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Kinetic Study of Phytochemical Extraction and Antioxidant Potential of Hibiscus cannabinus *L.*

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Abstract:

Kenaf Hibiscus cannabinus *L*.) is a valuable plant originating from Africa. It has multifunction among the old folk community and recently, it is employed in biochemical for functional food and structural engineering application in biocomposite materials. In Malaysia, kenaf plant is used as a material to the process of obtaining fiber for structural application. It also has excellent medicinal value for treating diseases through biochemical processing, but its profiling is still limited in the literature. This study aimed to (i) screen the secondary metabolite compound, and (ii) investigate the potential of kenaf leaf as a new functional food. The qualitative and quantitative phytochemical screenings of leaf extracts, as per the standard protocols were performed to identify the feasibility of kenaf leaf for use as a functional food. Bioactive compounds were found in both young and old kenaf leaves. In quantitative results, TPC, flavonoid and tannin, estimation of vitamin C, and DPPH test were carried out. This kinetic study showed that old leaf contained higher bioactive compounds than the young one. Kenaf leaf has the potential to be used as promising prototypes sources of functional food, and it is rich in antioxidants and bioactive compounds which are beneficial to human health.

Keywords: *Kinetic study; Antioxidant; Phytochemical; kenaf; Hibiscus cannabinus L.*

I. INTRODUCTION

An abundance of natural resources exists in the tropical rainforests of Malaysia. Kenaf (*Hibiscus cannabinus* L.) is a common wild plant that grows in the tropics. This plant is originating from the Malvacaea family [1]. Under optimum climatic conditions in Asian countries, it can attain a height of 5 meters within 6 to 8 months [2]. The application of kenaf was first begun in Malaysia in the early 1970s, and it has been grown as an inexpensive source of substance for generating panels' commodities. It is an advantageous plant and is suitably cultivated in location with the tropical climate [3]. This plant is classified as an agricultural crop, and it has been used as a promising source of plant fibre in making rope, gunny, thread, and carpets. The fibre from kenaf is also served as an essential raw material for the paper [4]. Kenaf plant has multiple applications in daily life. Young shoots and leaf are occasionally eaten as vegetables, while the seeds can be made as edible oil for cooking and various industrial applications. Industrial lubricant and biofuel production, to name a few are examples of industrial applications involving kenaf seed oil.

Kenaf is a well-known remedial plant that contains a variety of considerable groups of interesting bioactive components such as the anti-tumour compounds, phenolic compounds; anti-inflammatory, anti-proliferative activities which have been pharmacological tested [5]. Flora, which is rich in natural antioxidants, has significant influences on the rejuvenation of advantage in plant-based food and drugs. These natural antioxidants, typically made up of flavonoids, polyphenols, etc. are useful in diminishing the risks of developing cancer diseases. Note that the kenaf has also been widely embraced as a traditional remedy for treating injury since it contains many active ingredients like alkaloids, tannins, polyphenolics, saponins, steroids and essentials oils. So far, Kenaf is grown to a large extend solely for its fibre for paper pulp products. The application of its leafy portion is still lacking. The leaf is constantly treated as waste material. Zhao et al. [6], Basri et al. [5], Ayadi et al. [2] are among some of the notable researchers who had studied kenaf plant, especially the leafy portion recently. Zhao et al. [6] found that kenaf leaf is good for human health as it contains a high proportion of vitamin C, iron, nitrogen, phosphorus and calcium Besides, Basri et al. [5] reported that the leafy portion of the kenaf plant is one of the keys in healing of scurvy and jaundice, exciting the stomach and improving its action. As revealed by Ayadi et al. [2], the ascorbic acid contained in kenaf leaf extract can be used as a reducing agent. According to Ayadi et al. [2], kenaf leaf helps battle against blood and throat diseases.

A definite effort is necessary for nutritional analysis; product development and marketing since there is a limited number of functional food product in the market [7]. Many studies have

been made in formulating new functional food from waste over the years. To the best of our knowledge, no scientific report has been found to disclose the potential of kenal plant, specifically the leafy portion as a new potential source of functional food. This study was done to extract the secondary metabolites from the different varieties of kenaf leaf using distinct polarity of solvent using the Soxhlet method. Screening secondary metabolite compounds from different varieties of kenaf leaf was the next objective.

II. MATERIAL AND METHOD

A. Sample Pre – treatment

The leaf sample were dried using two types of drying method, which were oven drying and sunlight drying. The drying procedures were executed for 4 days for both drying methods. The leaf was dried for 96 hours continuously when using the oven drying. For sunlight drying, 32 hours were analysed which started from 9.00 a.m. until 5 p.m. All leaf was dried, and the young leaf was smaller in size as compared to the old leaf [8].

B. Liquid-Liquid Extraction

The leaf was dried using the oven at 40ºC, ground and the powdered leaf were extracted via Soxhlet extraction with different polarities of solvents, n-hexane and ethanol. The concentrated extracts were stored in the chiller freezer in airtight container for further use. Test for phenols, tannins, flavonoids, alkaloids and saponins were determined using the standard methods with some modifications [9]. Each sample was tested in triplicates. The extraction yield was calculated as follows:

$$\text{Total extraction yield: } \frac{\text{Mass of extraction x 100}}{\text{Mass of sample}}$$

C. Evaluation of antioxidant activities using 2,2-Diphenyl-1-Picrylhydrazyl Radical Scavenging Assay

Quantitative measurements of radical scavenging assay were measured following the method of Mohamad Nor [10] using 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) with slight modification. Firstly, reagent preparation was carried out. About 0.012 g of DPPH was dissolved in 100 mL of ethanol to generate DPPH stock solution (0.3 mM). The DPPH prepared was protected from light influence by keeping in the dark condition folded with aluminium foil. Antioxidant preparation was next conducted. About 0.1 mg/mL of ascorbic acid was prepared in ethanol; then the stock solution was diluted into different concentration for testing 10, 20, 30, 40, 50 µg/mL, respectively. Then, steps for DPPH quenching by antioxidants were performed. One mL of DPPH working solution was added into each micro centrifuge tube that readily contained the ascorbic acid solution. Each mixture was done for the DPPH radical scavenging activity by evaluating their absorbance at 515 nm with UV-spectrophotometer. The absorbance reading was recorded. The percentage inhibition of both standard and samples were calculated for each concentration and graphs were plotted (percentage inhibition against concentration). The percentage of DPPH free radical was estimated by Equation 3, where A0 and A1 are the absorbance of control Kenaf plant extract, respectively [11].

D. Bioprocess of Isolation on acid soluble collagen (ASC)

The pre-treated samples were extracted following the method of Huang et al. [12] with little modification. By continuous stirring sample was mixed with acetic acid (0.5M) and made into solution at the ratio of 1:2 (w/v) for 24 hours. The extract was then centrifuged at 10,000 × g for 15 minutes at 4ºC and the supernatant was separated. The residues were re-extracted with acetic acid (0.5M) with sample to solution ratio of 1:2 (w/v) for 12 hours. Then, it was centrifuged at 10,000 × g for 15 minutes at 4ºC. Both supernatants were combined and NaCl was added to salt out until the final concentration of the supernatant is 0.7M for precipitation to occur. The solution containing precipitate was centrifuged again at 2500 x g for 15 minutes for separation of precipitate from solution. The precipitates were then freeze-dried.

E. Total Phenolic Content

Phenolic content was measured following the method of Yadav and Agarwala [13] using the Folin-Ciocalteu colourimetric with some modification. One (1) mL of kenaf leaf plant extracts was inserted into a test tube and poured with 2.5 mL of Folin-Ciocalteu phenol (10%) and 2 mL of Na2CO3 solution (2%). The test tube was shaken using a mechanical shaker, and the solution was remained in the dark for 2 hours at room temperature. The blue-green colour appearance formed in the incubated test tube indicated the presence of phenolics which was recorded at 765 nm. Thus, gallic acid (0.1 mg/mL) was used as standard and serial dilutions were made ranging from 10 to 50 µg/mL. Calibration curve was plotted, and data was presented as gallic acid equivalents (GAE) (mg/g of the extracted compound).

F. Total flavonoid content

The total flavonoid content was analysed following the aluminium colourimetric method of [13] with some modification. Briefly, 1 mL of kenaf leaf plant extracts was added into a test tube containing 3 mL of ethanol, 0.2 mL $AlCl_3$ (10%), 0.2 mL of CH_3CO_2K (1 M) and lastly followed by 5.6 mL of distilled water. The test tube was shaken using a mechanical shaker before it was retained in the dark for 2 hours at room temperature. Appearance of light- yellow colour in the incubated test tube suggested the presence of flavonoids, which the absorbance was recorded at 420 nm against the reagent blank, distilled water. Quercetin (0.1 mg/mL) was set as standard and serial dilutions were made ranging from 10 to 50 µg/mL. Calibration curve was plotted, and the data was presented as quercetin equivalent (QE) (mg/g of the extracted compound).

G. Total Tannin Content

Tannin content was determined following the Folin-Ciocalteu colorimetric method of Mahmood et al. [8] with some modification. In a nutshell, 5 mL of Kenaf leaf plant extracts (0.1 mg/mL) was poured to the test tube followed by 1.25 mL of Folin-Ciocalteu reagent (100%) and 2.5 mL of Na_2CO_3 (20%). Then, the test tube was marked up until 20 mL of distilled water. The test tube was shaken using a mechanical shaker, and the solution was remained in the dark for 2 hours at room temperature. The light blue colour appearance was formed in the incubated test tube and it implied the presence of tannins. The absorbance was recorded for all standard solutions using UV-spectrophotometer at constant wavelength of 510 nm against the reagent blank, distilled water. Tannic acid (0.1 mg/mL) was used as the standard and serial dilutions were made ranging from 10 to 50 µg/mL. Calibration curve was plotted, and the data was presented expressed as tannin equivalent (TE) (mg/g of the extracted compound).

H. Vitamin C Estimation

The ascorbic acid of a sample was measured using the titration method as mentioned by Suntornsuk et al. [14]. This method was determined based upon the quantitative decolouration of DCPIP. The DCPIP was prepared by taking 0.05 g of DCPIP into 150 mL of hot distilled water and let the solution to cool. About 200 mL of distilled water was top up into the solution followed by adding 0.042 g of $NaHCO_3$.

Standard of vitamin C was prepared using ascorbic acid. Ten (10) mg of ascorbic acid was added in 100 mL of 3% metaphosphoric acid in 100 mL of distilled water. Ten (10) mL of ascorbic acid solution was added in a conical flask and the prepared dye DCPIP was titrated from a burette. A permanent light pink colour was formed in the titration medium, indicating that the titration was terminated. The burette reading was recorded, and the experiment was repeated three times. The experiment was continued using the kenaf leaf samples. Thus, the kenaf leaf samples were prepared, and the method used was similar to the preparation of standard vitamin C.

I. Statistical Analyses

The obtained data were analysed in triplicates, and the statistical analysis was done using analysis of variance (ANOVA) by SPSS Software. Data were considered to be significantly different at $p<0.05$.

III. RESULTS AND DISCUSSION

A. Comparative analysis of drying methods

Young leaf showed higher water loss over the old one (Table 1) because the former was thinner, smaller in size but had a larger surface area, which makes the water content evaporated faster than that of the later. This implied that loss of water content was affected by the surface area, and it is in line with the findings reported by Mercer [15].

Table 1: Weight and percentage of total extraction yield from different types of solvent

Type of Leaf	Type of Solvent				Total Extractable	
	95% Ethanol		Hexane			
	(g)	(%)	(g)	(%)	(g)	(%)
Old	15.75	10.50	2.55	1.70	18.30	12.20
Young	13.99	9.30	1.40	0.93	15.39	10.23
Total extractable	29.74	19.80	3.95	2.63	33.69	22.43

The results were found that the oven drying prevailed over the sunlight drying, as oven drying recorded the higher percentage of water loss. Oven drying took 6 hours, which was shorter than sunlight drying (32 hours). The sunlight drying method is a common method used in preserving food. This is because the high sugar content in food made them safe to dry in the sun. During the four days sunlight drying, hot and dry breezy days might affect the accuracy of the results [16]. The temperature during the experimental period was more than 40°C, and thence, it is suitable for drying the leaf samples [17]. Sunlight drying is a risky method as it depends on the weather. On the other hand, the oven is a thermally insulated chamber that enables the user to control the heating temperature. Furthermore, it is a closed chamber, which enables it to inhibit the growth of mould while preventing the presence of pests like ant, fly, etc. Based on these merits, the oven drying method is generally preferable compared to the sunlight drying method. Note that the oven drying method, such as the forced draft oven has received official approval by the Association of Official Agricultural Chemists (AOAC) [18]. However, several precautions must be taken before conducting the oven drying method. To obtain precise results, only the best quality of leaf should be selected as the sample and several crucial criteria, for example, overall appearance, zero mould and fresh look, to mention a few should be taken into consideration [19].

B. Total yield of crude extract

Kenaf extracts were obtained from two different solvents of different polarities which were 95% ethanol and hexane. The 95% ethanol has a higher polarity than the hexane. Extraction of plants was done through the Soxhlet extraction method at different temperatures which were for 95% ethanol, not more than 78°C and for hexane, not more than 68°C. Note that these temperatures are the boiling point for ethanol and hexane. Extraction yield shows the solvent's efficiency in extracting specific components from plants especially, the leaf part [20]. Table 1 shows the weight and percentage of crude extract content in Kenaf plant.

Table 1 shows the highest extraction yield was ethanol solvent (19.8%), while the lowest extraction yield was hexane solvent (2.63%). Polar functionalities contained in the ethanol make it

a superior solvent for organic compounds extraction, and it can attract polar and ionic molecules of bioactive compound from plants [21]. Thus, it can be seen that the bioactive compounds are easier to be extracted from plants that have polar property. Besides, developmental stages of leaf are another important factor that may affect the quality and quantity of bioactive compounds [22]. In this study, old leaf had shown higher extraction yield (18.3 g) than young leaf (15.39 g). Thus, the yield of extraction was influenced by several factors, for example, type of solvents used, either varying pH and polarities, extraction time, and the temperature that suitable with the chemical, physical and biological characteristics of the leaf sample [23].

C. Evaluation of the phytochemical constituents

The phytochemical screenings of all extracts were performed qualitatively. Table 2 shows the summary of phytochemical detected in each extract with two different solvents. Based on Table 2, note that positive results of phenols, flavonoids, alkaloids, tannin, and saponin were observed. Extracts from 95% ethanol showed a higher positive result compared to the extracts from hexane, irrespective of leaf types. For different types of leaf, same positive results were found for phenols, flavanoids, alkaloids, and tannins under 95% ethanol and hexane extracts, but the lowest positive results were observed only for the saponin test.

Table 2: Qualitative analysis of phytochemical in *kenaf* leaf

Phytochemical/ Solvent/leaf types	Solvent			
	95% Ethanol Extracts		Hexane Extracts	
	Old	Young	Old	Young
Phenols	+++	+++	++	++
Flavanoids	+++	+++	++	++
Alkaloids	+++	+++	++	++
Tannin	+++	+++	++	++
Saponin	+	+	+	+

D. Phenolic, flavonoid and tannin contents

In the current study, the total phenolic, total flavonoid, and tannin contents were represented in terms of gallic acid equivalent (mg of GAE/g) (the standard curve equation), quercetin equivalent (mg of QE/g) (the standard curve equation curve:), tannic acid equivalent (mg of TAE/g), respectively. Meanwhile, the standard curve equation obtained for the total phenolic, total flavonoid, and tannin contents were $y = 0.0306x + 0.0143$ with $R^2 = 0.9988$ at 765nm, $y = 0.0077x + 0.0391$ with $R^2 = 0.9687$ at 420nm, and $y = 0.0221x + 0.031$ with $R^2 = 0.9961$, respectively.

Table 3 presents the contents of phenolic, flavanoid and tannin in kenaf leaf. The phenolic content in the old and young leaf extracts was the lower followed by the flavonoid and tannin contents. In general, young leaf had a higher phenolic, flavonoid and tannin contents compared to the old leaf.

For the tannin content test, a high volume of dilution samples was needed to mix well so that a more accurate reading of absorbance can be achieved. The differences in total tannin content might be due to the changes in their chemical components during the extraction [24]. Tannin could not be extracted using solvents like water or alcohol [25]. A recent study by Ryu et al. [26] found that phytocompounds were present in the kenaf leaf and there were a total of 13 phytocompounds, including phenol, flavonoid and tannin in the leaf extract.

Table 3: Phenolic, Flavanoid and Tannin contents in *kenaf* leaf

Type of leaf	Phenolic (mg GAE/g)	Flavanoid (mg QE/g)	Tannin (mg TAE/g)
Old	0.6787 ± 0.0038	1.1742 ± 0.0063	8.46 ± 0.07
Young	0.5668 ± 0.0050	1.0255 ± 0.0316	7.37 ± 0.28
Total	1.2455 ± 0.0088	2.1997 ± 0.0379	15.83±0.35

E. Determination of vitamin C

Vitamin C was measured by titration of DCPIP method as described by Nigam [27]. Briefly, dye DCPIP was used as an oxidizing agent and it oxidized by the presence of ascorbic acid in the sample extracts. The titration of vitamin C was carried out with the aid of 3% metaphosphoric acid to prevent oxidation of other metallic ions that might present from the air or distilled water and also aid in stabilizing the solution pH. Thus, C=O bond in DCPIP molecule was very reactive, C=O acted as a good proton acceptor and eventually became C-OH after receiving electron. After the ascorbic acid in the solution has all been used, no more electrons remained to be reduced into HDCPIP. The HDCPIP formed from reduction DCPIP which appeared as a light pink colour. This condition showed the end point of the titration. Table 4 displays the vitamin C concentration in two samples, which were lower than the concentration of standard vitamin C used.

Table 4. Vitamin C (Ascorbic Acid) content in different types of *Kenaf* leaf

Old leaf (mg/mL)	Young leaf (mg/mL)	Standard vitamin C (mg/mL)
1.8	1.2	15.0

Disparities in vitamin C content between young and old leaf samples, however, were negligible. Young leaf contained higher vitamin C (0.0126 vs. 0.0108 mg/mL) than the old leaf.

The differences in ascorbic acid content between old and young leaf extracts might be due to the stage of the leaf, etc. To be more detail, the young leaf can directly get sunlight as compared to that of the old one. Besides, climatic conditions, species and climatic changes also influenced the content of ascorbic acid in the plants [28].

F. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Assay

DPPH radical scavenging assay is used to evaluate the ability of solvent extracts to donate or scavenge free radicals. DPPH was a stable free radical. When it reacts with an antioxidant compound, which can donate hydrogen, it is reduced to diphenylpicrylhydrazine. The changes in colour from deep-violet to light yellow can be measured spectrophotometrically. The antioxidant activity of different types of leaf was determined by using DPPH radical scavenging assay. Each sample conducted was prepared in concentrations of 10, 20, 30, 40, and 50 µg/mL, respectively.

Table 5. Antioxidant (DPPH scavenging) activity in *kenaf* leaf

Types of leaf	Concentration of plant extracts (µg/ml)	Antioxidant activity (%) (mean ± SD)
Old	10	52.54 ± 0.58^{a}
	20	59.39 ± 0.95^{b}
	30	65.87 ± 0.22^{c}
	40	77.29 ± 0.58^{d}
	50	81.13 ± 0.12^{e}
Young	10	49.83 ± 0.08^{a}
	20	54.93 ± 0.08^{b}
	30	64.73 ± 0.60^{c}
	40	70.23 ± 0.08^{d}
	50	80.47 ± 0.08^{e}

SD, standard deviation.

[a,b.c.d,e] Means with different superscript letters were differed significantly ($p<0.05$).

Table 5 shows the antioxidant activity increased with increasing concentration of the solvent. Fig 1 illustrates the scavenging activity in different types of *kenaf* leaf with five distinct concentrations, namely 10, 20, 30, 40, and 50 (µg/mL), respectively. Table 5 suggested that, the highest antioxidant activity was occurred in concentration 50 µg/mL of old leaf compared to the young leaf. The percentage of DPPH inhibition for old leaf also showed the highest antioxidant activity (81.13%) followed by the young leaf (80.47%). Young leaf extracts had a lower reading than the old leaf, which may arise mainly due to the presence of a low amount of total phenolic, flavonoid, and tannin contents in the leaf extracts. This antioxidant activity might be affected by the quantities of phenols and flavonoids present [29]. However, no information was found on the antioxidant activity of *kenaf* leaf in earlier published literature.

Meanwhile the IC50 for the ascorbic acid standard was determined between the concentrations of 10 to 50 µg/mL. The concentration of ascorbic acid needed to neutralize 50% DPPH radicals scavenging was 1.4559 µg/mL. The ascorbic acid standard curve showed linearity trend with R2 = 0.9425 (Fig 2), indicating that the DPPH solution used was working well since it was able to be reduced by the presence of antioxidant ascorbic acid. Through comparison of all sample extracts with the standard ascorbic acid, all leaf extracts showed less antioxidant activity. A lower IC50 value due to an increase in DPPH inhibition indicates a higher antioxidant activity. In terms of sample selection, as seen in Figure 1, young leaf extracts showed a more superior capacity to achieve 50% of DPPH inhibition compared to old leaf extracts. The IC50 value for young and old leaf was 11.67 and 7.03 µg/mL, respectively.

IV. CONCLUSION

The results and findings of this study conclude that extraction for yield shows that the bioactive compounds from this plant are higher in 95% ethanol (19.80%) compared to hexane. The old *kenaf* leaf contained higher antioxidant activity (81.13%) compared to young leaf. This plant contained bioactive compounds (phenol, flavonoids, tannin and saponin) implying that it has the feasibility to be developed as a new functional food with antioxidant properties like the herbal drink

Future researches should be made, to supplement the chemical profiling of *kenaf* leaf by identifying the details for the individual bioactive compound, comprising phenols, flavonoids, alkaloids, tannin and vitamin C, etc. These bioactive compounds are advantageous to human health as they may contribute to the activities of antioxidant, antimicrobial, anti-inflammatory, anti-diabetic and others. It is suggested to add lime or lemongrass flavour to the formulation to reduce the unpleasant odour of *kenaf* leaf.

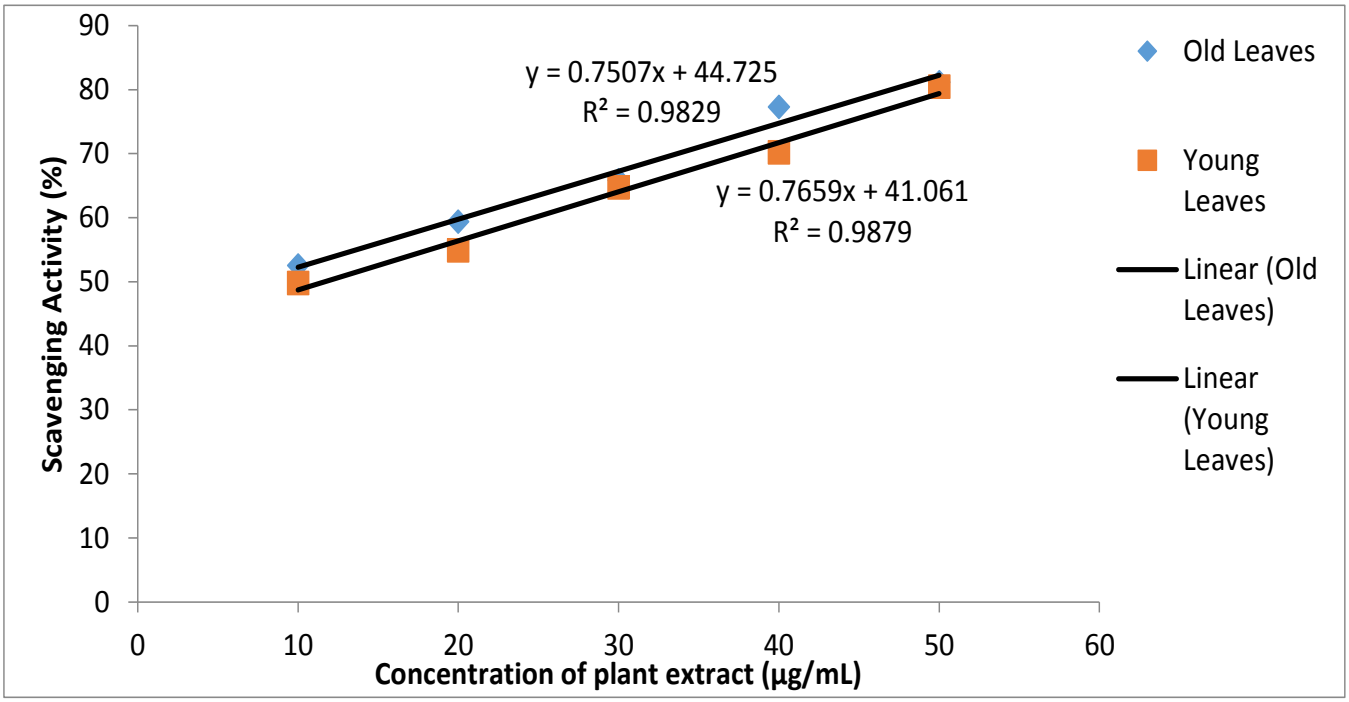

Fig. 1. Scavenging activity in different types of *kenaf* leaves with concentrations of 10, 20, 30, 40, and 50 (µg/mL), respectively.

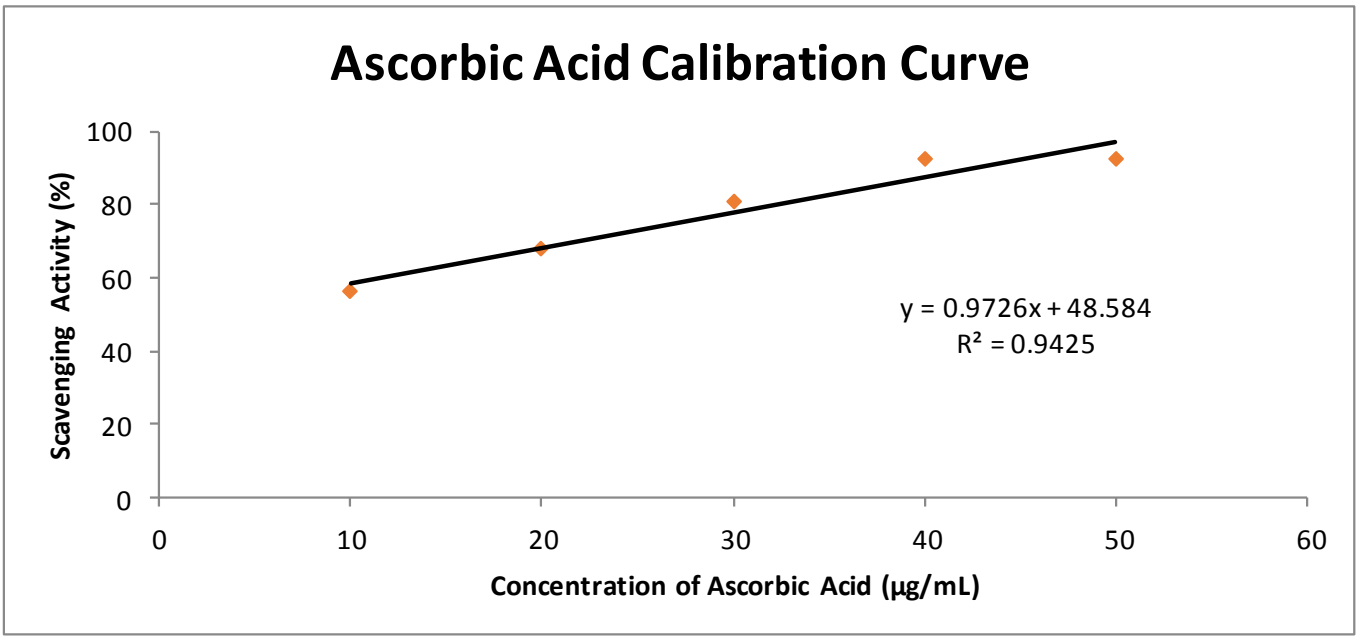

Fig. 2 Ascorbic Acid standard calibration curve at concentration from 10 to 50 µg/mL

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