.8, and 1 ml) is taken separately into a series of test tubes and Nelson's reagent (1 ml) is added to each tube and the tubes are placed in boiling water bath for 20 min and then cooled. Arsenomolybdic acid reagent (1 ml) is added to all tubes. The concentration of reducing sugar is expressed in terms of  $\mu\text{g}/\mu\text{l}$ . The concentration of reducing sugars was expressed as standard glucose equivalents by reference to the standard glucose calibration curve. The calibration equation for standard glucose curve was  $y = 0.0016x - 0.002$  ( $R^2 = 0.9941$ ), where  $x$  is the glucose concentration in  $\text{mg}/\text{L}$  and  $y$  is the absorbance reading at 765 nm. The aqueous extract showed more content of reducing sugars (0.231 $\mu\text{g}/\mu\text{l}$ ) than the other three extracts (Table1, Fig2).

#### Protein Estimation

The absorbance at 660nm is observed in a UV-visible spectrophotometer (Shimadzu, 1800). The unit of protein estimation is expressed in  $\mu\text{g}/\mu\text{l}$ . The concentration of proteins was expressed as BSA equivalents by reference to the standard protein calibration curve. The calibration equation for standard protein curve was  $y = 0.0045x - 0.002$  ( $R^2 = 0.9943$ ), where  $x$  is the glucose concentration in  $\text{mg}/\text{L}$  and  $y$  is the absorbance reading at 765 nm. All the values

were average of triplicates. The total protein content was found to more for the aqueous phase ( 1.213 $\mu\text{g}/\mu\text{l}$ ) than the other three organic phases (Table 1, fig 2)



**Fig2: Graph showing the values of reducing sugars and protein content of the extracts of cassava roots. The reducing sugars and total protein content are expressed in  $\mu\text{g}/\mu\text{l}$ . All the values are average of triplicates.**

**Table 1: Table showing the values of polyphenols, flavonoids, reducing sugar content and total protein content. All the values are average of triplicates. The values are expressed with  $\pm$  standard deviation.**

	Flavonoids	Polyphenols	Reducing sugars	Protein content
Aqueous	12.456 $\pm$ 0.12	19 $\pm$ 0.34	0.231 $\pm$ 0.08	1.213 $\pm$ 0.012
Methanol	8.456 $\pm$ 0.09	21 $\pm$ 0.35	0.114 $\pm$ 0.06	1.066 $\pm$ 0.02
Ethyl acetate	7.856 $\pm$ 0.89	23 $\pm$ 0.41	0.172 $\pm$ 0.05	0.866 $\pm$ 0.03
Hexan	7.34 $\pm$ 0.13	36 $\pm$ 0.31	0.187 $\pm$ 0.09	0.746 $\pm$ 0.05

#### DPPH radical scavenging activity

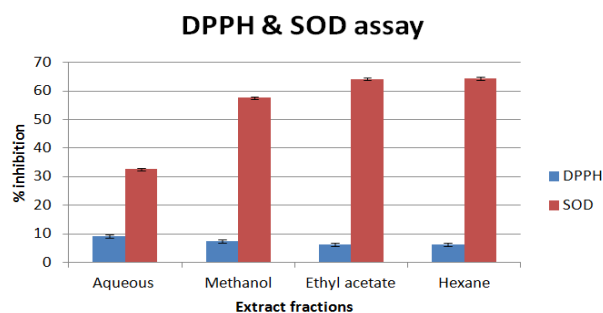
This assay was used to evaluate the radical scavenging activity of antioxidants in the plant extracts against a chemically-synthesised radical, 2,2-diphenyl-1-picryl-hydrazyl (DPPH). In this assay, 100 $\mu\text{l}$  of the extract (0–400  $\mu\text{g}/\text{ml}$ ) was added to 600  $\mu\text{l}$  of DPPH reagent (100  $\mu\text{M}$ ), mixed thoroughly and incubated in the dark at room temperature for 20 min.

The decrease in absorbance was measured at 517 nm. The experiment was carried out in triplicate using ascorbate as standard. The DPPH radical scavenging activity was calculated using the following equation: %of inhibition =  $(\text{Abs}[\text{blank}] - \text{Abs}[\text{sample}] / \text{Abs}[\text{blank}] * 100$ .

The DPPH free radical scavenging activity was found to more for aqueous phase (9.1%) than the organic phases (Table 2, Fig3).

#### SOD assay

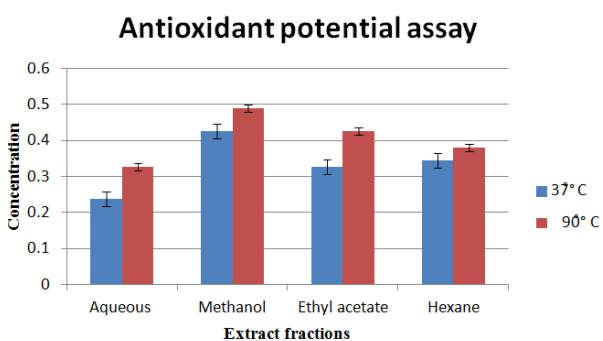
1 gm of dried powder of cassava Leaves are homogenized with extraction buffer (100 $\text{mM}$  potassium phosphate (pH 7.5), 2 $\text{mM}$  EDTA at 4 $^{\circ}\text{C}$ ) and used for SOD assay. For blank, 1.5 $\text{ml}$  of water is used as blank together with 1 $\text{ml}$  of 0.01 $\text{M}$  Tris-Cl (pH 8.5) and 0.5 $\text{ml}$  of pyrogallol. Absorbance at 420 $\text{nm}$  are recorded at 0 and 20 $\text{min}$  time intervals. The rate of increase on absorbance units (A) per minute for the blank and for the test sample are calculated by the equation  $(A_{420\text{nm}}/\text{min} = 0.D \text{ at last interval} - 0.D \text{ at first interval}/\text{time in min})$ . The % inhibition is given by the formula  $(A_{420\text{nm}}/\text{min}[\text{blank}] - A_{420\text{nm}}/\text{min}[\text{test}] / A_{420\text{nm}}/\text{min}[\text{blank}] * 100$ . In contrast to other results, the SOD activity was found to more for hexane fraction (64.66%) than the aqueous phase (Table2, Fig3).



**Fig3:** Graph showing the values of DPPH & SOD enzyme assay of the extracts of cassava roots. The %inhibition of DPPH & SOD are expressed in percentage. All the values are average of triplicates.

#### Antioxidant potential assays

The antioxidant power of the extracts has been assessed with the phosphomolybdenum reduction assay<sup>19</sup>. The samples were incubated for 60 min at 37°C or 90°C and the absorbance of the green phosphomolybdenum complex was measured at 695nm. The appropriate solutions of ascorbic acid have been used. The reducing capacity of the extracts has been expressed as the ascorbic acid equivalent. The fraction I showed highest reducing power at both 37°C and 90°C. At 90°C the activity was strongest for fraction II followed by fraction III and fraction I. But at 37°C the order was different for the fraction III and fraction I. At 37°C the highest being fraction II followed by fraction I and then fraction III. All the fractions showed dose dependent activity at 37°C and 90°C. The antioxidant potential activity was found to more for the organic phase and that too for the hexane fraction. This might be due the observation that the polyphenol contents are more in this hexane fraction (Table 2, Fig 4).



**Fig4:** Graph showing the values of antioxidant potential assay at 37°C & 90°C of the extracts of cassava roots. The antioxidant potential assay values are expressed in µg/ml. All the values are average of triplicates.

**Table 2:** Table showing the values of DPPH, SOD and the antioxidant potential assay. All the values are average of triplicates. The values are expressed with ± standard deviation.

	DPPH	SOD	Antioxidant potential assay	
			37° C	90° C
Aqueous	9.123 ± 0.05	32.574 ± 0.09	0.237 ± 0.12	0.326 ± 0.02
Methanol	7.329 ± 0.09	57.63 ± 0.089	0.243 ± 0.16	0.328 ± 0.08
Ethyl acetate	6.123 ± 0.12	64.23 ± 0.15	0.327 ± 0.97	0.425 ± 0.09
Hexane	6.234 ± 0.11	64.464 ± 0.19	0.345 ± 0.56	0.488 ± 0.123

Studies are on the pace to search for natural-based antioxidant containing compounds and which can be used as a nutritional supplement. In this paper, we have worked on isolating and estimating the nutritional qualities of the cassava roots. The parameters included not only included the sugars and protein content, but also the inhibitory effects of the cassava roots on the free radicals.

The flavonoid content was found to be more in case of the water extract and thereafter decreased for the organic extraction. But the flavonoid content was more or less similar for all the three organic phases (methanol, ethyl acetate and hexane). The reducing sugars and the protein content was also found to be more for the aqueous extraction than the organic extraction phase.

The antioxidant activities of cassava leaves have been reported in numerous studies but the roots of cassava were not reported for the aqueous or organic extracts. Even then, slight difference in the antioxidant activities do occur which solely depends on varieties, location and growth conditions.

Overall, in the estimation of the antioxidant capacities and the free radical scavenging assays showed positive results. The percent of inhibition of the DPPH was found to more for the aqueous extraction but in contrary, the hexane fraction showed more inhibition value for SOD assay. The antioxidant potential capacity was found to more for the hexane fraction than the other organic fractions and aqueous phase. Our correlates seems to support our study, in that the highest antioxidant potential activity was found to be due to more polyphenol content.

#### CONCLUSION

Cassava is the primary food of the tropics and the most staple food of the under developed and developing countries<sup>23</sup>. The nutritional values of the roots of cassava seems to be too important keeping in view of the nutritional deficiencies<sup>24</sup>. The roots are very poor in containing the vitamin C levels, which is the major cause of the malnutrition of the children fed on these staple crops<sup>26</sup>. In spite of its toxicity and low ascorbic acid levels, the plant is rich protein and antioxidant properties. The antioxidant potential content and the total polyphenols seem to be more for the aqueous extraction. The values seem to be promising for the crop to fight against the free radicals. The results confirm that the root extracts were strong antioxidants. In spite of its toxicity and low vitamin C levels, this crop can still be used as a powerful antioxidant containing food.

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