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TOXICITY TEST OF LIME PEEL EXTRACT (*Citrus aurantifolia*) USING THE BRINE SHRIMP LETHALITY TEST (BSLT) METHOD FOR DETECTION OF ANTI CANCER ACTIVITY

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ABSTRAK

Kanker termasuk salah satu dari berbagai masalah utama yang terjadi pada masyarakat Indonesia dan juga dunia. Kurangnya kesadaran masyarakat untuk deteksi dini menyebabkan terjadinya keterlambatan diagnosis. Salah satu tanaman obat yang banyak dijumpai di Indonesia yaitu jeruk nipis (*C. aurantifolia*). Pada ekstrak kulit jeruk nipis, terdapat minyak atsiri yang tersusun dari D-Limonene (38.94%), β-pinen (26.66%), α-terpineol (8.29%), dan terpinen-4-ol (8.29%). Senyawa-senyawa tersebut diketahui memiliki efek yang kuat sebagai anti kanker. Pada penelitian ini digunakan Rancangan Acak Lengkap (RAL) dengan 5 perlakuan dan 5 kali pengulangan, yaitu 0, 125, 150, 250, dan 1000 ppm. Metode penelitian yang digunakan yaitu uji fitokimia, analisis FTIR (*Fourier-Transform Infrared Spectroscopy*), dan uji BSLT. Nilai probit dianalisis dengan menggunakan SPSS untuk mendapatkan nilai LC_{50}. Ekstrak kulit jeruk nipis (*C. aurantifolia*) memiliki nilai LC_{50} 331,533 ppm termasuk kategori toksik dan berpotensi sebagai agen anti kanker. Berdasarkan pada uji fitokimia dan FTIR kulit jeruk nipis (*C. aurantifolia*) mengandung senyawa alkaloid, flavonoid, dan tanin yang diketahui memiliki sifat antioksidan dan dapat mengganggu keseimbangan *Reactive Oxygen Species* (ROS) sehingga dapat memicu apoptosis sel kanker.

Kata kunci: Anti kanker, *C. aurantifolia*, *Artemia salina*, *Brine Shrimp Lethality Test* (BSLT), FTIR

ABSTRACT

Cancer is one of the major problems affecting the people of Indonesia and the world. Lack of public awareness for early detection causes a delay in diagnosis. One of the medicinal plants that are commonly found in Indonesia is lime (C. aurantifolia). In lime peel extract, there is essential oil composed of D-Limonene (38.94%), β-pinen (26.66%), α-terpineol (8.29%), and terpinen-4-ol (4.32%). These compounds are known to have strong effects as anti-cancer. This study used a completely randomized design (CRD) with 5 treatments and 5 repetitions, namely 0, 125, 150, 250, and 1000 ppm. The research methods used were phytochemical

test, FTIR (Fourier-Transform Infrared Spectroscopy) analysis, and BSLT test. The probit value was analyzed using SPSS to obtain the LC_{50} value. Lime peel extract (C. aurantifolia) has an LC_{50} value of 331.533 ppm including the toxic category and has potential as an anti-cancer agent. Based on phytochemical and FTIR tests, lime peel (C. aurantifolia) contains alkaloid, flavonoid, and tannin compounds which are known to have antioxidant properties and can disrupt the balance of Reactive Oxygen Species (ROS) so as to trigger apoptosis of cancer cells.

Keywords: *Anti-cancer, C. aurantifolia, Artemia salina, Brine Shrimp Lethality Test (BSLT), FTIR*

INTRODUCTION

According to GLOBOCAN, the International Agency for Research on Cancer (IARC) there were 14,067,894 new cases of cancer and 8,201,575 deaths in 2012. According to the Health Research and Development Agency of the Indonesian Ministry of Health in 2018 through the Basic Health Research questionnaire that has been conducted, it was known that the prevalence of cancer in Indonesia is 1.8% at all age levels. Generally, this occurs due to a lack of public awareness for early detection, causing delays in diagnosis (Hidayati and Akrom, 2021).

Indonesia is known as an agricultural country that has various types of plants that are abundant. Various types of plants can be used as medicine because they contain active compounds and some types have the potential as anti-cancer agents (Elisya et al, 2022). One of the medicinal plants that are commonly found is lime. Lime contains saponin and alkaloid compounds and has an IC_{50} 49.589 ug/ml using DPPH assay. Total phenol content of lime is 116.5 mg GAE/100ml (Permata et al, 2018).

In lime peel extract, there are essential oils composed of D-Limonene (38.94%), β-pinen (26.66%), α-terpineol (8.29%), and terpinen-4-ol (4.32%). (Wahyudi et al., 2017). Lime peel contains flavonoids, alkaloids, saponins, and essential oils from phytochemical assay of ethyl acetate fraction (Rahmatullah et al, 2021).

Based on research by Sari et al (2021), the ethanolic extract of lime peel has antioxidant activity of IC_{50} 110.52 ppm with the ethanolic fraction method and is categorized as

moderate. Meanwhile, research conducted by Novriyanti et al (2022) lime peel extract has an IC_{50} 5.81 µg/ml which has very strong antioxidant activity using the DPPH assay. D-Limonene and β-pinen affects the apoptosis process through the Caspase-8 pathway that binds to death receptors on the surface of cancer cells based on in silico molecular docking assay (Hairunisa et al, 2019).

Brine Shrimp Lethality Test (BSLT) is one method that can be used to determine the toxicity of a material or compound of natural origin. The results of the toxicity test carried out with the BSLT method are determined based on the Lethal Concentration (LC_{50}) of the number of *Artemia salina* that die due to the effect of natural substances (Sepvina et al, 2022). This method is widely used because it is relatively easy, relatively cheap, fast, and also reliable.

This study was conducted to determine the level of toxicity of lime peel (*C. aurantifolia*) using 96% ethanol solvent so this research will be conducted toxicity test of lime peel ethanol extract (*C. aurantifolia*) to *Artemia salina* using BSLT method.

RESEARCH METHOD

Place and Time of Research

This research was conducted from December 2023 - February 2024 at the Botany Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Lampung.

Research Design

This research was conducted using a completely randomized design (CRD) with 5 treatments and 5 repetitions consisting of negative control (K-) 0 ppm, 125 ppm, 250 ppm, 500 ppm, and 1000 ppm. The negative control group with a concentration of 0 ppm only uses sterile seawater without the use of any extracts.

Sample Preparation

Lime was obtained from Gading Rejo sub-district, Pringsewu district. The lime peel was cleaned, air dried, then oven at 60^{o}C for ± 2 days (Sari and Asri, 2022). Then, pulverized with a blender to make simplisia. 500 g of simplisia was macerated in 96% ethanol solvent of 3.75 L for 5 days, then filtered and the filtrate was re-

soaked with 96% ethanol of 1.25 L for 2 days. The extract was then concentrated with a rotary evaporator at 50^{o}C (Sapitri et al., 2022).

Phytochemical Assay

Phytochemical assay refers to Okvianingsih et al (2023) which is shown in Table 1.

Table 1. Phytochemical Assay Procedure

Type of Test	Treatments	Indicators
Alkaloid	Method 1: 2 ml of extract + 2 drops of Dragendorff reagent Method 2: 2 ml extract + 2 drops HCl + 2 drops Mayer reagent	Forms orange precipitate on Dragendorff reagent and white precipitate on Mayer reagent
Flavonoid	2 ml extract + 0.1 g Mg powder + 5 drops HCl	Forms a reddish-orange color
Triterpenoid and Steroid	2 ml extract + 2 drops HCl + 2 drops H_2SO_4	Forms a red or purple color in the triterpenoid test and formed a bluish green color in the steroid test.
Tannin	2 ml of heated extract + 3 drops $FeCl_3$	Forms a greenish-brown or blue-black color

Identification of Compounds with FTIR Method

2 mg of lime peel extract and 198 mg of KBr were weighed and pulverized, then pressed into a transparent thin plate. Next, the sample was read using an FTIR tool. The resulting chromatogram was compared with the IR table.

***Artemia salina* Egg Hatching**

The aquarium was divided into two parts, the dark part and the light part. The light part was lighted with a lamp equipped with an aerator to dissolve oxygen. Then, the aquarium was filled with 500 ml of sterile seawater. *Artemia salina* eggs were weighed first for 100 mg. Next, Artemia eggs were put into the dark part of the aquarium and left for 48 hours.

Stock Solution Preparation

The stock solution was prepared by taking 100 mg of ethanol extract of lime peel (*C. aurantifolia*) and dissolved in 100 ml of sterile seawater to obtain a stock solution with a concentration of 1000 ppm. Then, the stock solution was re-diluted with concentrations of 125 ppm, 250 ppm, and 500 ppm by diluting sterile seawater to 5 ml (Widyasari et al., 2018).

Brine Shrimp Lethality Test (BSLT)

Test tubes were prepared for each concentration, which are 0 ppm, 125 ppm, 250 ppm, 500 ppm, and 1000 ppm and each was repeated five times. For negative control, the concentration of 0 ppm without the addition of ethanol extract of lime peel. A total of 10 48-hour-old *Artemia salina* larvae were put into each test tube. Then, 3 mg of yeast was dissolved in 5 ml of seawater and added as much as 1 drop into the test tube as a food source for *Artemia salina* larvae. After that, the larvae were left to stand for 24 hours. The number of dead larvae was calculated by subtracting the total number of larvae at each concentration from the number of surviving larvae.

RESULT AND DISCUSSION

Phytochemical Assay

Phytochemical assay conducted to determine the content of secondary metabolites contained in ethanol extract of lime peel (*C. aurantifolia*) can be seen in Table 2.

Table 2. Phytochemical Assay Results

Type of Test		Result
Alkaloid	Dragendorff	+
	Mayer	+
Flavonoid		+
Triterpenoid		-
Steroid		-
Tanin		+

Based on Table 2, it is known that the ethanol extract of lime peel (*C. aurantifolia*) contains alkaloid, flavonoid, and tannin compounds. This is indicated by the formation of an orange precipitate on the Dragendorff reagent and a white precipitate on the Mayer reagent which indicates that the lime peel (*C. aurantifolia*) contains alkaloids. Then, there is a change in color to reddish orange which indicates the presence of flavonoid compounds and in the tannin test there is a change in color to greenish brown.

Analysis of FTIR Result

The FTIR results of lime peel extract (*C. aurantifolia*) with 96% ethanol solvent can be seen in Figure 1.

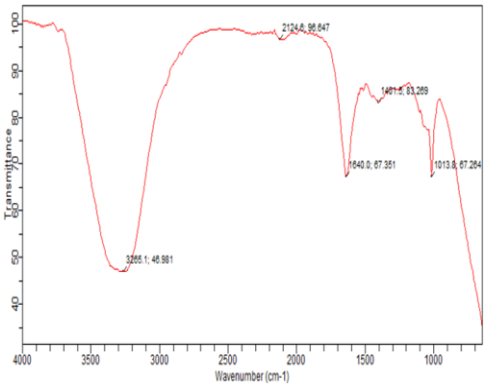

Figure 1. FTIR results of lime peel extract (*C. aurantifolia*)

Based on Figure 1, lime peel extract has several peaks, each of which has a wavelength frequency value described in Table 3.

Table 3. FTIR results of lime peel extract (*C. aurantifolia*)

Wave Numbers (cm^{-1})		Functional Groups	Compound
Reference Mustapha et al (2023)	Results		
3339.31	3265.1	Stretching vibrations O-H Stretching vibrations N-H	Flavonoid, Alkaloid
2319.35	2124.6	Stretching vibrations C-H	Flavonoid
1619.14	1640.0	Stretching vibration C=H	Carbonyl, Ester
1420.36	1401.5	Bending vibrations C-H	Flavonoid
1040.65	1013.8	Stretching vibrations C-O	Tannin

Functional group analysis was carried out using the FTIR method. At wave numbers 3000-3500 cm^{-1} there is a peak with a wide and sharp absorption at wave number 3265.1 cm^{-1} derived from O-H and N-H groups. The peak at wave number 2124.6 cm^{-1} interprets the stretching vibrations of the C-H bond. The presence of a peak at wave number 1640.0 cm^{-1} indicates the presence of C=H groups which are carbonyl and ester compounds. This compound gives a distinctive aroma to the lime peel (*C. aurantifolia*). The bending vibration at wave number 1401.5 cm^{-1} comes from the secondary alcohol group. the peak at wave number 1013.8 cm^{-1} shows the stretching vibration of the C-O group.

Flavonoids have functional groups composed of O-H, C-H, C=C, and C=O groups, alkaloids have N-H functional groups, and tannins have C-O functional groups (Berghuis et al., 2023). Based on this, lime peel extract (*C. aurantifolia*) contains flavonoid, alkaloid, and tannin compounds based on the FTIR. The results obtained are in accordance with the phytochemical assay presented in Table 2.

Brine Shrimp Lethality Test (BSLT)

Toxicity tests were conducted to determine the effect of lime peel

extract (*C. aurantifolia*) on *Artemia salina* larvae using the BSLT method. The average mortality of *Artemia salina* larvae can be seen in Table 4.

Table 4. Number of deaths of *Artemia salina* larvae after 24 hours

Concentrations (ppm)	Number of deaths	% Deaths	LC_{50} (ppm)
0	0,0±0,00[a]	0%	
125	3,4±1,14[b]	34%	
250	5,8±0,84[c]	58%	331,533
500	8,0±0,71[d]	80%	
1000	10,0±0,00[e]	100%	

Notes: letters a,b,c,d, and e indicate significant differences by LSD ($p<0.05$)

Based on Table 4, the results show that the concentration of 1000 ppm is the highest average larval mortality, while in the control there is no death of *Artemia salina* larvae. The calculation results using SPSS showed that the ethanol extract of lime peel (*C. aurantifolia*) has an LC50 value of 331.533 ppm. The higher the concentration of the extract tested, the higher the death of *Artemia salina* larvae which can be seen in Table 4. Based on research conducted by Setianingsih et al (2023) that the level of toxicity in a compound is categorized into highly toxic (LC_{50} < 30 ppm), toxic (LC_{50} < 1000 ppm), and non-toxic (LC_{50} > 1000 ppm). If the LC_{50} value < 1000 ppm, the compound has the potential as an anti-cancer agent. This indicates that the ethanol extract of lime peel (*C. aurantifolia*) is toxic to *Artemia salina* larvae and has potential as an anti-cancer agent.

Artemia salina has been widely used in many toxicity tests, such as heavy metals, pesticides, nanoparticles, bioactive molecules, natural extracts, and metal materials (Banti and Hadjikakou, 2021). This is because *Artemia salina* has the ability to survive in unfavorable conditions by forming dormancy cysts (Ntungwe et al., 2020). In addition, *Artemia salina* is known to have the Heat Shock Proteins 70 (Hsp70) gene or a group of proteins that respond to stress conditions, such as high temperature, ultraviolet light, inflammation, infection, and toxins (Junprung et al., 2019). Because it has the same type of genes as cancer cells that can be expressed under the same conditions, *Artemia salina* can be used as a toxicity test animal (Ntungwe et al., 2020).

Compounds found in lime peel (*C. aurantifolia*), such as alkaloids, flavonoids, and tannins act as stomach

poisoning that will disrupt the digestion of *Artemia salina* larvae. Flavonoids work by inducing an increase in the amount of oxidative stress in cancer cells so as to inhibit cell proliferation, suppress pro-inflammatory cytokines, trigger cancer cell apoptosis, necrosis, and activate autophagy (Kopustinskiene et al., 2020). Alkaloid compounds can encourage DNA damage and trigger apoptosis because they work by regulating the main signaling pathways that play a role in proliferation, cell cycle, and metastasis (Habli et al., 2017). Meanwhile, tannin compounds are known to activate intrinsic and extrinsic apoptosis by targeting several signaling pathways (Rajasekar et al., 2021).

The content of secondary metabolite compounds contained in the ethanol extract of lime peel (*C. aurantifolia*) is toxic to *Artemia salina* larvae because it can cause death. The smaller the LC_{50} value produced, the higher the content of secondary metabolite compounds produced.

CONCLUSION

Ethanol extract of lime peel (*C. aurantifolia*) contains alkaloid, flavonoid, and tannin compounds. The LC_{50} value of lime peel extract (*C. aurantifolia*) which is 331.533 ppm is classified as toxic and can act as an anti-cancer agent. The higher the concentration of lime peel extract (*C. aurantifolia*) tested, the higher the percentage of death of *Artemia salina* larvae.

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