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Determination of Antiradical Activity, Total Phenolic and Flavonoid Contents of Kamena-mena (*Clerodendrum paniculatum*. L) Leaves

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ABSTRACT

Antioxidants are compounds or molecules that can inhibit or delay-free radical reactions (initiation or propagation reactions) in the body. Kamena-mena (*Clerodendrum paniculatum*. L) is a plant that has the potential as an antioxidant. This plant is traditionally used to treat rheumatism, ulcers, neuralgia, inflammation, wounds, and antipyretics. This research purpose to investigate the antiradical activity of Kamena-mena leaves using the DPPH radical and ABTS assay and their correlation with phenolics and flavonoids. Kamena-mena leaf powder was extracted using the maceration method with methanol as the solvent. The methanol extract was added with warm water and diffraction with n-hexane, chloroform, and ethyl acetate solvents to obtain fractions. Antioxidant activity was determined using the DPPH and ABTS methods. Kamena-mena leaf extract and fraction showed very strong antioxidant activity using both DPPH and ABTS radicals. The ethyl acetate fraction showed the strongest antioxidant activity with IC_{50} value of 6,338 ± 0.005 µg/mL sample (DPPH) and 6,231 ± 0.031 µg/mL sample (ABTS), respectively. Phenolic and flavonoids compounds contributed to DPPH radical scavenging by 98.2% and 44.34%, respectively. whereas to ABTS radical, the contribution of phenolic and flavonoids was 98.78% and 36.26%, respectively. Kamena-mena leaves have very strong antioxidant activity, contain high amounts of phenolics and flavonoids, and have the potential to be developed as antioxidants and nutraceuticals.

Keyword: Antiradical activity, Kamena-mena leaves, ABTS, DPPH, phenolic, flavonoid

Introduction

Free radicals are free molecular species having unpaired electrons in atomic orbitals. The presence of unpaired electrons is a common feature of most radicals. Most radicals are unstable and highly reactive molecules. This makes free radical molecules very reactive and tends to bind with other molecules to make them stable. To be stable, the free radical atoms in the body will attack the body's cells, including normal cells.[1] Free radicals can react with several macromolecules (proteins, DNA and lipids) to cause new free radicals.[2] Free radicals can cause various diseases such as cancer, heart disease, cataracts, aging, and neurodegenerative disorders.[3] For that the body needs antioxidants in an effort to ward off excessive free radicals

Antioxidants are compounds or molecules that can inhibit or delay-free radical reactions (initiation or propagation reactions) in the body and reduce the surplus levels of free radicals to prevent oxidative stress.[4] Antioxidants can be synthetic antioxidants such as butylhydroxyanisol (BHA), butylhydroxytoluene (BHT), and terbutyl hydroxy quinone (BHQ). Besides synthetic antioxidants, natural antioxidants, come from plants such as leaves, fruits, and vegetables.[5] Because of the side effect of synthetic antioxidants, it is necessary to search antioxidants from natural materials.[6]

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Kamena-mena (*Clerodendrum paniculatum*. L) is a plant that has the potential as a source of natural antioxidants. Traditionally, Kamena-mena is used to treat rheumatism, ulcers, neuralgia, inflammation, wounds, antipyretics, eye diseases.[7]

Several studies have reported that *C. paniculatum* leaves have pharmacological activity, such as anti-bacterial activity,[8] antioxidant activity,[9] anti-inflammatory activity *in vitro* and *in vivo*,[10] and antioxidant activity *in vitro* and antiinflammatory *in vivo*.[11]

This study aims to evaluate the antiradical activity of extract and fractions of Kamena-mena (*Clerodendrum paniculatum* Linn) leaf using the DPPH (2, 2-diphenyl-1-picryhidrazyl) and ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) method and determination of phenolic and flavonoid contents.

Materials and Methods

Material

Fresh leaves of *C. paniculata* L were collected from Puuwatu District, Kendari City, Southeast Sulawesi Province , Indonesia in September - October 2019. Leaves material was authenticated at the Laboratory of Biology, FKIP, University of Halu Oleo, Indonesia by Mrs. Murni Sabilu and deposited in the herbarium of the same laboratory with a voucher specimen number BIO 258 (Figure 6), The fresh leaves (10 kg) were washed under running tap water to remove sand and debris, was airdried under shade for 10 days. The final weight after drying was 750 g, and powdered using a mechanical grinder. DPPH (Sigma-Aldrich®, USA), ABTS (Sigma-Aldrich®, USA), Galic Acid (Sigma-Aldrich®, USA), Quercetin (Sigma-Aldrich®, USA), methanol (E. Merck, Germany), ethyl acetate, n-hexane and aquades.

Extraction

750 grams of Kamena-mena leaf powder was macerated with 10 L methanol for 72 hours. The extract obtained was concentrated with a

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rotary evaporator to obtain crude extract. The crude extract (90 g) was partitioned with the aid of a separating funnel into hexane-soluble (20.5 g), chloroform soluble (5.1 g), ethyl acetate soluble (3.8 g), and water fractions (60.6 g).
Phytochemical screening is carried out according to standard procedures.[12-14]

Determination of anti-radical activity using the DPPH method
The methodology proposed by Garcia *et al*.[15] was used. Briefly, 1 mL of test sample from each concentration (1, 2, 3 , 4, and 5 µg/mL) were added 1 mL of DPPH solution. Then, 3 mL methanol added. Shaken until homogeneous, then incubated in a dark room for 30 minutes. Then the absorbance was measured using a UV-Vis spectrophotometer at 513 nm. The percentage inhibition was calculated using the formula:

$$\%\ inhibition = \frac{A_c - A_s}{A_c}\ x\ 100\%$$

Where:
% inhibition = percentage of DPPH radical inhibition
Ac = absorbance of control
As = concentration of sample

Value of antioxidant activity (IC_{50}), is calculated based on the linear regression equation between% inhibition and sample concentration or fraction, where the x-axis is the concentration while the y-axis is the % inhibition. So that the regression equation y = bx + a is obtained. Then the y value is replaced with 50. Where IC_{50} is defined as the concentration of the sample needed to inhibit 50% of the DPPH radical.

Measurement of antiradical activity using the ABTS method
The methodology proposed by Mistriyani *et al*.[16] was used. Briefly, The solution of ABTS 7 mM and potassium persulphate 2.45 mM were mixed in ratio 1:1 and allowed to stand in the dark for 12–16 h to produce stock solution of ABTS radical cation (ABTS +). This solution was further diluted with methanol to attain absorbance of 0.600-0.800 at 734 nm. The ABTS + working solution (3 mL) and 30 µL of blank, standard or sample were mixed and the absorbance was measured at 745.5 nm. The results were expressed as a percentage of inhibition of the ABTS●+ radical, according to the formula:

$$\%\ inhibition = \frac{A_c - A_s}{A_c}\ x\ 100\%$$

Where:
% inhibition = percentage inhibition of ABTS cation radicals
Ac = absorbance of control
As = concentration of sample

Determination of total phenolic content
The total phenolic content of extract and fraction of Kamena-mena (*C. paniculatum* L) were analyzed using the colourimeter method (Foin-Ciocalteau method).[17] 1 mL sample was added with 0.4 mL Folin-Ciocalteu Reagent (FCR) and allowed to stand for 5-8 min. After that, 4.0 mL sodium carbonate solution 7% (w/v) was added to the mixtures and diluted with water to 10 mL. The absorbance measured at 743 nm. Total phenolic content was expressed as Gallic Acids Equivalent (GAE) in mg/g sample

Determination of total flavonoid content
The flavonoid content of the extract and fraction of kamena-mena (*C. paniculatum* L) leaves was analyzed using the aluminium chloride colourimeter method. The procedure proposed by Zou *et al*.[18] 1 ml sample was added with 0.2 mL $AlCl_3$ 10%, and 0.2 mL of potassium acetate, then, 10 mL of distilled water added. After that, it was incubated for 30 min at room temperature, and the absorbance was measured at 443 nm. Total Flavonoids content was express as quercetin equivalent (QE) in mg/g sample.

Statistical analysis
Data were analyzed in triplicate and depicted as mean ± standard deviation calculated using the Excell program (Microsoft inc. USA) and *SPSS statistics version 24* program (IBM SPSS software).

Results and Discussion

Phytochemical screening
The results of phytochemical screening showed that the extract and fraction of *C. paniculatum* L leaf were positive for alkaloids, flavonoids, tannins, and terpenoids, but saponin not detected. As shown in Table 1.

Antiradical test
DPPH radical sacavenging activity
DPPH is a radical that accepts a proton or hydrogen to form a stable diamagnetic molecule. Besides, antiradical testing using the DPPH method is a method that is widely used to estimate the antiradical activity of antioxidant compounds. So, this method is also easy and economical.[19] DPPH method is a method of determining the potential of free radicals that are often used to evaluate antioxidant properties. Although these radicals have limited similarity with peroxide radicals, this test is usually used to measure the antioxidant strength of seeds, vegetables, conjugated linoleic acid, herbs, seed oils, and starches in various solvent systems such as ethanol, acetone, methanol, and benzene.[20] The DPPH method is one of the widely used test methods because it is simple, inexpensive, fast, and provides reproducible results. This method was originally introduced by Blois. DPPH radicals are organic radicals with nitrogen atoms as ligands, which absorbs the purple colour in the alcohol solution at 515 - 520 nm. When interacting with proton donor molecules (antioxidants), the radicals will be converted into DPPH-H, which results in a change in the colour of the solution from purple to yellow (diphenylpicrilhydrazin). This method is widely used to evaluate the antiradical potential of plant extracts.[21]
The results showed the extract and fractions of Kamena-mena leaf stabilized the DPPH radical. This can be seen by the change in the colour of the DPPH solution after the addition of the extract and fractions of Kamena-mena leaves which changed from purple to yellow after incubation for 30 minutes. The color change in the DPPH solution is due to the DPPH bond with the hydrogen atom donated by the antioxidant compounds contained in the extract and fraction of kamena-mena.
The reaction scheme between radicals and antioxidants can be seen in Figure 1.

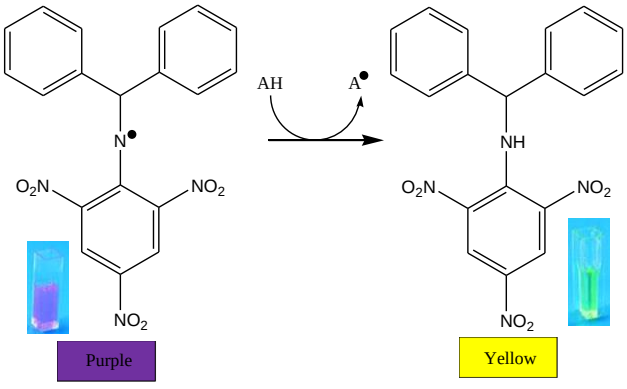

Figure 1: The schematic reaction of antioxidants with DPPH

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Table 2 shows the antioxidant activity (IC_{50}) of kamena-mena leaf of extract and fraction against DPPH radicals with Vitamin C as a positive control. Based on the IC_{50} value in Table 2, it shows that the Kamena-mena leaf extract and fraction have very strong antioxidant activity on DPPH radicals. The antioxidant activity for extracts and fractions of plant can be classified as strong (at IC_{50} value < 100 µg/mL), moderate (at IC_{50} value 101 – 150 µg/mL), and weak (at IC_{50} value 151 – 200 is µg/mL).[22] The data in Table 2 shows that the ethyl acetate fraction has a stronger antioxidant activity compared to methanol extract, chloroform fraction, n-hexane fraction, and water fraction with values of 6.338 ± 0.005 µg/mL, 6.846 ± 0.005 µg/mL, 7.086 ± 0.083 µg / mL, 8.354 ± 0.077 µg / mL and 9.291 ± 0.036 µg/mL, respectively. This is consistent with previous studies which showed that the ethyl acetate fraction was stronger, including the ethyl acetate fraction of Raghu (*Dracontomelon dao* (Blanco) Merr) stem bark,[23] ethyl acetate fraction of rambutan peels two cultivars: cultivar Aceh and Binjai,[24] ethyl acetate fraction of senggani (*Melastoma candidum* D. Don) leaves, fraksi ethyl acetate of Langsat (*Lansium domesticum* Coor.) Seeds[25] and the ethyl acetate fraction of the *Oroxilum indicum* Linn bark.[26] Therefore, it is suggested that ethyl acetate fraction is sub-fractionated further to obtain active isolate as antioxidant.

ABTS radical scavenging assay

One of the most widely used tests to screen for anti-radical peptides is the ABTS test. 2,2'-Azino-bis (*3-ethylbenzothiazoline-6-sulfonic acid*) is a diammonium salt ($ABTS^{\bullet+}$) cation radical, a stable free radical, often used to estimate the total antioxidant capacity (TAC) of natural substances, such as crude extracts, polyphenols, phenolic acids, flavonoids, others.[27] An antioxidant assay using the ABTS method by colourimeter method at 734 nm, and the rate of decolourization as a percentage of ABTS radical cation inhibition was determined as a function of concentration and time. ABTS • + chromophore is produced by the reaction between ABTS and potassium persulfate which converts ABTS into cation radicals. This cation radical is blue and absorbs light at 734 nm. ABTS • + is reactive to most antioxidants including phenols, thiols, and vitamin C.[28] In this test, the blue/green ABTS radicals produced by the oxidation of ABTS with potassium persulfate will be reduced in the presence of antioxidants. The commonly used incubation time is 6 - 30 minutes.[29,30] The absorbance measurement was measured at 743.5 nm.

ABTS is often used to measure antioxidant capacity in the food industry and agricultural research. ABTS assay was also used to measure the relative ability of antioxidants to scavenge ABTS radicals compared to the Trolox standard.[31]

Figure 2 shows the reaction of Oxidation of ABTS by potassium persulfate to generate radical cation ABTS + and its reaction with an antiradical compound (AOH).

Table 3 shows the IC_{50} value of extract and fraction of kamena-mena leaf against ABTS radicals. The results show that the extract and fractions provide very strong antioxidant activity. Ethyl acetate fraction showed the strongest antioxidant activity compared with methanol extract, n-hexane fraction, chloroform fraction, and water fraction.

Figure 3 shows the effect of concentration on the inhibition of ABTS cation radicals by antioxidants with as standard vitamin C.

Determination of Total Phenolic Content

The total phenolic content in an extract and fraction can be measured using the spectrophotometer method using the Folin-Ciocalteu reagent. The principle of the method is the reduced ability of phenol functional groups. Oxidation and reduction reactions of phenolic ions will take place in alkaline conditions. Reduction of the phosphotungstate-fosfomobildenum complex (Folin-Ciocalteu reagent) by phenolic ions will result in a blue colour change. If an extract or fraction contains a lot of phenolic compounds, the reduction ability will increase. As a result, the colour will be darker, and the absorbance will be higher.[33]

The strength of DPPH radical scavenging activity will increase with increasing levels of phenolic compounds such as flavonoids, phenolic acids, and phenolic terpenes. Where the flavonoid and phenol compounds are the main groups responsible for antioxidant activity.[34,35] This is because phenolic compounds have constituents such as hydroxyl groups, o-hydroxy groups which greatly affect free radical scavenging. The antiradical activity in the extract and fraction was characterized by a decrease in the absorbance value of the sample.[36]

Table 4 shows the value of the total phenolic content of extract and fraction of Kamena-mena leaf. The data indicate that the ethyl acetate fraction has a high phenolic content compared to methanol extract, n-hexane fraction, chloroform fraction, and water fraction with values of 25.775 ± 0.026 mg GAE/g sample, 23.237 ± 0.117 mg GAE/g sample, 14.651 ± 0.134 mg GAE/g sample, 20.392 ± 0.069 mg GAE/g sample, and 11.005 ± 0.090 mg GAE/g sample, respectively.

Determination of total flavonoid content

Flavonoids are a group of phenolic compounds that are most abundant and widely distributed in plants, which are characterized by a benzo-python structure.[37] Flavonoids are the most diverse group of natural compounds which possess a broad spectrum of chemical and biological properties Not only for the antioxidant activity, but they also have the properties as anti-carcinogenic and anti-arteriosclerosis.[38] Total flavonoid content was determined using aluminium chloride method. Aluminium chloride will form stable complex with carbonyl group at C4 and hydroxyls at C3 (flavonols) and C5 in flavonols and flavones. It could also form labile acid complexes with hydroxyls in the ortho position in B rings of flavonoids.[39] Total flavonoids in extracts and fractions were determined by colourimetric method, by reacting with sodium nitrite, then reacting with aluminum chloride to form a flavonoid-aluminum complex so that it can be measured by spectrophotometry at 513 nm. The total flavonoid content was expressed as mg quercetin equivalent (QE)/g of sample.

Table 1: Result of phytochemistry screen of Kamena-mena leaves

Sample	Test				
	Alkaloid	**Flavonoid**	**Tannin**	**Terpenoid**	**Saponin**
Methanol extract	+	+	+	+	-
n-hexane fraction	+	+	+	+	-
Chloroform fraction	+	+	+	+	-
Ethyl acetate fraction	+	+	+	+	-
Water fraction	+	+	+	+	-

Table 2: IC_{50} values of extract and fraction of Kamena-mena leaves using the DPPH method.

Sample	IC_{50} value (µg/mL ± SD)
Methanol extract	6.846 ± 0.005
n-hexane	8.354 ± 0.077
Chloroform fraction	7.086 ± 0.083
Ethyl acetate fraction	6.338 ± 0.005
Water fraction	9.291 ± 0.036
Vitamin C	4.139 ± 0.028

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Table 3: IC_{50} values of Kamena-mena leaves using the ABTS method

Sample	IC_{50} value (µg/mL ± SD)
Methanol extract	6.557 ± 0.012
n-hexane fraction	7.696 ± 0.016
Chloroform fraction	6.998 ± 0.138
Ethyl acetate fraction	6.231 ± 0.031
Water fraction	8.519 ± 0.031
Vitamin C	4.639 ± 0.035

Due to its antioxidant activity, the phenolic compounds provide beneficial health effects. The phenolic compounds have long been associated with a variety of biochemical and pharmacological properties including antioxidant, antiviral, anticancer, and anti-inflammatory activities. Phenolic group from plants have caught the attention of researchers. This is due to the ability of phenolic group to protect the human body from oxidation, aging, cancer, and diabetes.[40] Phenolic and flavonoid groups are concluded as compounds that have antioxidant activity that comes from plants because of their ability to provide hydrogen radicals in antiradical tests and because of their nature as reducing agents. Therefore the antioxidants activities are frequently correlated with the contents of phenolics and flavonoid.[16,41] Figure 4 and Figure 5, shown the correlation between (A) phenolic content and (B) flavonoid content of extract and fractions kamena-mena leaf (x-axis) using DPPH (Figure 4) and ABTS (Figure 5).

Table 4: Total phenolic content of Kamena-mena leaves

Sample	Phenolic content (mg GAE/100 g sampel)			Mean ± SD (mg GAE/ g sample)
	I	II	III	
Methanol extract	23.102	23.292	23.316	23.237 ± 0.117
n-hexane fraction	14.531	14.626	14.796	14.651 ± 0.134
Chloroform fraction	20.347	20.472	20.357	20.392 ± 0.069
Ethyl acetate fraction	25.755	25.805	25.765	25.775 ± 0.026
Water fraction	10.908	11.087	11.020	11.005 ± 0.090

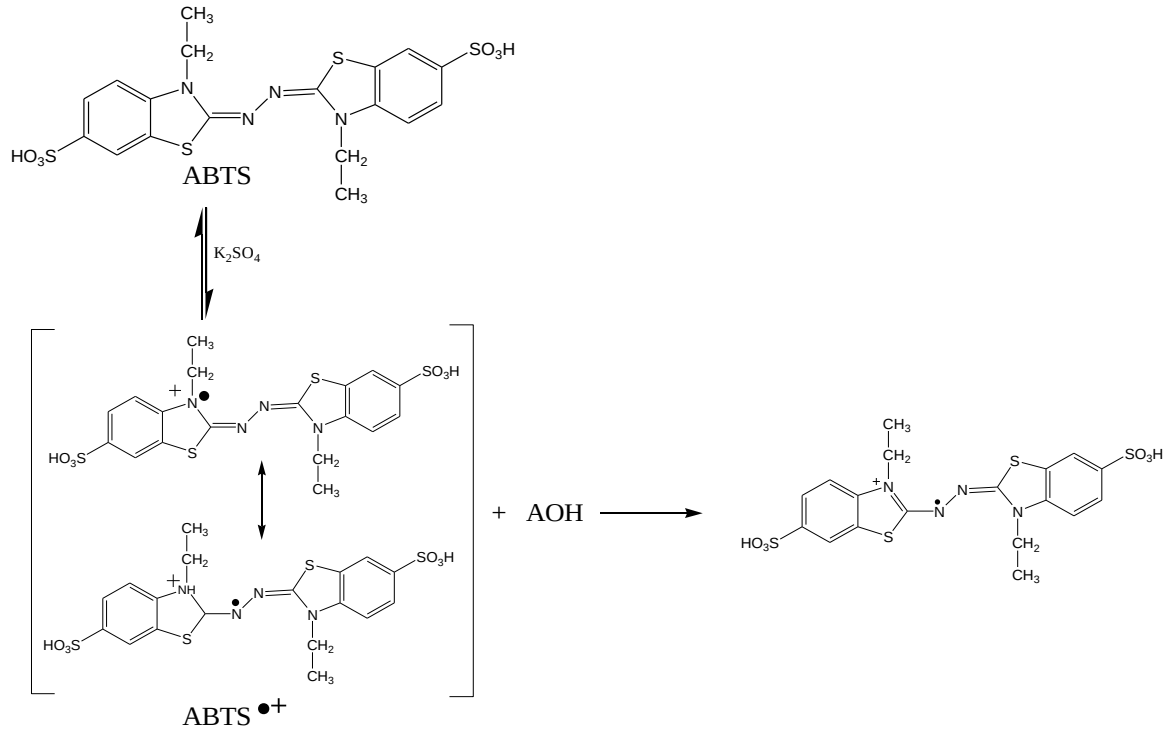

Figure 2: Oxidation of ABTS by potassium persulfate to generate radical cation ABTS + and its reaction with an antiradical compound (AOH).[32]

The coefficient of determination (R^2) is used as a tool to assess the relationship. The R^2 values obtained using the DPPH assay were 0.982 (phenolic) and 0.4434 (flavonoids), respectively. The R^2 values obtained using ABTS assay were 0.9878 (phenolic) and 0.3626 (flavonoids), respectively. This shows that phenolics and flavonoids contributed to DPPH radical scavenging by 98.2% and 44.34%, respectively.

In term of ABTS radical, phenolic and flavonoids contributed 98.78% and 36.26%, respectively. Based on these results, it shows that the DPPH and ABTS radical scavenging of extract and fraction of Kamena-mena leaf is not only limited to the phenolic and flavonoid groups. The antiradical activity can also be derived from other antiradical components such as alkaloids, vitamins, carotenoids, and lignins.

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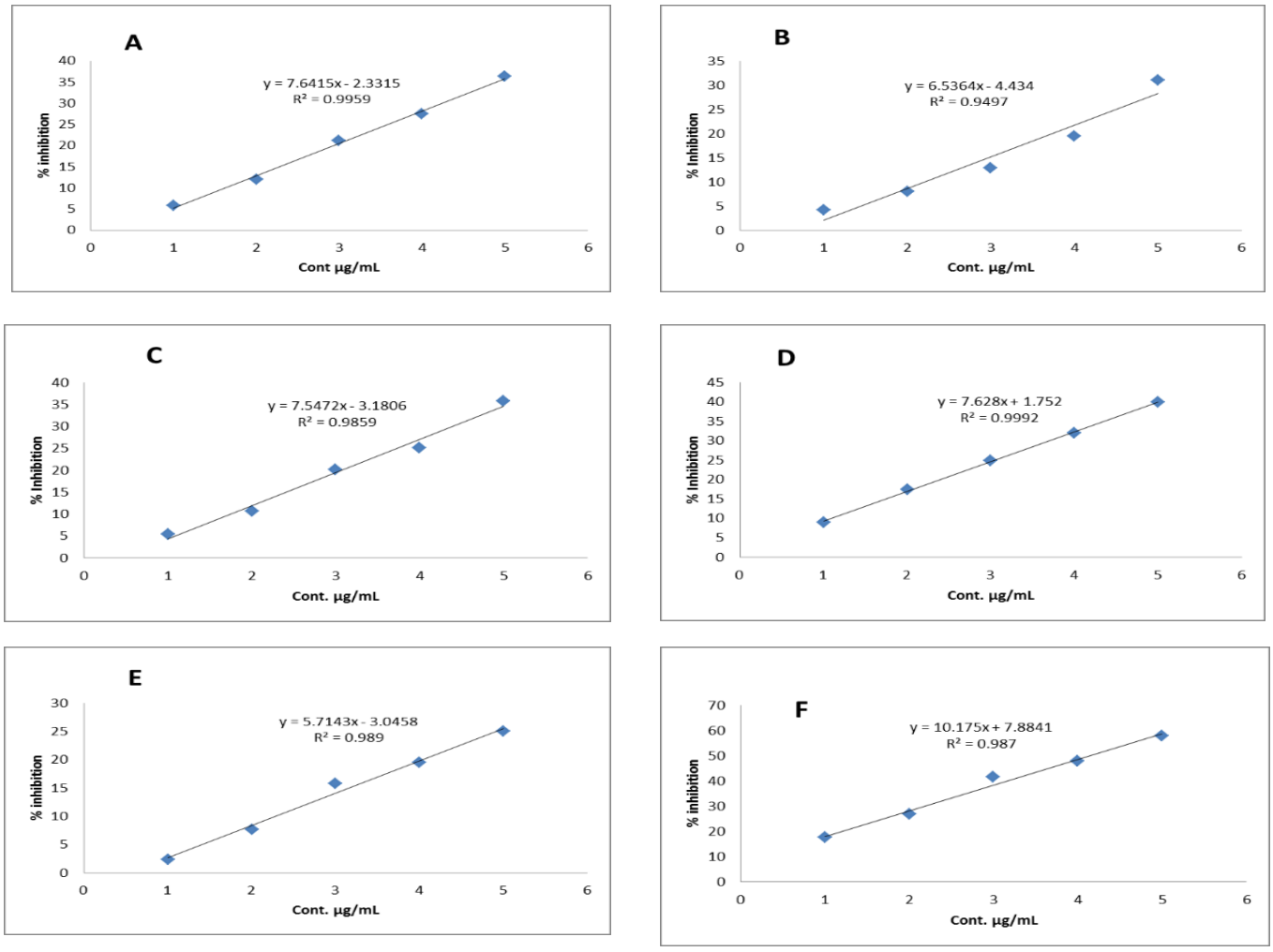

Figure 3: Effect of concentration on the inhibition of ABTS radicals.
(A) Methanol extract (R^2 = 0.9959), (B) n-hexane fraction (R^2 = 0.9497), (C) chloroform fraction (R^2 = 0.9859), (D) ethyl acetate fraction (R^2 = 0.9992), (E) water fraction (R^2 = 0.989), and (F) vitamin C (R^2 = 0.987)

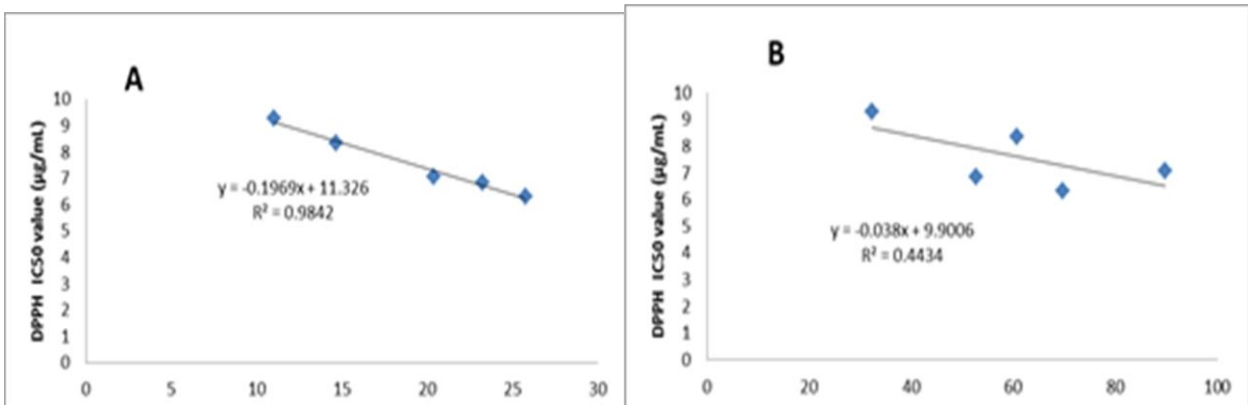

Figure 4: The correlation between **(A)** phenolic contents and **(B)** flavonoid contents (x-axis) with DPPH values (y-axis) of Kamena-mena leaves

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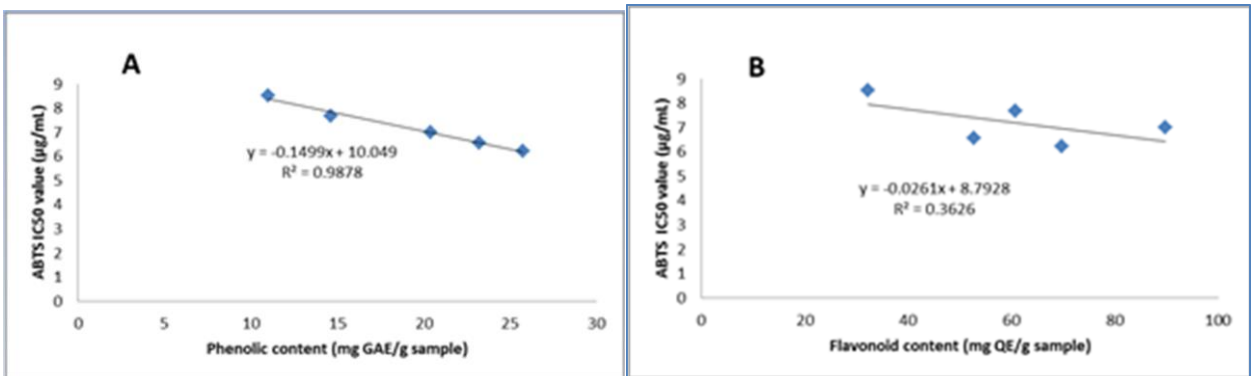

Figure 5: The correlation between **(A)** phenolic contents and **(B)** flavonoid contents (x-axis) with ABTS values (y-axis) of Kamena-mena leaves

Figure 6: (A) Plant Kamena-mena and **(B)** Kanmena-mena leaf

Conclusion

Kamena-mena leaves exhibited DPPH and ABTS antiradical activity. Ethyl acetate fraction showed the highest antiradical activity using DPPH and ABTS radical methods. The correlation of phenolics and flavonoid contents toward antioxidant activities are relatively moderat.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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