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Phytochemicals and toxicity of ketapang fruit flesh (*Terminalia catappa*. Linn) using the BSLT method

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ABSTRACT

Ketapang is widely used in traditional medicine. Parts of ketapang plant, such as roots, leaves, and bark, are known to have biological activities such as antioxidant, antimicrobial, anti-inflammatory, and anticancer. However, no biological activity has been reported on the flesh of the ketapang fruit. This research was conducted to obtain phytochemicals and toxicity information of ketapang fruit flesh as an anticancer medicine. Toxicity indicates the potential for a chemical compound to cause damage to living organisms, while phytochemicals indicate a group of compounds that have biological activity. Extraction of ketapang fruit flesh was carried out using various solvents, both polar and non-polar solvents, methanol, n-hexane, and ethyl acetate. The toxicity test used the Brine Shrimp Lethality Test (BSLT) method, while phytochemicals tests used the thin layer chromatography (TLC), in which identification of bioactive compounds of ketapang pulp extract used UV-Visible and FTIR spectroscopy. The results showed that the ketapang fruit flesh extract was toxic to *Artemia salina* Leach shrimp larvae. The most toxic was ethyl acetate extract, with an LC_{50} value was 17,171 ppm. The phytochemicals screening showed that ethyl acetate extract of the ketapang fruit flesh contained flavonoid, alkaloid, phenolic, terpenoid, and saponin compounds. Tracing using UV-Vis shows the presence of conjugated double bonds which refer to flavonoid compounds, as well as the IR spectrum which indicates the presence of a typical functional group of flavonoid compounds.

Keywords: *Terminalia catappa*. Linn, *Artemia salina* Leach, Brine Shrimp Lethality Test

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INTRODUCTION

A long time ago, humans used medicine from natural products, such as plants, animals, microorganisms, and marine organisms, to treat diseases. Approximately 80% of antimicrobial, cardiovascular, immunosuppressive, and anticancer drugs were of plant origin. One of the plants that potency as medicinal plant is ketapang (*Terminalia catappa* L.) (Chole and Ravi, 2020). Ketapang is a plant native to Southeast Asia and is widely found in Indonesia which is known to have efficacy as a medicinal plant because it contains compounds such as phenols, flavonoids, and carotenoids. Several studies reported that some part of this plant has antimicrobial, anti-inflammatory, antidiabetic, antioxidant, hepatoprotective, and anticancer activity, but the use of this plant as a medicine is still traditional (Anand et al., 2015). Ketapang leaves are reported to have antibacterial, anticandidal, antioxidant (Poongulali and Sundararaman, 2016), and anticancer activities (Lee et al. 2019; Zarredar et al., 2021). Ketapang fruit has been reported to have antioxidant activity (Krishnaveni, 2014) while the bark can lower blood sugar levels (Nia et al., 2017), and the roots have antibacterial activity (Pawar and Pal, 2002). However, the flesh of the ketapang fruit has never been reported to have certain bioactive compounds.

The research was conducted to determine the potential of ketapang fresh fruit as a medicine. Initial research to determine whether a plant has the potential as a drug is through toxicity tests and phytochemical screening. Toxicity is the ability of a substance to damage an organism. The method used for the toxicity test was the Brine Shrimp Lethality Test (BSLT) (Simorangkir et al., 2021). A toxicity test using the BSLT method is one method for screening medicinal plants that have the potential to be an anticancer. In comparison, phytochemical screening is a preliminary test to determine secondary metabolites in a sample. Phytochemical test on the most active extract fraction using Thin Layer Chromatography (TLC) (Banu and Cathrine, 2015) and identification using Ultra Violet (UV)-Visible and Fourier Transform InfraRed (FT-IR) spectroscopy.

MATERIALS AND METHOD

Materials

Ketapang fruit flesh from the ketapang plant in front of the Unsoed FMIPA campus, *Artemia salina* L eggs. The chemical was methanol, n-hexane, ethyl acetate, silica gel 60 F254, chloroform, glacial acetic acid, and acetone. All chemicals used are from Merck Ltd.

Methods

Extraction using methanol solvent and fractionation with organic solvents

2 Kg of ketapang fruit flesh powder was soaked (maceration) in methanol for 24 hours, stirring occasionally, and filtered to obtain methanol extract and residue. The maceration was repeated until the methanol extract was clear. The methanol extract was then concentrated and weighed. The concentrated methanol extract was partially stored (E1) and partly fractionated using n-hexane and obtained n-hexane fraction and residue. The n-hexane fraction was concentrated and weighed (E2), while the residue was dried for further fractionation using ethyl acetate solvent, and ethyl acetate and residue fractions were obtained. The ethyl acetate fraction was concentrated to obtain a concentrated ethyl acetate fraction (E3) and residue (E4). The fractionation process was repeated several times until a clear fraction was obtained.

Cytotoxic activity test against shrimp larvae of *Artemia Salina* Leach

The first step is the hatching of *Artemia salina* Leach eggs in a container using seawater. The container used is divided into two parts using a perforated partition, namely a light room and a dark room. The container is filled with 4 liters of artificial seawater. Then, in a dark room, ± 2.5 mg (one small spoonful) of shrimp eggs were added, which had previously been washed by soaking in distilled water for 1 hour. The dark room in the container is covered with aluminum foil and black duct tape, while the bright room is lit with lamps so that the hatching temperature is maintained at around 25-30

C. Shrimp eggs are left to soak for 48 hours until the eggs hatch. The eggs will hatch in the range of 24-36 hours and will move on their own to a bright room so that they are separated from the eggshell. The shrimp larvae that are actively moving indicate that they are ready for use (Umri et al., 2019).

The second step was the toxicity test of the ethyl acetate fraction of the methanol extract of the ketapang fruit flesh. Each fraction of 50 mg was added with 1 drop of DMSO solution and dissolved in 5 mL of seawater to form a stock solution of 10,000 $\mu\text{g/mL}$ of sample. Each fraction was diluted with various concentrations of 150, 100, 50, and 10 $\mu\text{g/mL}$. As a control, 5 mL of sea water was added with shrimp larvae, yeast, and DMSO. The extract that has been diluted into various concentrations is added as much as 5 mL into the test tube. Then added ten shrimp larvae and one drop of yeast solution as nutrients. The test tube was left at room temperature with light for 24 hours. The number of live shrimp larvae was calculated, and the percentage of larval mortality was determined to determine the LC50 value. The toxicity test data were analyzed by Probit Analysis and Microsoft Office Excel to find linear regression based on line graphs, and the LC50 (Lethal Concentration) value was obtained.

Phytochemical screening for secondary metabolite

The secondary metabolite test for flavonoids, alkaloids, terpenoids, phenolics, and steroids used TLC with silica gel 60 F254 as a stationary phase and an appropriate eluent as a mobile phase, while the saponin test was carried out by shaking using distilled water.

RESULT AND DISCUSSION

The process of extracting compounds contained in the flesh of ketapang fruit flesh by maceration method using methanol, n-hexane, and ethyl acetate as solvents. The maceration method is able to reduce the damage to the compounds contained in the sample due to heating. This method is very simple but is able to separate the desired chemical compounds using only certain solvents (Ćujića et al., 2016). In plant samples, there will be a breakdown of cell walls and membranes due to pressure differences inside and outside the cell so that compounds in the cytoplasm will be dissolved in organic solvents. The length of soaking time affects the extraction results. The longer the soaking time, the more extract produced.

The choice of solvent also affects the extraction results. Methanol was chosen as the first solvent because methanol can dissolve almost all groups of secondary metabolites. The second solvent extraction used n-hexane to attract non-polar compounds and solvent extraction used ethyl acetate to extract semi-polar compounds. The yield of each extract can be seen in Table 1.

Table 1. The extract of ketapang fruit flesh in various solvents

Extract	Color	Mass (gram)	Yield (%)
The methanol extract (E1)	Brownish green	14.84	2.10
n-Hexane fraction (E2)	Dark green	8.93	3.01
Ethyl acetate fraction (E3)	Brown	10.11	3.66
Ethyl acetate residue (E4)	Reddish brown	135.61	63.49

The toxicity test of the ketapang fruit flesh extract was carried out using the *Artemia salina* Leach larvae mortality method or the BST method. The advantage of the BST method is fast, cheap, simple, requires few materials that are easily obtained, and can be done repeatedly. The results of this BST test obtained primary data regarding a number of live shrimp larvae of *A. salina* Leach after incubation for 24 hours at various concentrations of each extract. The LC₅₀ value indicates the concentration that causes 50% mortality in test animals. The smaller the LC₅₀ value, the greater the toxicity. If the LC₅₀ value obtained is less than 1000 ppm, the test compound is said to be toxic and has the potential to be developed as a medicine (Simorangkir et al., 2021). The average mortality or mortality will increase as the concentration of each sample increases, and almost all bioactive components are toxic at high doses. BSLT test results showed that the methanol extract, ethyl acetate residue, ethyl acetate fraction, and n-hexane fraction were toxic to *Artemia salina* Leach shrimp

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larvae. The most toxic was the ethyl acetate fraction with an LC_{50} of 17.17 ppm. The regression equation along with the r value and LC_{50} value of the ketapang fruit flesh, is presented in Table 2.

Table 2. Regression equation with r and LC_{50} value of ketapang fruit flesh extract

Extract	Regression equation	r	LC_{50} (ppm)
Methanol	$y = 1.1222 x + 3.4188$	0.9939	25.64
n-Hexane	$y = 1.5397 x + 2.6486$	0.9980	33.67
Ethyl acetate	$y = 1.4103 x + 3.2585$	0.9737	17.17
Ethyl acetate residue	$y = 0.9777 x + 3.2445$	0.9912	62.44

The secondary metabolite compound test on the ethyl acetate fraction of the ketapang fruit flesh has the highest LC_{50} . Determination of the best eluent was carried out using TLC with a variety of solvent ratios to obtain the best eluent to be used in the secondary metabolite compound test with color reagents on the TLC plate. The advantages of the TLC test compared to the color test are that fewer samples and the position of compounds that are positive for the test for certain groups of compounds can be known. The eluent is a mixture of two or more pure solvents with varying volume ratios. TLC test results showed that the best eluent for the ethyl acetate fraction of ketapang fruit flesh was ethyl acetate: chloroform: glacial acetic acid (1:8:1). The results of this TLC obtained the most spots, namely seven spots with good separation. A chromatogram of the ethyl acetate fraction of ketapang fruit pulp which gives the best separation is presented in Figure 1.



Figure 1. Chromatogram of the ethyl acetate fraction of ketapang fruit pulp with ethyl acetate : chloroform: glacial acetic acid (1:8:1) as eluent

The best eluent is used for color testing using a TLC plate, except for the saponin test that is carried out using the shaking method. The results for the metabolites test in Table 3 showed that the ethyl acetate fraction of ketapang fruit flesh is positive for flavonoids, alkaloids, terpenoids, phenolics, and saponins. The positive ethyl acetate fraction contains flavonoids, indicated by the formation of purple color by spraying vanillin-HCl reagent and heating. Vanillin-HCl is a reagent to identify the presence of flavonoids. The principle of the vanillin-HCl test is that vanillin is protonated in an acid solution and produces a carbocation. These carbocations react with flavonoids. The resulting intermediate undergoes a dehydration reaction and produces a purple or red colored compound. Flavonoids are a class of compounds that are known to have various biological activities such as

analgesic, anti-inflammatory, antipyretic, and anticancer (Corcoran et al., 2012). The secondary metabolite test of alkaloid compounds was carried out by adding Dragendorff's reagent (Pant et al., 2017). The result is positive if an orange color is formed from the potassium-alkaloid complex. The terpenoid test showed a positive result if a brown stain appeared after contact with I₂ vapor. Double bond halogenation is a specific qualitative test to detect the presence of double bonds in terpenoids. The phenolic compound test showed positive results if the formation of brick red spots after being sprayed with 5% (w/v) FeCl₃. The addition of 5% FeCl₃ can only show the presence of phenolic compounds in general but cannot distinguish the polyphenol groups. The results of the saponin test were positive, as indicated by the formation of foam as high as 1 cm and remained stable for more than 15 minutes.

Table 3. Test results for secondary metabolites of the ethyl acetate fraction of ketapang fruit flesh using a TLC plate

Compound	Reagent	Visual Observation (before)	Visual Observation (after)	Rf	Results
Flavonoid	Vanillin-HCl	Brownish-yellow	Purple	0.54	+
Alkaloid	Dragendorff	Brownish yellow	Orange	0.47	+
Terpenoid	Uap I ₂	Brownish-yellow	Brown	0.59 0.84	+
Steroid	Liebermann-Burchard	Brownish-yellow	Brownish-yellow	-	-
Phenolic	FeCl ₃	Brownish-yellow	Brick red	0.51	+
Saponin	Distilled water	Brownish-yellow	Foaming	-	+

The secondary metabolite test results were supported by UV-Visible and FTIR spectroscopy analysis. Spectroscopic determination of UV-Visible spectroscopy was carried out to determine the maximum wavelength of the ethyl acetate fraction of ketapang fruit flesh. The UV-visible spectrum of the ethyl acetate fraction of ketapang pulp is presented in Figure 2. Figure 2 shows the main peak at a wavelength of 267 nm with an absorbance of 0.523. The transition that occurs at a wavelength of 267 nm shows an electron transition from $n \rightarrow \sigma^*$ or $\pi \rightarrow \pi^*$, which is conjugated so that it is suspected that the bioactive compounds in the ethyl acetate fraction contain flavonoids (Sisa et al., 2010). Most of the flavonoid compounds have two absorption maximums at wavelengths of 240-285 nm (band II) from the A ring system in the benzoyl part and 300-550 nm (band I) from the B ring system in the cinnamoyl part. Figure 2 showed that the peak in the band II region was clearly visible (267 nm) while the peak in the band I region was barely visible. However, based on the spectrum pattern of flavonoid types described by Markham (1988), the spectrum in this research corresponds to the spectrum of flavonoids. The characteristic of this spectrum is the presence of relatively low peaks in band I for hydroflavonoids, dihydroflavonols, and isoflavones, while chalcone, aurone, and anthocyanins have relatively high peaks. It is also supported by the positive results of the phytochemical test of the flavonoid group with the formation of a purple color after being sprayed with vanillin-HCl reagent and heating.

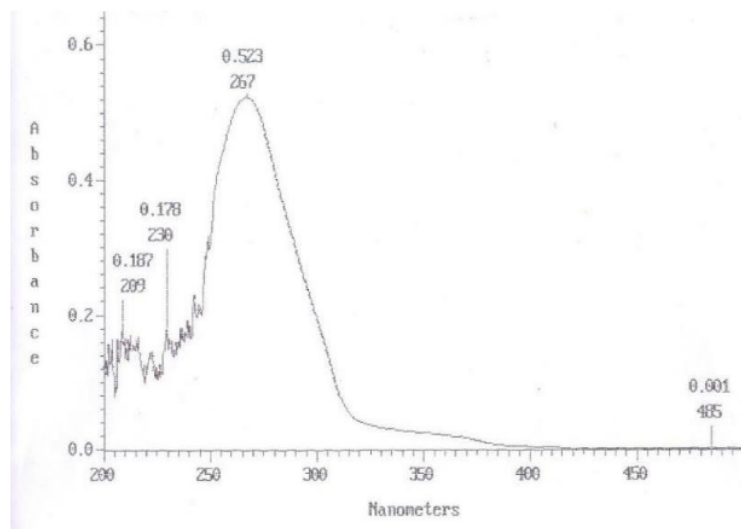


Figure 2. The UV-Visible spectrum of ethyl acetate fraction

Identification of compounds using FT-IR (Fourier Transform Infrared) spectrophotometer was carried out to determine the absorption band of the functional groups of chemical compounds in the sample. The infrared spectrum of the ethyl acetate fraction of ketapang pulp can be seen in Figure 3.

Figure 3 supports the identification using a UV-Visible spectrophotometer that the ethyl acetate fraction contains compounds from the flavonoid group. Characteristics of the infrared spectrum of flavonoid compounds are O-H, C-H aliphatic, C=O carbonyl, C=C aromatic, and C-O ether groups. Figure 3 shows that there are peak at 3186.46 cm^{-1} for O-H, 2931.85 cm^{-1} for aromatic C-H, 1735.96 cm^{-1} for C=O, 1604.80 cm^{-1} for C=C aromatics, 1242.18 and 1095.59 cm^{-1} for C-O ether, which are characteristic groups for flavonoid compounds (Tunnisa et al., 2018).

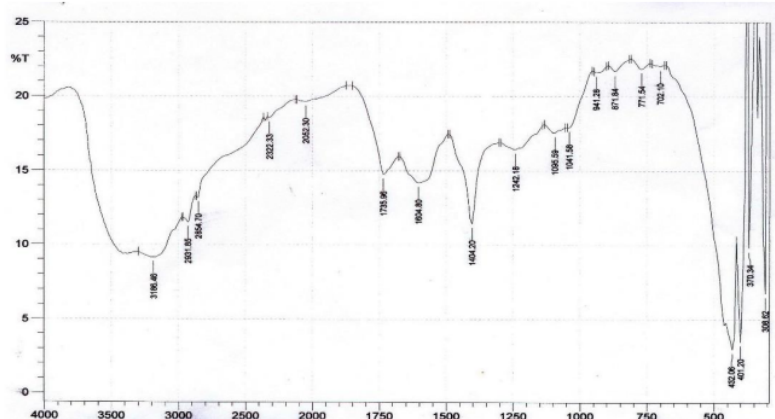


Figure 3. IR spectrum of ethyl acetate fraction from ketapang fruit flesh

CONCLUSION

Ethyl acetate extract of ketapang fruit flesh was the most toxic, contains flavonoid, alkaloid, terpenoid, phenolic, and saponin group compounds, and has potential as a medicine material compound.

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