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Estimation of nutritive value, total phenolic content and *in vitro* antioxidant activity of *Manihot esculenta* Crantz. (Cassava) leaf

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Abstract
In this research, *Manihot esculenta* Crantz. (Cassava) sample was collected from Kyon- pyaw Township, Ayeyarwady Region, Myanmar. It has been identified at Botany Department, Hinthada University. In order to find out the types of organic constituents present in the samples, preliminary phytochemical investigation was carried out by test tube and TLC screening methods. The nutritional values such as protein, fiber, fat and carbohydrates were also determined by AOAC method. The total phenolic content of different extracts was determined by Folin-Ciocalteu method. *In vitro* antioxidant activity was evaluated by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. Preliminary phytochemical tests have revealed that the presence of alkaloids, α-amino acid, carbohydrates, glycosides, tannins, phenolic compounds, cyanogenic glycosides, saponins, starch, steroids, terpenoids, flavonoids, and reducing sugar in the sample. Proximate composition of Cassava leaf has been found as 10.70 % of moisture, 3.19 % of crude fat, 29.28 % of protein, 16.25 % of dietary fiber, 33.71 % of carbohydrate, 6.87 % ash and 279 kcal /100 g based on dry sample. The higher extractable total phenolic was recorded in 70 % ethanol extract (748.22 µg GAE/mg) followed by aqueous extract (446.22 µg GAE/mg) for the leaf sample. Both 70 % ethanol and aqueous extracts showed significant DPPH radical scavenging activity. Ethanol extract of (IC50 = 17.69 µg/mL) leaf showed higher level of free radical scavenging activity and found to be the lower in aqueous extract of leaf (IC50 = 42.64 µg/mL). From these results, it can be inferred that this leaf possesses nutrients and antioxidant properties and has nutraceuticals potential for the treatment of malnutrition.

Keywords: *Manihot esculenta* Crantz. (Cassava) leaf, phytoconstituents, nutritional quality, total phenol content, antioxidant activity.

Introduction
Cassava (*Manihot esculenta* Crantz) is perennial shrub of the family Euphorbiaceae and usually 1.3–5 m tall with fleshy elongated tuberous roots or rhizomes. Cassava leaves also contain moderate level of phytochemicals that are important as natural antioxidant components of plant food products. Cassava leaves have the ability to provide a valuable supplement to the predominantly starchy diets [1]. Young green cassava leaves are consumed as vegetables in some Asian country such as Malaysia, Philippines and Indonesia [2]**.** In recent years, the aerial part of cassava plant, which had been treated as an agricultural by-product, but that nutritionally, presents great potential for human and animal consumption, has been gaining prominence. Furthermore, their use can provide an extra income to various producers that live on the cassava culture. Those leaves are rich in proteins and vitamins A and C, and minerals, especially Mg, Fe, Zn and Mn [3], obtained at a low cost, when compared to conventional leafy vegetables.

Experimental and epidemiological studies have been demonstrating that people who consume foods rich in antioxidants could have a reduced risk of many diseases, such as cancer, cardiovascular diseases, chronic diseases and aging, among others. The searches for antioxidants from natural resources have received much attention and efforts have been put into identify compounds that can act as suitable antioxidants to replace synthetic ones. However, the use of these synthetic antioxidants has been questioned due to their potential health risks and toxicity. In addition these naturally occurring antioxidants can be formulated to give nutraceuticals that can help to prevent oxidative damage from occurring in the body. Apart from food purposes, cassava leaf as also suspected to have bioactive compounds that exhibit antioxidant effect. Natural antioxidants or phytochemical compounds are secondary

metabolites of plants [4]. As polyphenolic compounds, flavonoids, have the ability to act as antioxidant by a free radical scavenging mechanism with the formation of less reactive flavonoid phenoxyl radicals.

Medicinally, the leaves juice is boiled down to syrup and used against many disorders, such as rheumatism, fever, headache, diarrhea and loss of appetite. Fresh leaves have shown the effect on inflammatory and microbial infectious disease [5]. It is reported that oral administration of an aqueous leaf extract to rats induced anti-inflammatory and analgesic effects [6-7]. According to the literature [8], methanolic extract of *Manihot esculenta* Crantz showed most potent anthelmintic activity. Cassava leaves are among the top three African indigenous vegetables rich in nutrients. They are the second in β-carotene after *Moringa oleifera*, the second in vitamin C after *Moringa stenopetala*, the third in vitamin E after *M. stenopetala* and *M. oleifera*, the third in zinc after *Pterocarpus mildbraedii* and *M. oleifera*, the third in antioxidant activity after *Adansonia digitata* and *Rorippa madagascariensis* and the third in total phenolic after *R. madagascariensis* and *A. digitata* [9].

In Myanmar, the northern part of Ayeyarwady region, cassava is widely grown and applied the tubers for nutritional and healthy purposes. The leaves are residue and worth mentioning due to their medicinal properties and nutrient value. The aim of the research was to study the nutritional quality, total phenolic content and antioxidant activity of *Manihot esculenta* Crantz. (Cassava) leaf that is locally applied as a human diet.

Materials and Methods

Sample Collection and preparation

The leaf sample of *Manihot esculenta* Crantz. (Cassava) was collected from Kyone-pyaw Township, Ayeyawady Region, Myanmar. After being collected, the scientific name of the sample was identified by authorized botanists at Botany Department, Hinthada University, Myanmar. The fresh samples were cleaned by washing with water and air-dried at room temperature to prevent some reactions of the phyto-organic constituents with sunlight. The air-dried samples were grounded using grinding machine. And then these powdered samples were kept in the sealed air-tight containers to prevent moisture changes and other contamination. It was then used without further purification or refining.

Nutrient Values and Physicochemical Characterization of Leaf Sample

0.5 kg of dried cassava leaf sample was used to determine the nutritional values: moisture, protein, fat, ash, carbohydrate, fiber and calorie content. The moisture content of sample was determined by AOAC method [10]. The total ash in the sample is the inorganic residue remaining after the organic matter has been burnt away. The fat content was determined by the soxhlet extraction method. In addition, the sample was also studied for fiber content by Fiber Cap method, protein content by AOAC method and ash content by using muffle furnace.

Phytochemical Investigation of Leaf Sample

In order to find out the types of organic constituents present in the dried powdered sample, phytochemical investigation was carried out according to the standard procedures [11, 12] and TLC screening methods.

The dried powdered sample (5 g) was percolated with methanol 50 mL for one week and filtered. This procedure was repeated for three times. The combined filtrates containing leaf constituents were evaporated by means of a water bath. Consequently, methanol soluble extract was obtained. In this way, pet-ether soluble extract from leaf sample was prepared. Thin layer chromatographic examinations on methanol and petroleum ether extracts were performed by using silica gel GF 254 precoated plate and a variety of solvent systems. After developing the chromatograms, these are viewed with the various spraying agents such as Libermann burchard reagent, I [2] solution, 5 % $FeCl_3$ solution to develop colour and classify the plant constituents.

Determination of Total Phenol Content in Crude Extracts by Folin-Ciocalteau's method

Folin-Ciocalteu method (FCM) was chosen to assess the total phenol content of plant materials. FCM based on the reduction of a phosphotungstate-phosphomolybdate complex by phenolic compounds to blue reaction products was used [13, 15] In this experiment, the total phenol content was studied on 70% ethanol extract, and aqueous extract Cassava leaf sample. 10 mg of gallic acid solution and 10 mL of distilled water were thoroughly mixed by vortex mixer. The mixture solution was filtered and the stock solution was obtained. Desired concentration 1000, 500, 250, 125, 62.5 and 31.25 μg mL^{-1} of solution were prepared from this stock solution of dilution with appropriate amount of methanol.

Each 1 mg of test sample (both extracts) and 1 mL of MeOH were thoroughly mixed by vortex mixer to get the 1 mg per mL of test sample solution. Blank solution was prepared by mixing the distilled water (0.5 mL) with Folin-Ciocalteu reagent (FC reagent) (5 mL). 5 mL of FC reagent was added to 0.5 mL of each solution (standard, test sample and blank solution) and kept for 5 min and then 4 mL of 20% sodium carbonate solution was added and made up to 10 mL with distilled water. The solutions were allowed to stand at room temperature for 30 minutes. After 30 minutes, the absorbance of these solutions was measured at 738 nm by using UV spectrophotometer. The absorbance of standard gallic acid solutions was also determined by UV-visible spectrophotometer at wavelength of 738 nm. The calibration curve obtained through this procedure is linear within the range 0–1000 μg mL^{-1} of gallic acid solution (Figure 1).

Absorbance measurements were done in triplicate for each solution and then mean values so obtained were used to calculate the total phenol contents as gallic acid equivalent (GAE) /mL from the calibration curve of standard gallic acid solution.

Screening of antioxidant activity of crude extracts by DPPH assay

DPPH (1, 1-diphenyl-2-picryl-hydrazyl) radical scavenging assay was chosen to assess the antioxidant activity of plant materials. This assay has been widely used to evaluate the free radical scavenging effectiveness of various flavonoids and polyphenols in food systems [16].

In this experiment, the antioxidant activity was studied on 70% ethanol extract, and aqueous extract Cassava leaf sample by DPPH free radical scavenging assay. DPPH (2.364 mg) was thoroughly dissolved in methanol (100 mL). This solution was freshly prepared in the brown coloured flask. Then it must be stored in the fridge for no longer than 24 hours. Each 2 mg of test sample (both extracts and isolated compounds) and 10 mL of Me OH were thoroughly mixed by vortex mixer. The mixture solution was filtered and the stock solution was obtained. Desired concentrations (200, 100, 50, 25 and 12.5 μg mL^{-1}) of solution were prepared from this

stock solution of dilution with appropriate amount of methanol. Blank solution was prepared by mixing the test sample solution (1.5 mL) with methanol (1.5 mL). 2 mg of Butylated Hydroxy Toluene (BHT) was dissolved in methanol and made up the volume to 100 mL. It was used as standard solution.

DPPH radical scavenging activity was determined by UV spectrophotometric method. The control solution was prepared by mixing 1.5 mL of 60 µM DPPH solution and 1.5 mL of methanol using vortex mixer. The sample solution was also prepared by mixing thoroughly 1.5 mL of 60 µM DPPH solutions and 1.5 mL of test sample solution. The solutions were allowed to stand at room temperature for 30 minutes. After 30 minutes, the absorbance of these solutions was measured at 517 nm by using UV spectrophotometer. Absorbance measurements were done in triplicate for each solution and then mean values so obtained were used to calculate percent inhibition of oxidation and then, IC_{50} (50% inhibitory concentration) value were also calculated by linear regressive excel program.

Results and Discussion

Nutrients and Phytochemical Constituents Present in Cassava Leaf

The nutritional values such as protein, fiber, fat and carbohydrates were also determined. Cassava leaves yielded an energy level of 278 kcal/100g, with a low content of moisture (10.7 %), fat (3.17 %) and ash (6.87 %). It makes a stable product for prolong period of time. In contrast, the content of carbohydrate (33.71 %), fiber (16.25 %) and protein (29.28 %) were found to be high level. This indicates that Cassava leaves are the good source of carbohydrate, fat, fiber and protein (Table 1).

After carrying out the nutritional quality determination, to separate the polar and non-polar organic constituents present in the samples, soluble matter contents were firstly prepared. From these experiments 14.67% of water, 17.72% of 50 % EtOH, 18.30 % of 70 % EtOH, 20.09% of 95 % EtOH, 25.23% of EtOAc and 29.34 % of Pet-ether extracts from Cassava leaf were obtained. It was found that the amount of non-polar constituents present in Cassava leaf was higher than that present of polar constituents.

Table 1: Some Nutritional Values of *M. esculenta* Leaf

No.	Parameter	Content (%)
1	Moisture	10.70
2	Protein	29.28
3	Fat	3.19
4	Ash	6.87
5	Carbohydrate	33.71
6	Fiber	16.25
7	Calorie Content (kcal/ 100 g)	279

In order to know the types of phytochemical constituents present in *M. esculenta* (Cassava), preliminary phytochemical tests were carried out. From these results, it was observed that steroids, terpenoids, flavonoids, glycosides, phenolic compounds, α-amino acids, reducing sugars, sponins, organic acids and tannins are present in *M. esculenta* (Cassava) leaf.

In addition, Thin Layer Chromatography (TLC) was also used to examine the types of compounds on methanol and petroleum ether extracts of Cassava leaf by using silica gel GF 254 precoated plate and a variety of solvent systems (Table 2). After the chromatogram was allowed to dry and was then sprayed with a solution of Liberman Burchard reagent. This reagent reacts with steroids, essential oils and terpenoids to give coloured compounds, mainly brown or purple. The chromatogram was again allowed to dry and then placed in an enclosed container (such as another beaker covered with a watch glass) along with a few iodine crystals. The iodine vapour in the container reacts with the spots on the chromatogram show up as yellowish spots indicating the presence of unsaturated group. 5 % $FeCl_3$ which sprayed onto the plate made clear the presence of phenolic compounds (Figure 1).

Table 2: Phytochemical Investigation on the Leaf of *M. esculenta* (Cassava) by TLC Screening

No	Types of compounds	Spray reagents	Solvent system	Observation	Remark
1	Phenolic compounds	5% $FeCl_3$	PE: EtOAc	Reddish brown/ Bluish black	+
2	Steroids Terpenoids Essential Oil	Liebermann-Burchard	Chloroform only	Various colour intensity	+
3	unsaturated compounds	Iodine vapour	PE: EtOAc	Yellow colour	+

(+) present

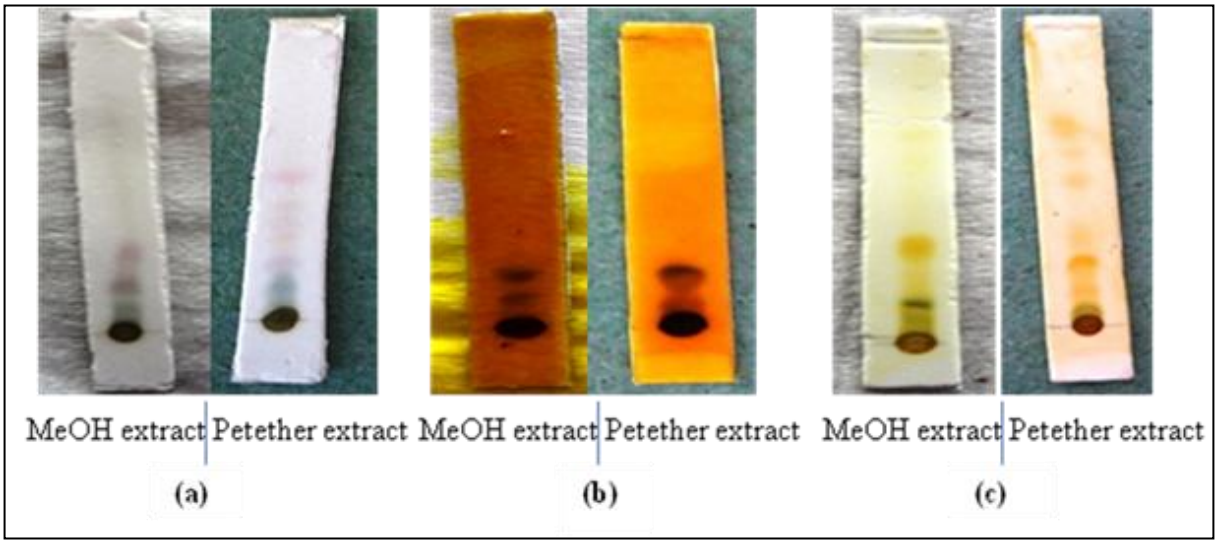

Fig 1: TLC Screening on the MeOH and PE extracts of *Manihot esculenta* Crantz. (Cassava) Leaf using different spraying reagents (a) Liebermann reagent (b) 5% $FeCl_3$ reagent (c) I_2 reagent

Total Phenol Contents in 70 % EtOH and Aqueous Extracts of Cassava Leaf

The determined contents of phenolic compounds are presented in Table 3. Folin-Ciocalteu method for the determination of phenolic compounds was standardized using the standard gallic acid. Five-point calibration using various concentrations ranging from 0- 1000 µg/mL gallic acid as the standard was linear ($R^2 > 0.995$) (Figure 2).

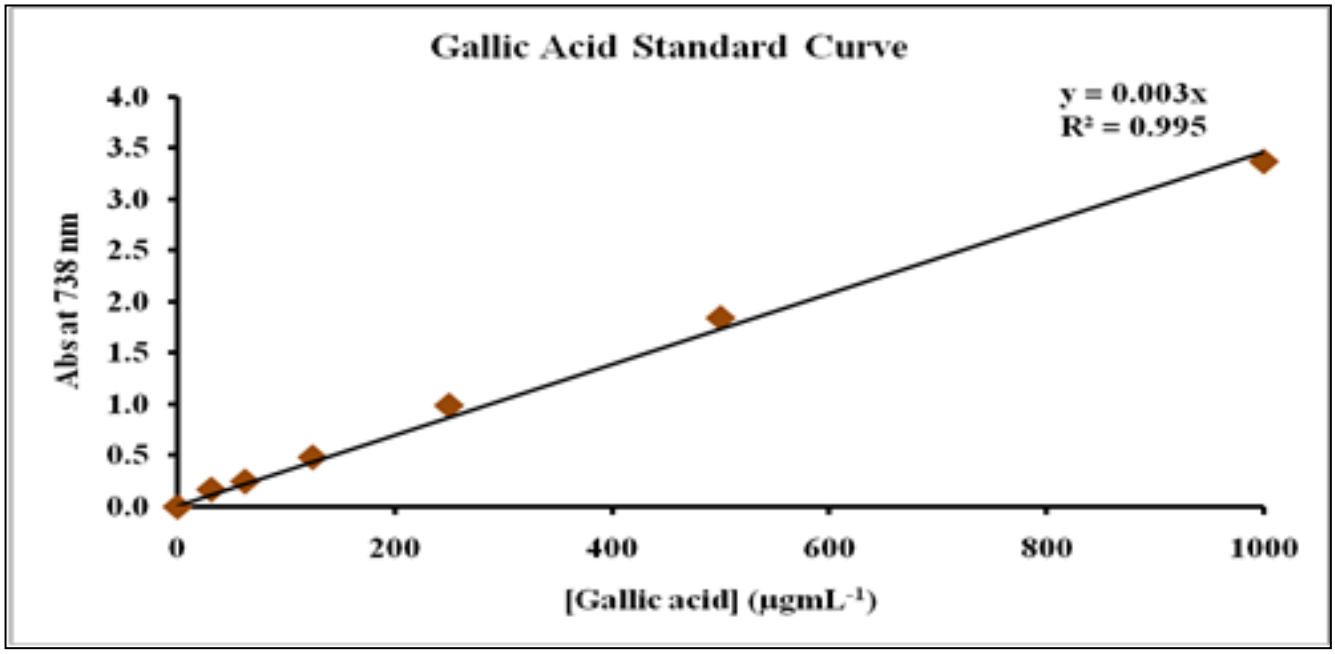

Fig 2: Calibration curve for Standard Gallic acid solution (using UV/Vis spectrophotometer at 738 nm)

The determined values were expressed as gallic acid equivalents (GAE). Highly repeatable results for standards and samples were obtained. The average values of phenolic compounds content in ethanol extract (784. 22 µg GAE per mg) were approximately two times higher than that in aqueous extract (446. 22 µg GAE per mg).

The great differences in the contents of phenolic compounds in different extracts indicate that less polar phenolic compounds form the most important part of the phenolic compounds in ethanol extract.

Table 3: Total Phenol Content (µg GAE/mg) of Aqueous and 70 % EtOH Extracts from *M. esculenta* Leaf

Extract	Total Phenol Content (µg GAE/mg)
Aqueous	446.22±7.72
70 % EtOH	748.22±0.76

Screening of antioxidant activity of crude extracts from Cassava Leaf

The antioxidant activity was studied on the aqueous and 70 % ethanol extracts from Cassava leaf by DPPH free radical scavenging assay method. DPPH (1, 1-diphenyl-2- picryl hydrazyl) method is the most widely reported method for screening of antioxidant activity on many plant drugs. This method is based on the reduction of coloured free radical DPPH in ethanolic solution by different concentration of the samples. The activity was expressed as 50% inhibitory concentration (IC_{50}).

The present study was carried out to investigate the radical scavenging activity of two crude extracts such as ethanol and aqueous extracts from Cassava leaf by using DPPH assay according to the spectrophotometric method. In this experiment, five kinds of different concentrations (12.5, 25, 50, 100 and 200 µg/mL) of each crude extract were prepared with ethanol solvent. Determination of absorbance was carried out at wavelength 517 nm using UV-visible spectrophotometer. Each experiment was done triplicate.

The percent oxidative inhibition values of crude extracts were measured at different concentrations and the results are summarized in Table 4. From these experimental results, it was found that as the concentrations were increased, the absorbance values were decreased, i.e. increase in concentration and increase in radical scavenging activity of crude extracts usually expressed in term of % inhibition. From the average values of % inhibition, IC_{50} (50% inhibition concentration) values in µg/mL were calculated by linear regressive excel program.

Table 4: Oxidative % Inhibition and IC_{50} Values of Aqueous and EtOH Extracts from *M. esculenta* Leaf

Extracts	% Oxidative Inhibition in different concentration (µg/mL)				
	12.5	25	50	100	200
Aqueous extract	25.18±0.95	34.92±0.70	56.33±1.05	66.53±1.83	81.53±1.15
70 % Ethanol extract	35.46±1.85	70.47±2.30	87.37±1.05	87.52±1.15	89.50±2.99

From these results, it can be clearly seen that IC_{50} values were found to be 42.64 µg/mL and 17.69 µg/mL for aqueous and ethanol extract (Figure 3). Among these extracts, radical scavenging activity of ethanol extract was found to be the higher than that of aqueous extract. Since the lower the IC_{50} showed the higher the free radical scavenging activity, it was observed that all of these extracts have the lower antioxidant activity than standard BHT (IC_{50}= 1.17 µg/mL).

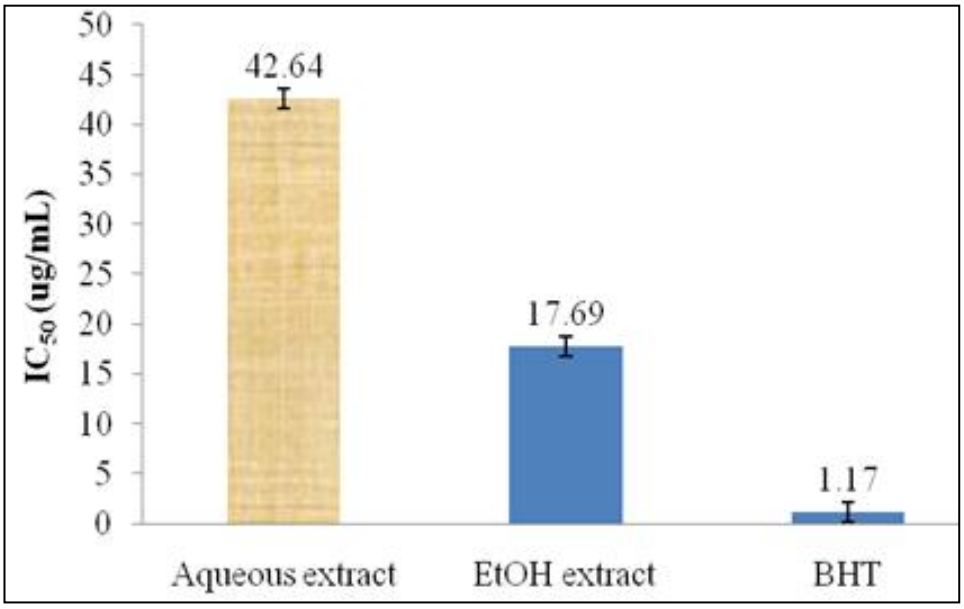

Fig 3: Bar graph of IC_{50} values of aqueous and 70 % ethanol extracts from Cassava leaf

The phenolic antioxidants in food include flavonoids, anthocyanins, catechins, chalcones and hydroxybenzoic and hydroxycinnamic acids. Many of these are found to be present in Cassava leaf. In this study, the total phenolic contents of the aqueous and ethanol extract react an oxidizing agent phosphomolybdate in FC reagent under alkaline conditions and result in the formation of blue colour complex; the molybdenum blue which is measured at 738 nm colourmetrically. The antioxidant activity of aqueous and ethanolic extracts was shown to be influenced by the total phenolic also. Cassava leaf containing high phenolic contents have been found to exert high antioxidant potential.

DPPH free radical scavenging method is a widely used method to evaluate the free radical scavenging ability of various samples. The DPPH radical is stable organic free radical with absorption maximum band around 517 nm. In this assay, the antioxidants reduce the DPPH radical (purple colour) to a yellow coloured compound, diphenylpicrylhydrazine. The extent of colour change depends on hydrogen donating ability of the antioxidants. It has been documented that ascorbic acid, tocopherol, cysteine, glutathione, gallic acid and few other compounds can reduce and decolourize DPPH radical.

Conclusion

Some indigenous people in Myanmar, *Manihot esculenta* Crantz. (Cassava) green leaves are not so applied as herbal medicine but consumed as vegetables. It is very essential to have a scientific evidence of Casava leaves and to know their phytochemical constituents and potential for the improvement of health and hygiene. In this study, Cassava leaf was found to be possessed the high nutrient value and could be applied as a good source of carbohydrate, protein and crude fiber but limited in fat. Moreover, total phenolic contents and antioxidant activity were observed significantly in both EtOH and aqueous extract of Cassava leaf. Consequently, it could be inferred that Cassava leaf is a nutritious antioxidant vegetable and provide nutraceuticals potential for the treatment of malnutrition.

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