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Antioxidant Activity, Cytotoxicity, and Identification of Secondary Metabolites of *Kigelia africana* from Waterpark Platinum Riau

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Abstract

Antioxidants can prevent reactive oxygen-associated diseases, which trigger carcinogenesis, cardiovascular disease, and premature aging. India and Africa have mostly practiced sausage trees (Kigelia africana) as traditional medicine, whereas Indonesia is still limited. The research aims to determine the phytochemistry and bioactivity of the n-hexane fractions from crude methanol extract of the leaves and bark of the sausage tree as antioxidants and their toxicity. The sausage tree is derived from Waterpark Platinum Riau. The sausage trees were macerated with methanol and fractionated by n-hexane and ethyl acetate. All samples were tested for their antioxidant to 1,1-diphenyl-2-picrylhydrazyl (DPPH) and cytotoxicity to Artemia salina Leach larvae. Phytochemical results of methanol crude extract, n-hexane, and ethyl acetate fractions of leaves and sausage bark showed the presence of all secondary metabolites except alkaloids in the n-hexane fraction. Antioxidant and cytotoxic activity of stem and leaf bark ethyl acetate fraction was stronger than crude methanol extract and n-hexane fraction. Based on LC-MS/MS data, the secondary metabolite components that have contributed strongly antioxidant activity of this study are flavonoid compounds such as kaempferol and the derivatives, lignans (cubebin), and steroids (pregnant).

Keywords: Kigelia africana, DPPH, Artemia salina, flavonoid, lignan, steroid

INTRODUCTION

Indonesia is an archipelagic country located on the equator and is known as a country with a high level of biodiversity. Around 30,000 species of Indonesian plants have medicinal properties (Jumiarni & Komalasari, 2017). The Bignoniaceae family is a plant that plays a role in traditional medicine. Kigelia africana, also known as the sausage tree, is a species of this family that has many benefits as traditional medicine (Grace et al., 2002; Nabatanzi et al., 2020b).

Each part of the Kigelia africana plant is reported to have various biological activities, including parts of the bark and leaves. The bark of this species has analgesic, anti-inflammatory (Owolabi & Omogbai, 2007), antifungal (Owolabi et al., 2007), antibacterial (Agyare et al., 2013; Hussain et al., 2016; Idris et al., 2013; Owolabi et al., 2007), antioxidant (Agyare et al., 2013; Akanni et al., 2014; Bakare et al., 2015), and anti-cancer (Wambua Mukavi et al., 2020). In addition, the leaves have bioactivity as antioxidants (Atolani et al., 2013; Hussain et al., 2016; Nabatanzi et al., 2020a), antibacterial (Hussain et al., 2016; Idris et al., 2013), anti-cancer (Atolani et al., 2013), hepatoprotective (Shama et al., 2013), and anti-inflammatory (Namita et al., 2012).

One of the bioactivities of the bark and leaves of Kigelia Africana is antioxidants. The methanol extract from the leaves of a sausage tree grown in Nigeria was reported to have the potential as an antioxidant with an IC_{50} value of 0.32 ± 0.02 μg/mL (Nasiru & Oluwasegun, 2014). Methanol extracts of the bark and leaves of the sausage tree grown in Ghana are known to have antioxidant activity with IC_{50} values of 13.7 μg/mL and 56.9 μg/mL, respectively (Agyare et al., 2013). Ethyl acetate and chloroform extracts of sausage tree bark from Ghana are known to have antioxidant activity with IC_{50} values of 1.4 μg/mL and 56.41 μg/mL, respectively. In addition, chloroform extract from the leaves of the sausage tree grown in India was reported to have cytotoxic activity against colon cancer (HCT-116) with an IC_{50} value of 80 μg/mL (Ravi & Krishnan, 2016).

The characteristics of the biological activity of medicinal plants are determined by the content of the active compounds they contain. The compounds identified from the methanol extract of stem bark by GC-MS which had been carried out indicated the

presence of ester compounds, limonene, terpinene-4-ol, mirtenol, tetradecane, and palmitic acid. Meanwhile, the hexane fraction identified as monoterpenoids with the main component α-terpineol (39.21%), and other compounds such as hydrocarbons (14.32%), diterpenoids (4.54%), fatty acids and fatty acid esters (3.65%)) and alkaloid derivatives of benzo[a] [4,7]-phenanthroline-9-one (8) (1.71%) (Obafemi et al., 2017). Fabgohun's research (2020) identified the eicosane, pitol, and 9-octadecinamate compounds in the ethanol extract of sausage leaves. The ethyl acetate fraction of sausage fruit from PT Alam Indah Bunga Nusantara Cianjur using LC-MS identified 5,7-dimethoxy-4-methyl coumarin; 3-Hydrosi-β-lapakon; 3',6-dimethyl flavone; 2-(3,4-dihydroxy phenyl) - 5,7- dihydroxy-3-chromeniumyl hecopyranoside; 7H,8H–dipenalen-9,2-b:2,1-difuran-7,8-dion and D-leukyl-D-phenylalanyl-D-phenylalane-yl-D-valyl-N-isopropyl-D-leucinamide (Yani, 2017).

In African and Indian countries, research on sausage leaves and bark as antioxidants has been widely carried out, while in Indonesia, their use is still limited to ornamental plants. Differences in growing places, geographical locations, physiological variations, evolution, genetic changes, storage, and other environmental conditions will affect the presence of secondary metabolites in a plant (Figueiredo et al., 2008). Extracts of methanol, chloroform, and ethyl acetate have potential as antioxidants, and research on their cytotoxic properties is still limited. Therefore, it is necessary to explore the secondary metabolites of the n-hexane and ethyl acetate fractions from the crude methanol extract of leaves and sausage bark which contribute as antioxidants, and at the same time evaluate their toxicity properties. Therefore, in this study, phytochemical, cytotoxic, and antioxidant tests were carried out on the n-hexane and ethyl acetate fractions from the crude methanol extract of the leaves and bark of the sausage tree growing at Waterpark Platinum Riau. Cytotoxic and antioxidant tests were carried out using the Brine Shrimp Lethality Test (BSLT) method and 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent. Furthermore, the two fractions were identified for their secondary metabolites using the GC-MS and LC-MS/MS methods.

METHODOLOGY

Materials and Instrumentals

The materials used were leaves and stem bark of K. africana from Waterpark Platinum Riau, filter paper, concentrated HCl, 2% FeCl3, concentrated H_2SO_4, tween 80, shrimp larvae, seawater, distilled water, Wagner reagent, Meyer reagent, Dragendorff reagent, magnesium powder, chloroform, ethanol, acetone, DPPH reagent, thin layer chromatography (TLC). The solvents used are technical solvents that have been distilled once, namely methanol, ethyl acetate (EtOAc), and n-hexane (H). The tools used are glassware, rotary evaporator, vials, analytical balance, separating funnel, porcelain cup, oven, centrifugation tube, spatula, capillary tube, distillation apparatus collection, gas chromatography-mass spectrometry (GC-MS) QP2010S SHIMADZU, microplate spectrophotometer reader.

Sample Preparation

Samples of leaves and bark of the sausage tree were collected, then dried at room temperature and ground into 80 mesh.

Determination of Water Content

The porcelain cup is dried for 30 minutes in the oven at 105 °C, then cooled in a desiccator and weighed. As much as 1 g of each leaf powder and bark of the sausage tree was put into a cup and heated in an oven at 105 °C for 3 hours. The cup containing the sample was cooled in a desiccator for 30 minutes, then weighed. Heating and weighing the sample was carried out repeatedly until a constant weight was obtained (AOAC 2016). The percentage of water content is calculated based on the following Equation 1.

$$\text{Water Content (\%)} = \frac{A-B}{A} \times 100\% \quad (1)$$

Note: *A = wet sample weight (grams),*
B = dry sample weight (grams)

Maceration of The Leaves and Bark of The Sausage Tree of *K.africana*

The powder from the leaves and bark of the sausage tree was taken as much as 2 kg each, then macerated separately between the leaves and bark of the sausage tree using methanol with a ratio of 1:3 (sample: methanol) for 24 hours at room temperature with three repetitions. The maceration results were filtered, concentrated, and weighed as a concentrated methanol extract. The yield of sausage tree maceration results can be calculated by Equation 2.

$$\%\text{yield} = \frac{weight\ of\ macerated\ extract\ (g)}{Macerated\ sample\ weight} \times 100\% \quad (2)$$

Partition of Sausage Tree Methanol Extract

Partitioning of the methanol extract of the leaves and bark of the sausage tree was carried out using the liquid-liquid extraction method using n-hexane and

ethyl acetate as solvents. Concentrated extracts of each leaf and bark of the sausage tree (25.87 g and 43.76 g) were dissolved in methanol. Then each was extracted with n-hexane solvent with a ratio of 1:2 (methanol extract: n-hexane) using a separate funnel and let stand to form two layers. The top layer (n-hexane) is separated from the bottom layer (n-hexane insoluble compounds). The insoluble n-hexane phase was re-extracted with ethyl acetate to obtain the ethyl acetate soluble compound fraction. The liquid-liquid extraction results obtained soluble fractions of n-hexane and ethyl acetate and insoluble fractions of n-hexane and ethyl acetate (remaining methanol fraction). The three fractions are calculated in yield using Equation 2.

Phytochemical Testing

Phytochemical testing refers to the research of Shaikh and Patil (2020). The samples tested were the crude methanol extract and the three fractions resulting from the fractionation: n-hexane, ethyl acetate, and residual methanol.

Alkaloid Test. The sample was dissolved in chloroform, added a few drops of concentrated ammonia, and then shaken and filtered. 2M H_2SO_4 was added to the filtrate and shaken until two layers were formed. The top layer was divided into three equal parts, and a few drops of Mayer's, Dragendorff's, and Wagner's reagents were added to each. Alkaloid group compounds were detected by observing the formation of a white precipitate when Mayer's reagent was added, a brown deposition in Wagner's reagent, and an orange-red precipitate in Dragendorff's reagent.

Saponin Test. The sample in 5 mL of distilled water is heated and shaken vertically in a test tube the formed a stable foam layer (±1 cm high), indicating saponins.

Phenol and Tannin Test. The sample in 2 mL of distilled water was heated, and then a few drops of 5% $FeCl_3$ solution were added. Bluish green or black colour indicates the presence of phenols and tannins.

Terpenoid-Steroid Test. The sample was dissolved with 2 mL of chloroform and divided into two parts. Each sample solution was added with a few drops of Liebermann-Burchard reagent and concentrated H2SO4. The colour of the bluish-green solution formed with the Liebermann-Burchard reagent indicates steroid compounds, and the brownish-red colour with concentrated H_2SO_4 indicates the presence of terpenoids.

Flavonoid Test. The sample was dissolved with 2 mL of 50% methanol and heated. The solution is added with magnesium powder and a few drops of concentrated HCl. Then the solution was shaken, and 5 mL of amyl alcohol was added. An orange or red color formed indicates the presence of flavonoids.

Antioxidant Activity Testing with the DPPH Method

Determination of antioxidant activity refers to the literature of Salazar-Aranda et al. (2011). Each sample was made at 32.5-1000 µg/mL concentrations, namely 1000; 500; 250; 62.5; 32.5 µg/mL. To the solution of each concentration, 100 µL of 125 µM DPPH was added. The mixture was homogenized and incubated at 37 ºC for 30 minutes in the dark. The absorption of the solution was measured with a microplate reader spectrophotometer at a wavelength of 517 nm. Ascorbic acid was used as a positive control. The value of % DPPH inhibition can be calculated by Equation 3.

$$\%\text{Inhibition} = \frac{Blank\ Abs - Sample\ Abs}{Blank\ Abs} \times 100\% \qquad (3)$$

Note:

Blank abs = Absorbant 125 µM DPPH
Sample Abs = Absorbance of Test Sample

Antioxidant activity was determined using the IC_{50} value. The IC_{50} value of each sample was calculated using the formula of the linear regression equation, which states the relationship between the concentration of the antioxidant fraction expressed as the x-axis and the % inhibition expressed as the y-axis from the measurement replication series.

Toxicity Measurement with The BSLT Method

Toxicity test with BSLT refers to the literature Irmawan et al. (2018) and Hanafi et al. (2020). Artemia salina eggs were hatched in a container filled with aerated seawater under sufficient light conditions with a 40-60 watt incandescent lamp for 48 hours. Each sample was prepared with a stock solution with a concentration of 2000 µg/mL in 0.02% tween 80 (in seawater) and then diluted into 1000, 750, 500, 250, 100, and 75 µg/mL concentrations. Ten A. salina shrimp larvae were put into 5 mL of the test solution and used as a blank as a control. Each concentration was repeated three times and compared with the control. Observations were made for 24 hours. The number of dead larvae was counted, and the average was determined from three replications. The percentage of shrimp larvae mortality is calculated by Equation 4.

$$\%\text{Mortality} = \frac{Number\ of\ death\ larvae}{Number\ of\ test\ larvae} \times 100\% \qquad (4)$$

The LC_{50} value is obtained using the linear regression equation y = ax + b. the y value represents

the probit value at 50% mortality of larvae according to the Miller Tainterprobit method. In contrast, x represents the logarithmic value of the concentration of the test solution. The LC_{50} value was obtained from the concentration of the solution, which caused the death of 50% of the larvae.

GC-MS Analysis of The N-Hexane Fraction from The Leaves and Bark of The Sausage Tree

Compound analysis was done by injecting one µL of sample into the injecting column on the GC-MS instrument. The chromatographic conditions used in the Shimadzu GC-MS-QP2010S system were as follows: the column used was DB-5MS with a length of 30 m, a diameter of 0.25 mm, and a thickness of 0.25 um. The oven temperature used is between 70–300 ºC. The carrier gas used was helium at a pressure of 15.5 kPa, a total rate of 28.8 mL/min, and a ratio of 1:49. The eluted components will be detected on the mass detector. The spectrum of the components of the compounds analyzed was compared with the National Institute of Standards and Technology (NIST) literature (Rahayu et al., 2019).

LC-MS/MS Analysis of The Ethyl Acetate Fraction from The Leaves and Bark of The Sausage Tree

LC-MS/MS analysis was performed with an Ultra Performance Liquid Chromatography (UPLC) system and a XEVO-G2SQTOF mass spectrometer. The conditions used were the stationary phase C18 column (1.8 µm 2.1×100 mm), temperature 50 °C (column) and 25 °C (room), injection volume 250 µL, desolvation temperature 350 °C, detection at 50 eV, the mobile phase with a graduated gradient system using water + 5mM ammonium formate (A) and acetonitrile + 0.05% formic acid (B), and a flow rate of 0.2 mL/min was eluted for 23 minutes.

RESULTS AND DISCUSSION

Water Content

Testing the percent moisture content of the leaf powder and bark of the sausage tree obtained an average yield of 6.39% for the leaves and 6.96% for the bark. The percentage of water content obtained meets the standard as herbal raw material because it is less than 10%. These results indicate that the sample can avoid contamination caused by fungi, and the sample storage time will be relatively longer (Poós & Varju, 2017).

The Yield of Methanol Crude Extract and Fraction Results

Methanol is used as a maceration solvent which is polar with a high dielectric constant. Increasing the polarity of the solvent will increase the percentage of extract yield produced by the extraction process because non-polar to polar compounds will dissolve into the solvent (Firdayani et al., 2015). Methanol can extract polar compounds such as phenolic compounds, proteins, and carbohydrates (Felhi et al., 2017). The yield percentage of methanol extract from the leaves and bark of the sausage tree is shown in Figure 1. The methanol extract of the bark had a higher percentage than the leaves. According to Dewatisari et al. (2018), the amount of yield shows the number of bioactive components contained in the sample. Nabatanzi et al. (2020b) reported that the bark has a higher phytochemical diversity than other plant parts. In addition, the bark and fruit of the sausage tree are traditionally better known for their medicinal uses than the leaves (Bello et al., 2016).

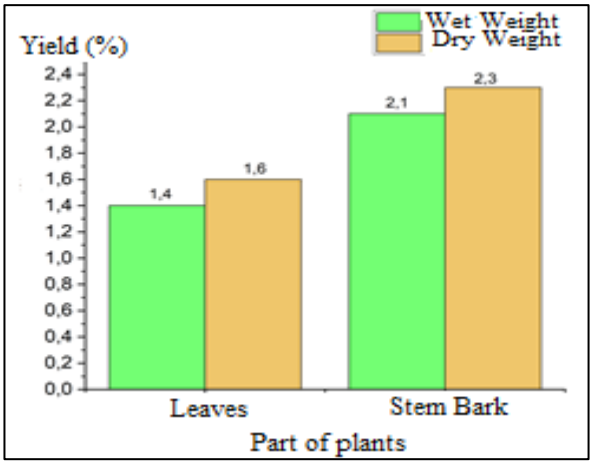
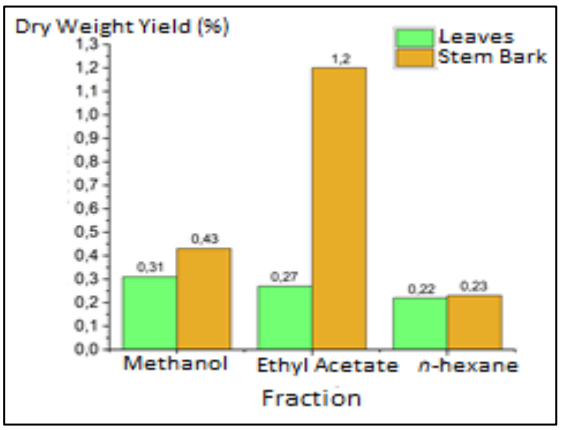


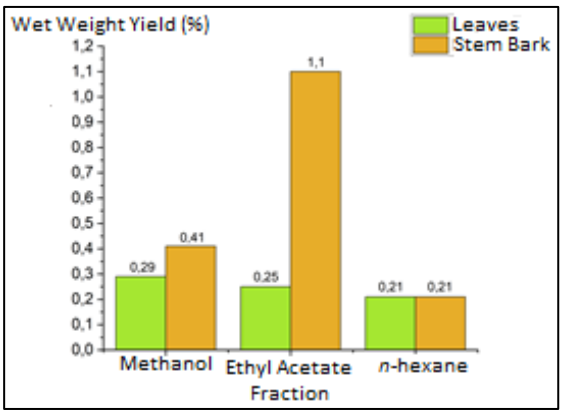
Figure 1. The yield curve of the methanol extract of the leaves and bark of the Indonesian sausage tree

The extract partitioning process separated the compounds based on their polarity level. The methanol extract from the leaves and stem bark was partitioned by liquid-liquid extraction using solvents with different polarities, namely methanol, ethyl acetate, and n-hexane. The dielectric constants of methanol, ethyl acetate and n-hexane at 25 ºC are 33, 6, and 2, respectively (Li & Du, 2011). Based on the dielectric constant, the order of solvent polarity is methanol, ethyl acetate and n-hexane. Extract partition yield results can be seen in Figure 2.

Figure 2 shows that the yield of the ethyl acetate fraction of sausage bark, calculated in terms of dry and wet weight, is the highest compared to other samples, including leaves. It is caused by ethyl acetate is a semi-polar solvent that can dissolve polar and non-polar compounds, including flavonoids, phenols, terpenoids, alkaloids, aglycones, and glycosides (Kumar et al., 2023), while n-hexane is a non-polar solvent, which dissolves chemical compounds such as waxes, lipids, and volatile terpene essential oils. The remaining compounds in methanol are considered very polar and insoluble in the ethyl acetate fraction.



(a)



(b)

Figure 2. Yield curves for the dry weight (a) and fresh weight (b) of the remaining methanol fraction, n-hexane fraction, and ethyl acetate from the crude methanol extract of leaves and bark of a sausage tree

Phytochemical Testing

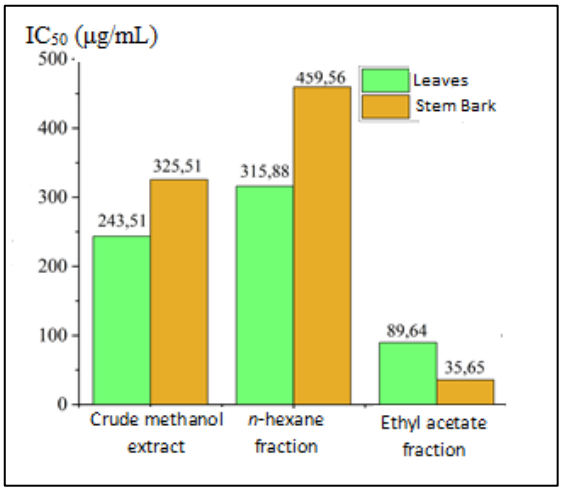
The results of phytochemical tests on leaves and bark of Indonesian sausage trees on methanol extract, n-hexane fraction, ethyl acetate, and methanol residue in Table 1 mostly showed the presence of flavonoids, phenolics, alkaloids, steroids, triterpenoids, saponins, and tannins. The results of the phytochemical fraction of the n-hexane fraction of the leaves and bark of the sausage tree did not contain alkaloids. This result is indicated by the absence of a white precipitate which means that there is no reaction to form the compound [alkaloid+]n[HgI4]n with Mayer's reagent (Djenar et al., 2020), there is no brown precipitate of potassium-alkaloids with Wagner's reagent (Awaluddin et al., 2022). No red precipitate [alkaloid+]n[BiI4]n was formed with Dragendorf reagent (Raal et al., 2020). At the same time, the remaining methanol fraction did not show the presence of steroids which was indicated by the absence of a green colour when the sample was added with Liebermann Buchard reagent.

Antioxidant Activity and Cytotoxicity of Methanol Crude Extract and n-hexane and Ethyl Acetate Fractions of Leaves and Bark of Sausage Tree

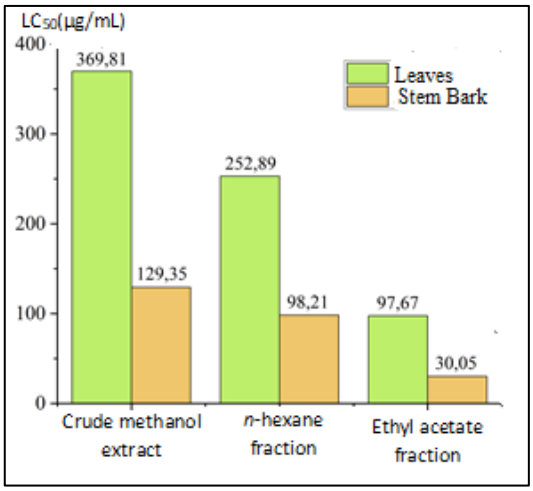
Antioxidants are compounds with a molecular structure that can give electrons to free radical molecules without being disturbed at all and break free radicals' chain reaction (Meigaria et al., 2016). The DPPH method is a method used to determine antioxidant activity with the principle of donating a hydrogen atom (H^+) from the test substance to the DPPH free radical, which is characterized by a color change from purple to yellow (Fadiyah et al., 2020; Taufik & Sulfiani, 2023).

The antioxidant power of a compound or extract is divided into four categories based on its IC50, namely strongest (<50 ppm), strong (50–100 ppm), moderate (100–150 ppm), and low (>150 ppm) (Souhoka et al. al., 2021). The BSLT test is a preliminary test used to detect the toxicity of extracts or compounds. The percentage of larval death is proportional to the sample's toxicity, which indicates the biological activity of bioactive ingredients that can be developed as anti-cancer compounds (Irmawan et al., 2018). Sample toxicity is expressed as LC_{50}, the concentration required to kill 50% of the animal population tested. There are three categories of toxicity based on the concentration, namely very toxic (LC_{50} <30 µg/mL), toxic (LC_{50} = 30–1000 µg/mL), and non-toxic (LC_{50} >1000 µg/mL) (Reymon et al., 2021).

The antioxidant test results from Figure 3a show that the ethyl acetate fraction has extreme activity on stem bark (IC_{50} 35.65 µg/mL) and strong on leaves (IC_{50} 89.64 µg/mL). Based on the antioxidant IC_{50} value, the ethyl acetate fraction of the leaves and bark of the sausage tree has the potential as an antioxidant agent. This value is in line with the ethyl acetate fraction of sausage bark, which was reported by Bakare et al. (2015) originating from Nigeria had the strongest antioxidant activity using the DPPH method (IC_{50} 0.01 µg/mL) compared to methanol extract (IC_{50} 0.08 µg/mL) and the n-hexane fraction (IC_{50} 0.02 µg/mL).



(a)



(b)

Figure 3. Antioxidant (a) and cytotoxic (b) activity curves of methanol, leaf, and bark crude extracts of sausage tree

In addition, the ethyl acetate extract of stem bark from Ekiti State (Nigeria) was also reported to have extreme activity with DPPH and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) with IC_{50} 1.4 µg /mL and 10.55 µg/mL compared to chloroform (IC_{50} 56.41 µg/mL and 25.79 µg/mL) and water (IC_{50} 17.84 µg/mL and 26.68 µg/mL) (Obafemi et al 2017. Sausage stem bark showed stronger antioxidant activity than the ethanol and n-hexane extracts of Java bark (Lannea coromandelica) with IC_{50} of 3.996 and 2193.043 mg/L, respectively, as measured by the DPPH method (Taufiq & Sulfiani, 2023).

The results of the antioxidant activity of the methanol crude extract of sausage leaves had a lower IC_{50} than the stem bark; the same was found in the n-hexane fraction, while for the ethyl acetate fraction, the IC_{50} of the stem bark was, on the contrary, lower than the leaves (Figure 3a). In the comparison, the ethyl acetate fraction of leaves and stem bark showed a lower IC_{50} than the crude methanol extract and its n-hexane fraction in the range (35-89) µg/mL. They were classified as strong antioxidants (Souhoka et al., 2021).

Strong inhibition of free radicals is associated with polyphenolic compounds and flavonoids, which can donate electrons or transfer hydrogen atoms to neutralize free radicals. Several studies have reported the total content of phenolic and phenolic compounds in sausage tree plants. Bakare et al. (2015) said that the ethyl acetate fraction of sausage tree bark from Nigeria had the highest total phenol and flavonoid activity of 292.63 ± 2.63 mg GAE/g and 136.34 ± 4.5 mg QUE/g compared to the n-hexane fraction (71.06 ± 0.25 mg GAE/g and 107.72 ± 4.10 mg QUE/g) and methanol extract (92.98 ± 0.21 mg GAE/g). This result confirms that the ethyl acetate fraction has high antioxidant potential due to the presence of hydroxyl groups

Table 1. Results of phytochemical testing of extracts and fractions from the leaves and bark of the sausage tree

Test	Extract/Fraction							
	Crude Methanol		*n*-hexane		Ethyl Acetate		Residual Methanol	
	Leaves	Stem Bark	Leaves	Stem Bark	Leaves	Stem Bark	Leaves	Stem Bark
Alkaloids	+	+	-	-	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+
Phenolic	+	+	+	+	+	+	+	+
Triterpenoids	+	+	+	+	+	+	+	+
Steroids	+	+	+	+	+	+	+	-
Saponins	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+

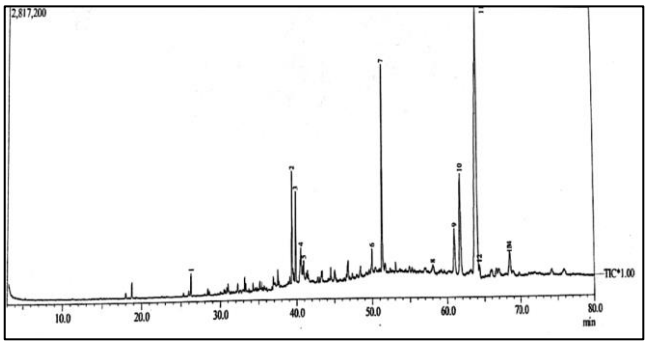
derived from polyphenolic and flavonoid compounds and is more efficient against diseases associated with free radicals. The results of the cytotoxic test of the crude extract of methanol, leaves, and bark of the sausage tree are shown in Figure 3b. Based on the BSLT test, the crude methanol extract, the n-hexane fraction, and the ethyl acetate fraction of the sausage bark showed a lower LC_{50}, meaning that it was more toxic than the sausage leaves and the ethyl acetate fraction of the stem bark was the most toxic (Reymon et al., 2021). This result is in line with what was reported by Adoum (2016); ethanol extracts of stem and leaf bark showed low toxicity with $LC_{50} > 1000$ µg/mL against Artemia salina.

Fredrick et al. (2014) reported that the ethanol extract of sausage tree leaves from Nigeria showed low or non-toxic toxicity to white albino rats with an LD_{50} of 1264.9 mg/kg. In addition, the methanol extract of leaves and stem bark from Bode and Ibadan (Nigeria) showed an LD_{50} >5000 mg/kg orally in rats. This result indicates that the sub-acute toxicity test of the methanol extract of the leaves and bark of the sausage tree did not result in a significant decrease in the erythrocyte index, so the extract was not toxic to red blood cells (Oyebanji et al., 2015). Different things were reported by Viol et al. (2016) that the methanol extract of sausage tree bark from Zimbabwe is toxic to A. salina with an LC_{50} of 262.20 ± 25.07 µg/mL.

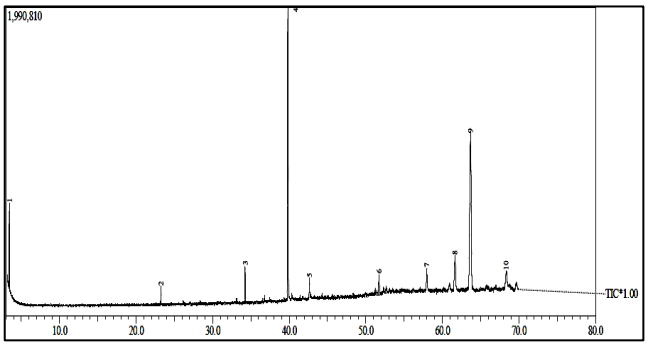
Chromatogram and GC-MS Spectrum of The N-Hexane Fraction of The Leaves and Bark of The Sausage Tree

The n-hexane fraction of leaves and sausage bark was subjected to GC-MS testing to identify their secondary metabolites. Figure 4 shows the GC-MS chromatogram of the n-hexane fraction of bark (a) and leaves (b) of a sausage tree. The results of the GC analysis of the n-hexane fraction of stem bark in Figure 4a within the time range (26.250-68.474) minutes showed 14 peaks (Table 2). The compounds identified include hydrocarbons, alcohols, phenols, fatty acids, terpenoids, and steroids. This strengthens the phytochemical results that the n-hexane fraction of the stem bark does not contain alkaloids. Based on the mass spectrum, several dominant peaks were detected, namely the compound estran - 3 - on - 17 - (acetyloxy) - 2 - methyl - (2-alpha-5-alpha17-beta) identified as the main component (51.67%), followed by 9-octadekenamide (11.76%), and cholesta-8-en-3-ol (11.42%). Steroids represented the highest composition in the n-hexane fraction of stem bark (71.72%), while fatty acids contributed 15.05%, alcohol 6.97%, hydrocarbons 1.33%, and only one diterpenoid with a composition of 3.91%.

The compound estran-3-on-17-(acetyloxy)-2-methyl-(2-alpha-5-alpha17-beta) belongs to a group of steroid compounds that are reported to have antioxidant activity (Rozirwan et al., 2022). Compound 9-Octadekenamide or oleamide is a long-chain primary fatty acid amide compound that can affect endogenous mechanisms that encourage humans to sleep and function as analgesics, antidepressants, and anti-hypolipidemic (Akanmu et al., 2007; Cheng et al., 2010; Vock et al., 2008). 4α-zimosterol is a cholesterol-derived compound that functions in steroid biosynthesis and metabolism of purines-pyrimidines, nicotinates, and nicotinamides (Turanli et al., 2019). The sterol compound cholesta-8-en-3-ol has been reported as anti-cancer, anti-eczematic, hypolipemic, and anti-infertility (Ragi et al. 2018).



(a)



(b)

Figure 4. Chromatogram of GC-MS results of the n-hexane fraction of bark (a) and leaves (b) of sausage trees growing at Waterpark Platinum Riau

The sitostenon compound is a stigmastan steroid compound that has activity as an antibacterial, antifungal, anti-tuberculosis, anti-cancer, anti-melanogenic (Ahmad et al., 2016; Chu et al., 2015;

Isah et al., 2016; Truong et al., 2011). Phenol is an antiseptic, antibacterial compound (Giri et al., 2016; Sabbineni, 2016). Phytols are diterpenes of long-chain unsaturated acyclic alcohols and are widely used as fragrance ingredients. This compound has biological activity as an antioxidant, anti-inflammatory, antimicrobial, and anti-cancer (Islam et al., 2018). Compound 1-Hexadecanol is an alcohol compound as an antioxidant (Amudha et al., 2018). Eikosana is reported as anti-inflammatory, analgesic, antipyretic, and antifungal (Okechukwu 2020). The compound dicholesteryl succinate is a cholesterol ester, and no one has reported its bioactivity, but other derivatives, such as cholesteryl hemisuccinate, have been reported to have antitumor activity (Fariss et al., 1994). Most of the bioactivity of compounds reported only a few functions as antioxidants. This phenomenon follows the results of the antioxidant fraction of n-hexane stem bark with low activity.

Table 2. The GC-MS results on the n-hexane fraction of sausage tree bark

Peak	Retention Time (minutes)	% Area	Compounds	Molecular Formula	Molecular Weight
1	26.250	1.02	Phenol	$C_{14}H_{22}O$	206
2	39.359	4.91	1-Hexadecanol	$C_{16}H_{34}O$	242
3	39.836	3.91	Fitol	$C_{20}H_{40}O$	296
4	40.528	3.29	Oleic Acid	$C_{18}H_{34}O_2$	282
5	40.915	1.04	9-Octadecanal	$C_{18}H_{34}O$	266
6	49.984	1.33	Eikosana	$C_{20}H_{42}$	282
7	51.317	11.76	9-Octadekenamide	$C_{18}H_{35}NO$	281
8	58.100	0.98	Dicholesteryl succinate	$C_{58}H_{94}O_4$	854
9	61.008	4.67	5α,6α-epoxycolest-7-en-β-ol	$C_{27}H_{44}O_2$	400
10	61.807	11.42	Colesta-8-en-3-ol or 14-Metil-5α-colest-8-en-3β-ol	$C_{28}H_{48}O$	400
11	64.041	51.67	Estran-3-on-17-(asetiloxy)-2-methyl-(2α,5α,17β)-	$C_{21}H_{32}O_3$	332
12	64.349	1.12	4α-Methylcolesta-8,24-dien-3β-ol or 4-α-Methylzimosterol	$C_{28}H_{46}O$	398
13	68.442	1.03	9,12,15- octadecatrionic acid-2-fenil-1,3-dioxan-5-yl ester	$C_{28}H_{40}O_4$	440
14	68.474	1.86	Stigmast-4-en-3-on	$C_{29}H_{48}O$	412

Table 3. Results of GC-MS analysis of the n-hexane fraction of sausage tree leaves

Peak	Retention time (minutes)	% Area	Compounds	Molecular Formula	Molecular Weight
1	3.450	4.39	Epichlorohydrin	C_3H_5ClO	92
2	23.252	1.59	Benzene, 1,2-dimetoxy-4-(2-propen-1-yl)-	$C_{11}H_{14}O_2$	178
3	34.253	3.43	2-Dodecanona	$C_{12}H_{24}O$	184
4	39.845	26.96	Oktadecanal	$C_{18}H_{36}O$	268
5	42.653	2.45	1,2-epoxytetradecane or oxirane, dodecyl-	$C_{14}H_{28}O$	212
6	51.747	2.10	Farnesol or 2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-	$C_{15}H_{26}O$	222
7	57.993	4.47	Vitamin E	$C_{29}H_{50}O_2$	430
8	61.665	8.68	4-α-Methylzimosterol	$C_{28}H_{46}O$	398
9	63.714	42.03	Dicholesteryl succinate	$C_{58}H_{94}O_4$	854
10	68.350	3.92	Stigmast-4-en-3-on atau Sitostenon	$C_{29}H_{48}O$	412

GC results (Figure 4b), n-hexane fraction of sausage leaves within a time range (3.450-68.350) minutes identified ten peaks (Table 3) which are suspected to be compounds of the aldehyde, ketone, epoxide, aromatic hydrocarbon, cyclic ether, cholesterol (lipid) groups. , steroids, vitamin E, and sesquiterpenes. The mass spectrum study detected several dominant peaks of dicholesteryl succinate as the main component (42.03%), followed by octadecanal (26.96%) and 4-α-methylzimosterol (8.68%). The cholesterol derivatives (lipids) group represents the main composition by contributing 42.03% and followed by aldehydes (29.41%), steroid derivatives (12.6%), cyclic ethers (6.84%), vitamin E (4.47%) %), ketones (3.43%), aromatic hydrocarbons (1.59%) and contain only one sesquiterpene compound (2.10%). Based on the literature, the compounds identified in the n-hexane fraction of Indonesian sausage tree leaves were reported to have various bioactivities.

Octadecanal is a long-chain aldehyde compound as a Curculionidae aggregation pheromone (dos Santos Neta et al., 2021). This compound is also reported as

anti-inflammatory and anti-apoptotic (Kumar et al., 2018). Oxyrane compounds were reported as antimicrobial and insect repellent (Thirunarayanan & Vanangamudi, 2016). Turanli et al. (2019) reported 4α-zimosterol, a cholesterol-derived compound, which functions in steroid biosynthesis and metabolism of purine-pyrimidine, nicotinate, and nicotinamide.

Farnesol, an acyclic sesquiterpene alcohol commonly found in essential oils, is reported to have bioactivity as an antioxidant, anti-inflammatory, anti-cancer, antimicrobial, antifungal, and can treat allergic asthma, gliosis, and edema (Bezerra et al., 2020; Jung et al., 2018; Palanisamy et al., 2020; Su et al., 2015). Vitamin E, an essential micronutrient phenolic compound in the body, is a strong antioxidant and is fat soluble to protect tissues from uncontrolled lipid peroxidation. Vitamin E is also reported to have anti-cancer potential and prevent cardiovascular disease, immune complications, and hematologic disorders (Galli et al., 2022). Steroid compounds such as sitostenon, 4α-methylzimosterol, cholesterol succinate, and terpenoids such as farnesol contribute as antioxidants. However, they are weak, and these compounds are also reported to have anti-cancer properties, so they are thought to support the toxicity of the n-hexane fraction.

Chromatogram and LC-MS/MS Spectrum of The Ethyl Acetate Fraction of The Leaves and Bark of The Sausage Tree

The ethyl acetate fraction of leaves and sausage bark was subjected to LC-MS/MS testing to identify the secondary metabolites. Figure 5 shows the LC-MS/MS chromatogram of the ethyl acetate fraction of the bark (a) and leaves (b) of a sausage tree. The results of the LC analysis of the ethyl acetate fraction of the stem bark in Figure 5a within the time range (1.26-17.98) minutes contained many peaks, and four peaks could be identified based on their mass spectrum they were detected as lignans, alkaloids and steroids (Table 4).

Based on Table 4, it is known that in the bark of the sausage tree, there are aminopregnan compounds (a steroid) which have been identified in the dichloromethane extract of Chrysophyllum carnito leaves and have antimalarial activity (Ma'arif et al., 2019). The N-benzylnactyloctanamine compound is an alkaloid compound found in the Marsilea crenata Presl plant. (Rukiana, 2018). Kubebin (a lignan) from the ethanol extract of *Piper cubeba Lf* fruit. was reported to have activity as an antioxidant, anti-cancer, antimicrobial, anti-inflammatory, and antidiabetic (Setyani et al., 2021). The picraquassin compound was identified in the bark of the Picrasma quassinoids tree and had anti-cancer activity (Jamil et al., 2020).

Table 4 Results of LC-MS/MS analysis of the ethyl acetate fraction of sausage tree bark

Retention Time (minutes)	Molecular Weight	m/z $(M+H)^+$	Prediction
7,52	$C_{20}H_{21}O_6$	357,1373	Kubebin
11,95	$C_{31}H_{54}O_3$	529,2125	Picraquassin I
12,13	$C_{21}H_{37}N$	304,33004	Aminopregnane
13,32	$C_{23}H_{41}N$	332,3299	N-Benzyln actyloctanamine

The results of the LC analysis of the ethyl acetate fraction of sausage leaves in Figure 5b within the time range (1.19-17.75) minutes there were many peaks, and five peaks could be identified, based on their mass spectrum they were detected as flavonoid, terpenoid and heterocyclic groups (Table 5). Based on Table 4, it is known that in the leaves of the sausage tree, there is the compound 3-O-methyl kaempferol, which is a flavonoid compound that has been identified in the methanol extract of Annona muricata leaves and has activity as an antioxidant (Asbanu et al., 2019). The 3,4,7-trihydroxyflavone compound belongs to the flavonoid group and has been reported to have activity as an anti-inflammatory agent (Islam et al., 2018).

Table 5 Results of LC-MS/MS analysis of the ethyl acetate fraction of sausage tree bark

Retention Time (minute)	Molecular Formula	m/z $(M+H)^+$	Prediction
7,87	$C_{16}H_{13}O_6$	301,0741	3-*O*-Metilkaempferol
7,67	$C_9H_{11}N_4O_6$	271,0629	3,4,7-Trihidroxyiflavone
5,91	$C_{11}H_{17}O_3$	197,1186	Loliolida
6,91	$C_{15}H_{11}O_6$	287,0555	Kaemferol
12,57	$C_{12}H_9NO$	184,0719	Phenokxazine

Loliolida is a monoterpene reported to have antibacterial activity (Ghazali et al., 2021), antioxidant, and can treat nervous disorders (Silva et al., 2021). Kaempferol is a class of flavonoids that have been reported to have activity as an anti-inflammatory (Candra & Wijaya, 2021), antioxidant, and antiviral (Aisa et al., 2021). The presence of the identified compounds, both the ethyl acetate fraction of the stem bark and sausage leaves, is thought to have a very

strong contribution to their toxicity and antioxidant activity.

CONCLUSIONS

The results of phytochemical testing of methanol crude extract, n-hexane and ethyl acetate fractions of leaves and sausage bark showed the presence of all secondary metabolites except alkaloids in the n-hexane fraction. The antioxidant and cytotoxic activity of the ethyl acetate fraction of stem and leaf bark was stronger than the crude methanol extract and n-hexane fraction. Secondary metabolite components in the n-hexane fraction of K. africana stem bark were identified as estran-3-on-17-(acetyloxy)-2-methyl-(2α,5α,17β); 4-α-methyl-zimosterol and 9-octadecinamide, while in the leaves identified dicholesteryl succinate, farnesol and octadecanal which are thought to contribute as antioxidants and are toxic although weak. The secondary metabolite components in the ethyl acetate fraction of the sausage tree were identified as kaempferol compounds and their derivatives, lignans, and terpenoids, which are thought to contribute strongly to their toxicity and antioxidant activity.

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