



Antioxidant Activity, Toxicity Test and Antimicrobial Test of *Streptococcus mutans* and *Candida albicans* Extract Of Ultrasound Assisted Extraction of Java Chili (*Piper retrofractum* Vahl) Leaves

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Abstract

P. retrofractum leaves are widely cultivated by the community to be used as traditional medicine, this is because of its secondary metabolite content which has several bioactive components such as steroids, flavonoids, alkaloids, saponins, tannins and has pyhtol and piperine compounds as dominant compounds that have antimicrobial potential. This study aims to determine flavonoid content, antioxidant activity and cytotoxic test of *P. retrofractum* leaf extract. The extraction method used was *Ultrasound Assisted Extraction* (UAE) with 3 solvents of different polarity, namely n-Hexan, ethyl acetate and 70% ethanol. Determination of flavonoid content using UV-Vis spectrophotometric method, determination of antioxidant activity using DPPH method and cytotoxic activity test using Brine Shrimp Lethality Test (BSLT) method. Antibacterial activity test was conducted by measuring the Minimum Inhibitory Concentration (KHM) and Diameter of Inhibitory Area (DDH). KHM test was done by agar dilution method, DDH test by disc paper diffusion method. The results of the extraction of *P. retrofractum* leaves produce the highest yield is ethanol extract which is 9.92%. The result of determining the highest flavanoid content is 70% ethanol extract at 1.2702%. The strongest antioxidant activity is 70% ethanol extract with an IC₅₀ value of 40.7475 ppm with a very strong category. The results of the cytotoxic test showed that the three types of extracts were toxic because they had LC₅₀ less than 1000 µg/mL. The best KHM result for *S. mutans* is 10% n-hexane extract and 10% ethyl acetate and for *Candida albicans* is 5% n-hexane extract. The best DDH of *S. mutans* was 20% n-hexane extract with a value of 17.37 mm approaching Susceptible (Intermediate) and the best DDH of *Candida albicans* was obtained by 20% n-Hexan extract with an average DDH of 17.12 mm approaching Susceptible (Intermediate).

Keywords: Javanese chili leaves (*piper retrofractum*), flavonoid content, antioxidants, antimicrobial, *candida albicans*.

1. Introduction

Piper retrofractum Vahl or Java chili or chili puyang belongs to the Piperaceae family, widely distributed in the tropics. More than a thousand species are included in the genus *Piper*. *P. retrofractum* is a plant species native to Southeast Asia and is mostly cultivated in Indonesia, Thailand, Malaysia, Bangladesh, Vietnam, and India. *Piper retrofractum* in Indonesia is found on Java Island, Sumatra Island, Bali Island, Nusa Tenggara Island, and Kalimantan Island, where the main production center is on Madura Island (Sudarmaji et al., 2019). The elongated fruit shape with an upward growth direction resembles cayenne pepper (Evizal, 2020; Hariyani, et al., 2022; Silalahi, 2020). The stem is cylindrical with a blackbrown to blackish-brown color with a length of 2.93-9.82 cm, a diameter of 2-7 mm (Wardani et al., 2021). *P. retrofractum* grows a number of adhesive roots (4-9 pieces) 0.7-1.0 mm in diameter and 1-2.5 cm long. The newly emerged adhesive roots are white in color and turn brown. Roots that do not find a place to stick will dry out.

Piper retrofractum has monopodial branching with two types of branches, namely orthotropic and plagiotropic. Orthotropic branches or climbing branches grow vertically with a length of 6-8 cm, have stronger roots attached to the books, and when mature have rough spots. Plagiotropic or fruiting branches grow horizontally, have shorter internodes that are 2.08-8.02 cm long. Plagiotropic branches are fruit-bearing, green to dark green in color. In addition to these two branches, there are branches from the main stem that grow on the ground and have adventitious roots on each

book. This branch has smaller leaves and stems, and if it finds a place to climb, it will grow into an orthotropic branch to form a new tree (Haryudin et al., 2009; Evizal, 2013). The leaf shape is lanceolate to ovate with a length of 4.33-14.22 cm, a width of 2.35-8.28 cm, and a petiole length of 0.2-1.72 cm with pinnate leaf bones.

The leaves of *P retrofractum* grown in Indonesia are wider and shorter than those found in Thailand, India and other countries in Southeast Asia (Chaveerach et al., 2006; Peter, 2006; Guzman et al., 1999). Leaves on orthotropic branches are symmetrically curved, while leaves on plagiotropic branches are asymmetrically curved (Haryudin et al., 2013). The fruit grows in various sizes and shapes such as short, round, elliptical (conical), long flat (filiform), and long tiny (cylindrical). There are three short stigmas with subglobose to obovoid globose *P retrofractum* seeds with a length of 2 mm to 2.5 mm (Taufik et al., 2020; Wardani et al., 2021). *Piper retrofractum* Vahl has many potentials, one of which is as a significant antioxidant thanks to the content of secondary metabolite compounds such as alkaloids, flavonoids, phenolics, tannins, saponins, triterpenoids, steroids, and has piperine, saponin, cavinic, and volatile oil content. These compounds have strong antioxidant activity, helping to fight free radicals and prevent cell damage in the body (Prakash, 2001).

The biological activity of plants is influenced by the type of secondary metabolites contained therein. Molecular groups affect biological activity because they are related to the mechanism of action of the compound on receptors in the body. These compounds mostly contribute to plant interactions with the ecosystem, such as antibiotics, antifungals, antivirals, and allelopathy (Bougaud, 2001). Antioxidant activity is closely related to the content of secondary metabolites that function as antioxidants such as phenol compounds (Zubia, 2007), one of which is flavonoids. Research conducted by Jadid et al. (2016) and Mulia et al. (2016), states that *P retrofractum* extracted with different solvents produces extracts that have different antioxidant activities. N-hexane and ethyl acetate extracts have moderate antioxidant activity and methanol extracts have weak antioxidant activity. Mahaldar et al. (2019) in their study showed that different organs showed different antioxidant activities, ethanol extracts of *P retrofractum* roots and stems showed different antioxidant activities with LC50 values of 133 and 91 µg/mL, respectively, while ascorbic acid was 14 µg/mL. The antioxidant activity of the root is categorized as moderate and the stem activity is categorized as strong. Flavonoids belong to the class of plant secondary metabolites, which have a polyphenolic structure and are widely found in fruits, vegetables and certain beverages. Flavonoids have a variety of beneficial biochemical and antioxidant effects associated with various diseases such as cancer, Alzheimer's disease (AD), atherosclerosis, and others (Ovando et al., 2009; Lee et al., 2009).

Piper retrofractum is also known to have anticancer activity. According to the findings reported by Ekowati et al. (2011) the results of cytotoxic activity of *P retrofractum* extract against Hela cells were 33 µg/mL and t47D cells were 53 µg/mL. According to Ekowati et al. (2012) the cytotoxic activity of *P retrofractum* extract against myeloma was 36 mg/mL and WiDr cells was 158 mg/mL. Thus *P retrofractum* can be assumed to have potential as a chemopreventive agent against breast cancer cells (MCF-7). *P retrofractum* extract protects normal cells from cancer cells by binding to proteins in cancer cell mitochondria to trigger apoptosis without damaging surrounding (Selvendiran et al., 2003).

Phumat et al. (2017) species from the Piperaceae family are widely explored as antimicrobial and antifungal, one of which is the leaves of *P retrofractum* which has great potential for medicinal use. Jamal et al. (2013), stated that *P retrofractum* leaves have antibacterial activity. Research shows that fruit and leaf extracts effectively inhibit the growth of bacteria such as *Staphylococcus aureus*, *Staphylococcus mutans*, *Escherichia coli* and *Candida albicans*. The use of medicinal plants as a source of screening for new antimicrobial agents has several advantages regarding safety, availability, and minimizing the risk of side effects and addiction (Dong-sun et al., 2003). While there are currently many cases of drug resistance to infectious microorganisms that have been reported worldwide (Rello et al., 2019). One of them is the case of dental caries caused by *S. mutans* bacteria which is one of the most common infectious diseases in humans, still a significant public health problem in several countries (Gomez et al., 2003). Caries is damage to the tooth structure caused by bacteria that cause acid discharge from oral bacteria (*S. mutans* and *S. sobrinus*) that ferment on the tooth surface (Fraser, 2004).

S. mutans is one of the bacteria that plays a major role in causing dental caries in humans. These bacteria usually coexist with *S. sobrinus* on decayed teeth. Biofilms of both organisms are formed from a mixture of sugars, formed plaque, and colony growth due to ideal growth conditions at a pH between pH 3.0 to pH 7.5. The lactic acid from the sugars fermented by the bacteria and the plaque formed will eat away at the protective layer of tooth enamel, resulting in the formation of cavities (Samaranayake, 2005). Epidemiological studies have reported that *S. mutans* is more common than *S. sobrinus* in dental plaque (Loesche, 1986; Bader, 2001). In addition to cases of infectious microorganisms there are also cases of fungal infections caused by *C. albicans* that can affect areas such as the skin, genitals, throat, mouth, and circulatory system called candidiasis. It is caused by an overgrowth of *C. albicans*. Candidiasis is difficult to treat and may recur after treatment. In people with weakened immune systems, candidiasis can be life-threatening if it enters the blood and spreads to vital organs (Harvard Health Publication, 2017; Panphut et al., 2020).

Given this, the search for new antimicrobial agents continues. *P. retrofractum* has been used as a source of medicinal compounds to treat various disease conditions, such as fever, flu, diarrhea, beriberi, anemia, and cholera (Musthapa, 2016; Mahaldar et al., 2020; Syafitri et al., 2023; Syafitri, 2024). The plant has potential nutritional and phytochemical properties, which support its pharmacological uses. The medical uses of this plant are related to its phytochemical composition, which includes alkaloids, saponins, tannins, flavonoids, steroids, terpenoids, and glycosides (Chaveerach et al., 2006; Sholehah et al., 2023). In addition, *P. retrofractum* is also commonly used as a spice (Lim, 2012).

Choosing an extraction method to maximize the potential of the secondary metabolite compounds contained is very important. The UAE method is an extraction technique that involves the application of ultrasonic waves at frequencies above 20 kHz and a little heating at 40°C is one option. Ultrasonic waves can break cell walls which will help the release of active compounds. The vibration frequency in UAE is 20000 Hz in 1 second on the extracted material (Chemat & Khan, 2011; Utami et al., 2020). The UAE method has advantages compared to the maceration method, namely a shorter extraction process and more efficient use of solvents.

The potential of *P. retrofractum* extract as antibacterial and antifungal can be known through the research stage. The test method that can be done in research is the agar dilution method to determine KHM and disc diffusion method to determine DDH, in this study will use PDA media for fungi and media (NA) for bacteria with 1% Tween solvent in the test solution. Toxicity test is needed to determine the pharmacological potential of *P. retrofractum* leaves, while the method used is BLST. The Brine Shrimp Lethality Test (BSLT) method is proposed as a simple bioassay to monitor the pharmacological activity of a natural extract. This method has several advantages, namely, fast, cheap, relatively few samples needed and simple. This method can be utilized as a preliminary bioassay before proceeding to a more complicated test stage for certain pharmacological activities (Mc Laughlin & Rogers, 1998; Susilowati, 2017). By using the BSLT method, the safety limit of its use for medicinal purposes will be known (Carballo et al., 2002).

This research is expected to produce alternative drugs from natural materials as antioxidants, anticancer, antifungal and antibacterial and is expected to provide maximum therapeutic effects with small side effects. In the extraction process, three solvents with different polarity are used, so that the most effective solvent will be seen in extracting bioactive compounds. Ethanol solvent can attract flavonoid compounds, tannins and alkaloids, ethyl acetate solvent can attract phenols, saponins and n-hexane solvent can attract terpenoid compounds (Shirsath, 2012).

2. Materials and Methods

The research was conducted in April-December 2023 at the Pharmacy Laboratory of the Faculty of Mathematics and Natural Sciences, Pakuan University. The materials used include *Piper retrofractum* Vahl leaves from the IPB Biopharmaca Collection Park and have been determined by the National Research and Innovation Agency (BRIN) Cibinong, Bogor. Isolates of *C. albicans* fungus and *S. mutans* bacteria came from the Microbiology Laboratory of IPB University. The positive control in the *S. mutans* test used penicillin and *C. albicans* used and nystatin, both brands of Kimia Farma, as a negative control using tween 80. Nutrient agar, PDA, 70% ethanol (C(2) H₅OH), 96% ethanol (AMSURE®), ethyl acetate (C₄H₈O₂), n-hexane (C₆ H₁₄), DPPH (2,2-diphenyl-1-picrylhydrazil), vitamin C, quinine, concentrated HCl, Aquadest, AlCl₃, methanol p.a, Na Acetate, Magnesium (Mg) powder, Bouchardat reagent, Dragendroff reagent, Mayer reagent, Chloroform (CHCl₃), sulfuric acid (H₂SO₄) 1%, Aquadest, anhydrous acetic acid, iron (III) chloride solution (FeCl₃), barium chloride BaCl 1%, BaCl₂ 21%, physiological NaCl.

Tools used included analytical balance, digital scale (LabPRO®, KERN®), grinder, 40 mesh, waterbath, vaporizer cup, dropper pipette, measuring cup (Pyrex®), oven (Memmert®), autoclave (ALL AMERICAN®), glass cup (Pyrex®), filter paper, erlenmeyer (Pyrex®), incubator (NUVE®), desiccator (Iwaki®), ultrasonic device (Elmasonic® 180 H), rotary evaporator, glass funnel (Pyrex®), test tube (Pyrex®), tube rack, spatula, silicate chair, micropipette, UV-Vis spectrophotometry, aluminum foil, furnace, cuvette, volumetric flask, Whatman no 1 filter paper and other glassware.

2.1. Extraction

P. retrofractum leaves as much as 3.5 kg were washed with running water, then dried in an oven at 45° C for 48 hours, after drying, they were pulverized using a grinder and sieved using mesh 40 to get powdered dried leaves. Next, the powdered dried leaves were weighed as much as 200g, 2000mL of n-hexane solvent (1:10) was added. Extraction using the UAE method at a frequency of 40 kHz, a temperature of 40°C and an extraction time of 15 minutes. The filtrate obtained was filtered with whatman no.1 filter paper and the powder dregs were air dried and re-extracted with ethyl acetate solvent with the same treatment, then the dregs were dried again and re-extracted with 96% ethanol solvent. Then each filtrate obtained was evaporated with a rotary evaporator at 50°C until a thick extract was obtained.

2.2. Extract Characteristics

Dried leaves powder and viscous extracts of n-hexane, ethyl acetate and 96% ethanol of *P retrofractum* leaves were then characterized including organoleptic tests (shape, color, aroma and taste), determination of water content and ash content. The water content test was carried out using the gravimetric method, which is weighed as much as 2g of the sample and then put into a cup that has been marked. Then dried in an oven at 105°C for 5 hours, cooled in a desiccator and weighed. Drying is continued and weighed at a distance of 1 hour until the weight is constant or the difference between the last two weighings is no more than 0.25% (MOH RI, 2017). The ash content test was carried out by weighing 2g sample and placing it in a silicate crucible that had been marked, then incinerated in a furnace at 600°C, then cooled in a desiccator and weighed. Weigh until the weight is constant or the difference between the last two weighings is no more than 0.25% (MOH RI, 2017).

2.3. Phytochemical Screening

Qualitative phytochemical screening tests were carried out on powdered dried leaves and *P retrofractum* leaf extracts which included alkaloid compounds, flavonoids, tannins, saponins, and triterpenoids/steroids with the reaction method of color changes, the emergence of deposits and changes in shape that occur after being reacted with each reagent using a tube test.

2.4. Determination of Flavonoid Content

Determination of flavonoid levels using the AlCl_3 colorimetric method, the initial stage is the preparation of a standard curve of quercetin as much as 10 mg put into a 100 ml volumetric flask, then added methanol p.a to 100 ml (100 $\mu\text{g/ml}$ mother liquor). To determine the ideal stable time, absorption was measured with the maximum wavelength using standard solution with a concentration of 6 ppm. Taken as much as 0.6 ml of 100 ppm quercetin solution and then put into a 10 ml volumetric flask added methanol 3 ml AlCl_3 10% as much as 0.2 ml, Na acetate 0.2 ml and distilled water until 10 ml and then homogenized, then measured the absorption for 60 minutes at a maximum wavelength of 400-500 nm. Then a standard solution of 2 $\mu\text{g/ml}$, 4 $\mu\text{g/ml}$, 6 $\mu\text{g/ml}$, 8 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ was made in a 10 mL volumetric flask and added with 3 mL methanol, 0.2 mL AlCl_3 , 0.2 mL Na acetate and distilled water. The solution was homogenized after which it was allowed to stand for the incubation time and then measured the absorbance using UV-Vis spectrophotometry at the maximum wavelength (Rivai et al., 2011). The extract content (ppm) can be calculated using a linear regression equation, where the Y value is the absorbance of the extract. The linear regression equation ($y = bx + a$) was obtained by making a curve between the concentration of standard quercetin solution and the absorbance value obtained. In the extract weighed as much as 25 mg of n-hexane, ethyl acetate and 70% ethanol extracts of *P retrofractum* leaves dissolved with methanol up to 25 mL (1000) and then taken 1 mL was put into a 10 mL volumetric flask after which 3 mL methanol was added, 0.2 mL AlCl_3 , 0.2 mL Na acetate and distilled water, then read the absorbance at the maximum wavelength (Rivai et al., 2011). The absorbance value is entered into a linear regression equation ($Y = bx + a$) obtained from a standard curve (with flavonoid standards, such as quercetin or rutin), to obtain C (flavonoid concentration in ppm). The C value is used in the flavonoid percentage formula:

$$\% \text{ Flavonoid content} = \frac{C (\text{ppm}) \times \text{Volume (mL)} \times fp \times 10^{-6}}{\text{Sample weight} - (\text{Sample weight} \times \% \text{Water content})} \times 100\% \quad (1)$$

Description:

C : Flavonoid concentration (ppm)
 V : Volume of Extract Used (ml)
 fp : Dilution Factor

2.5. Antioxidant Activity Testing

Testing the antioxidant activity of *P retrofractum* leaf extract with DPPH, weighed as much as 39.432 mg and then put into a 100 mL volumetric flask. Then dissolved with methanol p.a until the limit mark (measuring flask has been coated with aluminum foil). Next, a blank solution was made in the form of 1mm DPPH solution as much as 1 mL and then added methanol to 10 mL then homogenized and incubated for 30 minutes at a temperature of 25-30° C with a wavelength of 400-600 nm. Weighed 50 mg of vitamin C mother solution, then dissolved with methanol into a 50 mL volumetric flask with a concentration of 1000 ppm and then pipetted 10 mL of vitamin C (1000 ppm) into a 100 mL volumetric flask and dissolved with methanol until the limit mark. The wavelength was measured at 10, 20, 30, 40, 50, 60 minutes to obtain a stable optimum absorption time to ensure a stable ideal absorption time. Standard series

solution of vitamin C was made standard series with concentrations of 2, 4, 6, 8 and 10 ppm from the parent solution of 100 ppm in a 10 mL volumetric flask, then added 1 mL of DPPH solution and methanol until the limit mark of 10 mL (volumetric flask coated with aluminum foil) then incubated at the optimum incubation time and measured the absorption using UV-Vis spectrophotometry at the maximum wavelength. *P. retrofractum* leaf extracts of n-hexane, ethyl acetate, and 70% ethanol, weighed as much as 10 mg each and then put into a 100 mL volumetric flask which will be dissolved with methanol until the limit mark (measuring flask lined with aluminum foil). A series was made, namely 20, 40, 60, 80, 100 ppm and then pipetted from the parent solution as much as 0.2; 0.4; 0.6; 0.8; and 1.0 mL, then put into a 10 mL volumetric flask. Then added DPPH solution as much as 1 mL homogenized and allowed to stand for 30 minutes. Each solution was measured for absorbance using UV-Vis spectrophotometry at the maximum wavelength. The percentage value of DPPH inhibition was calculated using the following:

$$\% \text{ Inhibition} = \frac{\text{Blank absorbance} - \text{Sample absorbance}}{\text{Blank absorbance}} \times 100 \% \quad (2)$$

The linear regression equation was used to obtain the IC₅₀ value with the amount of sample concentration as the x-axis and % inhibition as the y-axis, from the equation: $y = a + bx$. The IC₅₀ value can be determined by calculating the value of the y line equation of the percent (%) inhibition. The IC₅₀ value can be determined by calculating linear regression after obtaining the equation $y = a + bx$ with the x-axis is the concentration (µg/mL) and the y-axis is the percentage of inhibition (%) (Martiningsih et al., 2016).

2.6. Toxicity Testing

The method used for toxicity testing is BSLT, using *Artemia salina* Leach larvae. The first stage was made a parent solution of extracts with a concentration of 1000 ppm, then diluted to 100 ppm, then from the parent solution made concentrations of 50, 25, 10, and 5 ppm in 10 ml of seawater. Extracts that are difficult to dissolve are given the addition of a few drops of tween 80 before being diluted. *Artemia salina* L. eggs were hatched in an aquarium filled with artificial seawater, as much as 15 grams of salt without iodine mixed with seawater as much as 500 mL. Hatching was assisted using aeration and lamplight to hatch perfectly. After the larvae were 48 hours old, 10 larvae were put into the test vial and then added to the extract solution with the concentration according to the treatment. Each concentration was done with three replicates, while the control was done without the addition of extract solution. Then the shrimp larvae that died after 24 hours of incubation were counted, and the data obtained were analyzed using Probit Analysis Method to determine LC₅₀ with 95% confidence interval.

2.7. Antimicrobial Testing

Antimicrobial testing starts from sterilizing the tools and materials used using an oven and autoclave. Antimicrobial test preparation of pure microbial culture was carried out rejuvenation in NA media for *S. mutans* bacteria and PDA media for *C. albicans* fungi. The media was incubated at 37°C for 24 hours for bacteria and for 2x24 hours for fungi. Furthermore, a suspension of *S. mutans* and *C. albicans* cultures was made, by mixing with 0.9% physiological NaCl, to obtain the same turbidity as the 0.5 McFarland standard solution. Preparation of Mc Farland 0.5 standard solution is as much as 0.05 ml of 1% Barium Chloride in distilled water mixed with 9.95 ml of H₂SO₄ 1%. Anti-microbial testing is carried out in two stages, the first stage is the KHM test and the second stage is the DDH stage.

2.8. KHM testing

KHM testing of *S. mutans* and *C. albicans* used the agar dilution method. Testing was carried out by entering 15 mL of NA media into a Petri dish, then adding each extract for *S. mutans* (2.5, 5, 10, and 20% in 1% tween 20) and the concentration of each extract for *C. albicans* (5, 10, 15, and 20% in 1% tween 20). Furthermore, into 1 mL of bacterial suspension was inserted and homogenized to form a figure 8 and incubated for 24 hours at 37°C. KHM testing for *C. albicans* was carried out in the same way as bacterial testing, but using PDA media added *C. albicans* culture and then incubated for 2x24 hours at 37°C. Furthermore, observations were made after completion of incubation, test media that remained clear and there were no fungal or bacterial colonies called KHM.

2.9. DDH testing

DDH testing uses the Kirby-Bauer plate method with the aim of determining antibacterial activity by measuring the clear zone formed around the paper disk. Paper disks were soaked for 15 minutes in *P. retrofractum* leaf extract according to the concentration tested with reference to the KHM results. As a positive control for the *S. mutans* test, 1000 ppm penicillin was used, and for *C. albicans*, 100,000 IU/mL nystatin was used, and the negative control for both

used 1% tween 80. The cultures were incubated in an incubator at 37°C for 24 hours for *S. mutans* and 48 hours for *C. albicans*. The parameter measured was the DDH value in the form of a clear zone formed around the disk using a push rod. The clear zone formed indicates the presence of antimicrobial power in the test solution against *S. mutans* bacteria and *C. albicans* fungi.

2.10. Data Analysis

Data analysis was conducted to determine the comparison of flavonoid content and antioxidant activity of UAE extracts with different solvent polarity. The data obtained were analyzed statistically using Analysis of Variance (ANOVA) with complete randomized design (RAL) using SPSS program with 95% confidence level ($\alpha \leq 0.05$). If there is a difference then continued with Duncan's further test.

3. Results and Discussion

3.1. Characteristics of Dried leaves and Extract

The results of making dried leaves powder from 3.5kg of fresh *P retrofractum* leaves produced 905 g of dried leaves powder, so that the yield of dried leaves powder obtained was 25.86%. The characteristics of *P retrofractum* leaf powder from the results of organoleptic testing are presented in Figure 1 and the characteristics of *P retrofractum* leaf extract with three solvents are presented in Figure 1.



Figure 1: Dried leaves *P retrofractum*

In general, each extract, namely n-Hexan extract, ethyl acetate extract and 90% ethanol extract, is almost the same color, namely greenish black and smells typical of *P retrofractum* leaves with a slight innate weak odor from the solvent used. The provisions of dried leaves powder and *P retrofractum* leaf extract have not been determined in the Indonesian herbal pharmacopoeia so that it cannot be compared with the standardization of *P retrofractum* leaf extract.

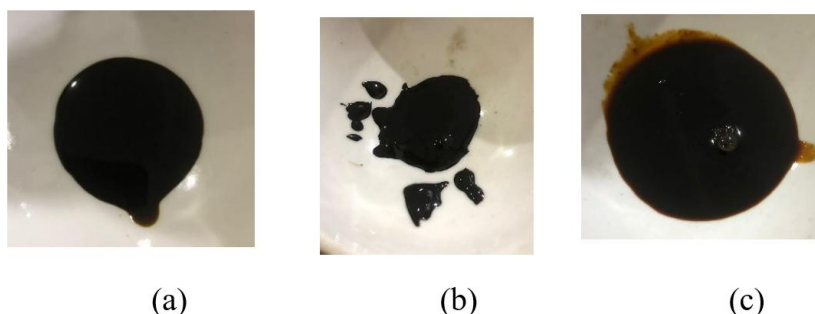


Figure 2: *P retrofractum* leaf extracts with various solvents n-Hexan (a), Ethyl Acetate (b), and 90% Ethanol (c).

The yield is the ratio of the dry weight of the product produced to the weight of the raw material (Yuniarifin, et.al, 2006), the yield value is related to the amount of bioactive material attracted during extraction (Dewatisari et al, 2020). The extraction results show that different types of solvents produce different extract yields. Senduk, et.al (2020) stated that the extraction of bioactive compounds is related to the amount of bioactive substances in the dried leaves and the solvent used. The higher the extraction yield value, the higher the content of extracted substances (Dewatisari, et.al., 2020). The results of the calculation of yield in the extraction process from 3.5kg of *P retrofractum* leaf dried leaves powder with different solvents are presented in (Table 1).

Table 1: Characterization results of dried leaves Powder and *P retrofractum* leaf extract with different solvents

Material Type	Yield (%)	Moisture Content (%) $\pm$ SD	Requirement (%)	Ash Content (%) $\pm$ SD	Requirement (%)
Powder	25.86	4.51 $\pm$ 0.046	$\leq$ 10	13.5638 $\pm$ 0.137	$<$ 10
n-Hexane Extract	1.58	22.7931 $\pm$ 0.139	5–30	9.7617 $\pm$ 0.112	$\leq$ 16.6
Ethyl Acetate Extract	1.64	23.6804 $\pm$ 0.177	5–30	8.3495 $\pm$ 0.069	$\leq$ 16.6
70% Ethanol Extract	9.92	24.7554 $\pm$ 0.170	5–30	9.2445 $\pm$ 0.035	$\leq$ 16.6

From Table 1, it shows that the amount of extract yield produced is influenced by the polarity of the solvent and the bioactive components in the extract. The highest yield is found in ethanol extracts. Noviyanti (2016) stated that 70% ethanol solvent is often used in the extraction process because it is cheap, easy to obtain and many have the same level of polarity as bioactive compounds or substances, so it is appropriate to extract phenolic and flavonoid compounds. Kanifah, et.al (2015) stated that differences in yield can be caused by differences in the boiling point of each solvent. The solvent n-hexan has a boiling point of 69°C, ethyl acetate 77°C and ethanol 78.32°C, solvents with high boiling points will produce high yields as well. Ngo, et.al., (2017) solvent polarity affects the extraction results and the type of bioactive compounds produced. The use of non-polar solvents produces metabolite compounds with low yields, and for polar solvents produces metabolite compounds with high yields (Wijaya, et.al., 2023). Sayuti (2017) also reported that the amount of yield depends on the solubility of the bioactive component. This illustrates that the compounds in *P retrofractum* leaves are more polar than semipolar and nonpolar such as alkaloids, saponins and flavonoids, polyphenols which are polar.

The results of determining the water content of *P retrofractum* leaf dried leaves powder produce a percent water content that meets the quality requirements of $\leq$ 10 (Ministry of Health RI, 2000). The extracts of *P retrofractum* leaves from three different types of solvents also meet the standard requirements for thick extracts, namely 5-30% (Voight, 1994). Moisture content is a parameter that shows the quality of dried leaves powder as well as for extracts. The water content for n-hexan viscous extract was 22.7931%, for ethyl acetate extract 23.6804% and for ethanol extract 24.7554%. The 70% ethanol extract is the extract that has the highest residual water content because it contains polar compounds that evaporate faster. The results of ash content determination showed that the ash content of *P retrofractum* leaves obtained in dried leaves powder was 13.5638%, n-hexane extract 9.7617%, ethyl acetate extract 8.3495% and 70% ethanol extract 9.2445%. The results show that the ash content for dried leaves powder and all extracts still meet the requirements according to the Indonesian Ministry of Health, (2017) and Hanif et al., (2018) that the standard for ash content of extracts is $\leq$ 16.6%.

3.2. Phytochemical Screening of Dried leaves and Extracts

Qualitative phytochemical tests were carried out for compounds of the alkaloid, flavonoid, terpenoid, saponin and tannin groups. The flavonoid compound group test was carried out to prove its potential as an antioxidant and other compound tests because it has anti-microbial activity such as its role as an antibacterial and fungal. Qualitative polyphenol content of this compound is based on the potential of these compounds as antibacterial and antifungal. According to Junairiah et al. 2020 the dominant compounds in *P retrofractum* leaf extract are terpenes (phytol and germekren D isomers) and alkaloids (piperine) which have antibacterial and antifungal properties. The results of qualitative identification of compounds in the thick extract of *P retrofractum* leaves are presented in Table 2.

Table 2: Phytochemical screening results of raw material powder and *P retrofractum* leaf extract

Phytochemical Test	Color (+)	N-Hexan Extract	Ethyl Acetate Extract	70% Ethanol Extract
Alkaloids	Yellowish white	-	-	+
Flavonoids	Red, orange	-	+	+
Terpenoids	Red, purple	+	-	-
Saponins	Foam 1-10 cm $<$ 10'	-	-	+
Tanins	Blue, green	-	+	+

Notes: + (Contains the tested chemical compounds), - (Does not contain the tested chemical compounds)

The identification results carried out on *P retrofractum* leaf extracts using three different solvents, especially for ethanol extracts containing compounds that have antimicrobial activity, namely alkaloids, flavonoids, terpenoids, tannins and saponins. In the phytochemical test only ethanol extract is positive for alkaloids. Alkaloids are semi-polar to polar and according to previous research (Junairiah, 2020) ethanol extracts contain alkaloid compounds in the form of piperine. The flavonoid compounds are semi-polar to polar so that they are positively identified in ethyl acetate and ethanol extracts. Concentrated HCl and magnesium in the alkaloid test function to reduce plant glycoside bonds with flavonoids so that they can be identified to form an orange color. Then terpenoid compounds are non-polar so that they are only identified with nhexane solvents which are characterized by the formation of a red color. Specific compounds from the terpenoid group are phytol and germakren D which have antimicrobial activity (Junairah, 2020). Saponin compounds were identified in the 6% ethanol extract because of the same polarity which was marked by the formation of foam with a height of 1 cm which lasted consistently due to saponins having hydrophile groups that can bind to water and hydrophobic groups that bind oxygen in the air so that it hydrolyzes into aglycone and glycone. Furthermore, tannin compounds are only identified in ethyl acetate extracts that form a green color where FeCl₃ reacts with one of the hydroxyl groups in tannin compounds.

3.3. Determination of Flavonoid Levels and Antioxidant Tests

Flavonoid determination aims to determine the levels of flavonoid compounds contained in *P retrofractum* leaf extracts using spectrophotometry with AlCl₃ reagent. Quercetin as a standard solution to determine the flavonoid content of n-Hexan, ethyl acetate and ethanol extracts of *P retrofractum* leaves. Quercetin belongs to the flavonoid flavonol group that has a ketone group at the C-4 atom and there is a hydroxy group at the C-3 and C-5 atoms that are close together (Azizah et al., 2014). Determination of flavonoid content was determined at the wavelength of quercetin which showed that the absorption of the reaction compound formed had a maximum absorption at a wavelength of 425.5 nm with an absorbance value of 0.6355. The maximum wavelength is used because it can provide the maximum absorbance so that it can provide an absorption value with the highest measurement sensitivity. Determination of the optimum incubation time aims to determine the length of time required for the solution to reach a stable absorbance.

Incubation time was determined by measuring the absorbance of quercetin standard solution at the maximum wavelength using UV-Vis spectrophotometer. Based on the results of the research that has been done with a duration of 60 minutes, stable absorbance is found in the 30th minute with an absorbance value of 0.6702. Determination of the standard series curve obtained with the absorbance value of quercetin standard solution concentrations of 2, 4, 6, 8, 10 ppm which shows the absorbance value is in the range of 0.2-0.8 range of good absorbance values that range from 0.2-0.8 in the ultraviolet region and obtained a linear regression equation of $Y = 0.0525x + 0.2083$ with $r = 0.9998$. The average results of the determination of total flavonoid content are presented in Table 3.

Table 3: Results of total flavonoid content determination and antioxidant test of leaf extract of *P retrofractum*

Extract	Flavonoid content (%)	Antioxidant test	
		IC50 (ppm)	Categorization
N- Hexanes	0,3500 ± 0,0101	68,2247 ± 0,4149	Strong
Ethyl acetate	0,6356 ± 0,0331	66,2354 ± 0,0013	Strong
70% Ethanol	1,2702 ± 0,0329	40,7475 ± 0,2493	Very strong

The measurement of flavonoid content showed that the n-hexane extract contained 0.3500%, ethyl acetate extract 0.6356% and ethanol extract 1.2702%. The highest flavonoid content is ethanol extract, this indicates that *P retrofractum* leaves contain more active compounds that are polar, the higher the flavonoid content, the higher the benefits of flavonoids as antioxidants. In this study, the wavelength of 516nm with an absorbance of 0.7304 is the maximum wavelength used for antioxidant testing with the DPPH method. Determination of the optimum incubation time is done to determine the most optimum time a sample reacts with the maximum, the optimum incubation time is determined based on the time when the absorbance begins to stabilize (Sadeli, 2016).

In this study, at 5 minutes to 30 minutes there was a significant decrease in absorbance. The optimum incubation time was obtained at 30 minutes with an absorbance value of 0.6202. As a positive control using vitamin C in testing antioxidant activity (Njus et al., 2020). The results obtained in the linearity equation of the vitamin C standard are $y = 6.0629x + 0.4605$ with $R^2 = 0.9992$.

The IC₅₀ value states the concentration of the test compound that can capture free radicals by 50% and the smaller the IC₅₀ value indicates the better it is in counteracting free radicals. Antioxidant activity test results against *P retrofractum* leaves showed the best results were ethanol extracts with IC₅₀ of 40.7475 ppm classified as very strong, then n-hexan IC₅₀ of 68.2247 ppm and ethyl acetate extract of 66.2354 ppm, both included in the strong category.

4. Toxicity Test Results

The BSLT method is one of the methods used to determine the toxic ability to cells (cytotoxic) of a pure compound or compound extracted from plant material using *Artemia salina* Leach shrimp larvae as bioindicators. With the BSLT method, the cytotoxic test can be done by counting how many *Artemia salina* Leach shrimp larvae die due to the effects of plant extracts or natural material compounds (Nasution et al., 2023). The results of the cytotoxic test of *P retrofractum* leaf extracts with different solvents are presented in Table 4.

Table 4: Average larval mortality for each concentration of *P retrofractum* leaf extracts

Extract Type	Extract Concentration (µg/mL)			LC50 (µg/mL)
	1000	100	10	
n-Hexane Extract	45,657	43,222	40,444	20,3245
Ethyl Acetate Extract	83,324	81,322	63,522	9,7354
70% Ethanol Extract	90,124	87,876	85,773	6,7867

The results of the toxicity test of *P retrofractum* leaf extract showed differences in larval mortality at each concentration. The highest larval mortality was found in 70% ethanol extract with a concentration of 1000 ppm with an average mortality value of 90.124%, at a concentration of 100 ppm the average mortality value was 87.876%, and at a concentration of 10 ppm the average mortality value was 85.773%. Based on the results of probit LC 50 analysis on all types of leaf extracts *P retrofractum* showed to have cytotoxic properties with LC50 <30µg/mL, this is in accordance with the categorization by Meyer et al. (1982) which states that extracts or pure compounds are toxic if they have an LC50 value < 1000 µg/mL.

4.1. Minimum Inhibitory Concentration (KHM) Test Results

The results of the KHM test on *P retrofractum* leaf extract using the agar dilution method are presented in Table 5. The extract solvent used for extract dilution is 1% tween 80.

Table 5: Minimum inhibitory concentration test results of *P retrofractum* leaf extracts

Target Microbs	Extract	KHM (%)
Streptococcus mutans	n-Hexane	10
	Ethyl Acetate	10
	70% Ethanol	20
Candida albicans	n-Hexane	5
	Ethyl Acetate	10
	70% Ethanol	10

The KHM test results against *S. mutans* can be attributed to the phytochemical screening results that terpenoid compounds were only identified in the n-hexane extract. The specific compounds found as antibacterials from the terpene group are phytol and germakren D, the mechanism of terpenoids as antibacterials is to react with porin (transmembrane protein) on the membrane on the outside of the bacterial cell wall so that it forms a strong polymer bond and porin becomes.

The compounds identified in the ethyl acetate extract are flavonoids and tannins. Flavonoids in the form of enzymes can work by denaturing proteins that will cause cell metabolic activity to be disrupted. Flavonoids have the ability to form complexes with extracellular proteins dissolved from cell walls, so microorganisms cannot attach and invade cells. Tannin compounds work by precipitating proteins through reactions with cell membranes, inactivating enzymes and genetic material functions and then inhibiting reverse transcriptase and DNA topoisomerase enzymes so that bacterial cells cannot form (Pakaya et al., 2021).

Ethanol extract can inhibit the growth of *S. mutans* at a concentration of 20%, in the extract identified alkaloid compounds, flavonoids and saponins. The mechanism of alkaloids as antibacterial is to interfere with the constituent components of peptidoglycan in bacterial cells, so that the cell wall layer is not formed intact so that the cell will die.

The saponins act as a chemical barrier in plant defense against pathogens, saponins can cause leakage of certain proteins and enzymes in bacterial cells.

KHM test results against *C. albicans* fungi from n-hexan extract is 5% while ethyl acetate and ethanol extracts obtained 0%. N-hexan extract can inhibit fungal growth with smaller concentrations, terpenoid content in n-hexan extract interferes with the growth and development of fungal spores through the cytoplasmic membrane thus causing damage to the cytoplasmic membrane, cell coagulation, and proton disruption in fungal cells. Ethyl acetate extract contains flavonoids and tannins, flavonoids can act as antifungals because they have phenol groups that can denature proteins and damage cell membranes that are irreversible. The more lipophilic the flavonoid is, the easier it is to adhere to the fungal cell wall and damage the fungal cell. The tannins as antifungal inhibit the biosynthesis of ergosterol (the main sterol constituent of fungal cell membral) so that the fungus cannot grow. Ethanol extracts were identified as containing alkaloids, flavonoids, and saponins. Alkaloids inhibit fungi by disrupting DNA synthesis, while saponins damage fungal cells due to their ability to bind to hydrophilic and lipophilic molecules and then form complexes with sterol enzymes in the fungal cell wall so that cell wall permeability is lost. From this test it can be concluded that the more non-polar the solvent used, the smaller the concentration needed to inhibit microbes. And the greater the concentration of extract used, the more effective as an antimicrobial. The concentration for bacteria is needed greater because the cell wall is not easily lysed due to bacterial immunogenicity.

4.2. Diameter of Inhibition (DDH) Testing

The results of DDH testing research using the disc paper diffusion method will show a clear area around the disc paper which is an indication of the ability of the test antimicrobial agent to inhibit bacteria or fungi. The diameter of the inhibition area in *S mutans* and *C albicans* testing is presented in Table 6 and Figures 3 and 4. In Table 6 shows the concentration of n-Hexan extract, ethyl acetate extract and 70% ethanol extract concentrations of 2.5%, 5% and 10 have the same DDH in the resistant category (Hombach, et.al 2013) and 20% concentration shows DDH in the Intermediate category, however, the highest DDH obtained by 20% concentration of n-Hexane extract reached 17.37 mm. The results of the DDH test against the fungus *C. albicans* concentrations of 5%, 10% and 15 also have the same DDH in the resistant category and 20% concentration shows DDH in the Intermediate category, the highest DDH is obtained by n-Hexan extract. 20% with a DDH of 17.12 mm From the results of the DDH test which is indicated by a clear zone around the disc measured using a vertical and horizontal vernier with units of mm on *S. mutans* and *C. albicans* can be seen in Table 6 and Figures 3 and 4.

Table 6: Average DDH value (mm) of *P retrofractum* leaf extract

Target Microbes	Sample Extract	Concentration (%)	Average DDH (mm)	Category
<i>S mutans</i>	n-Hexsane	2.5	6.67b ± 0.09	Resistant
		5	8.19c ± 0.13	Resistant
		10	11.23d ± 0.03	Resistant
		20	17.37f ± 0.43	Intermediate
		K+	25.42g ± 0.95	Susceptible
		K-	0	0
	Ethyl Acetate	2.5	7.93c ± 0.40	Resistant
		5	8.33c ± 0.08	Resistant
		10	11.57d ± 0.20	Resistant
		20	15.37e ± 0.77	Intermediate
		K+	25.08g ± 0.52	Susceptible
		K-	0	-
	70% Ethanol	2.5	6.75b ± 0.12	Resistant
		5	7.58c ± 0.24	Resistant
		10	11.10d ± 0.26	Resistant
		20	14.77e ± 0.45	Intermediate
		K+	25.42g ± 0.79	Susceptible
		K-	0.00a ± 0.00	-
<i>C albicans</i>	n-Hexsane	5	8.35c ± 0.44	Resistant
		10	10.20d ± 0.83	Resistant
		15	12.45e ± 0.40	Resistant
		20	17.12g ± 0.63	Intermediate
		K+	24.03h ± 0.85	Susceptible
		K-	0.00a ± 0.00	-
	Ethyl Acetate	5	6.62b ± 0.42	Resistant
		10	10.87d ± 0.69	Resistant
		15	12.90e ± 0.15	Resistant

	20	16.98g $\pm$ 0.23	Intermediate
	K+	26.65j $\pm$ 0.51	Susceptible
	K-	0.00a $\pm$ 0.00	-
70% Ethanol	5	6.55b $\pm$ 0.22	Resistant
	10	8.60c $\pm$ 0.18	Resistant
	15	12.75e $\pm$ 0.66	Resistant
	20	15.18f $\pm$ 0.25	Intermediate
	K+	24.85i $\pm$ 0.41	Susceptible
	K-	0.00a $\pm$ 0.00	-

Numbers followed by the same superscript letter in the same column and row indicate an effect that is not significantly different from DDH. Categorization: Susceptible (acceptable as antibacterial, Intermediate (intermediate as antibacterial), Resistant (not acceptable as antibacterial).

Table 6 also shows that the higher the concentration of the extract and the more nonpolar the nature of the solvent used in the extraction, the higher the chance of being used as an antimicrobial. And the most effective extract is n-hexane with a concentration of 20% with a diameter of 17.37 mm including the intermediate category and is the diameter closest to the positive control. From these data it can be concluded that nonpolar solvents with low concentrations will have the same effect as high concentration polar solvents. The most effective extract in inhibiting the growth of *S. mutans* bacteria and *C. albicans* fungus is 20% n-hexane extract with DDH 17.37mm for *S. mutans* and 17.12mm for *C. albicans*. *P. retrofractum* extract has active compounds in the form of flavonoids that have antibacterial activity. Flavonoids are the largest phenol compounds in nature found in plants that have antibacterial properties. The possibility of flavonoid antibacterial activity can change the physical and chemical properties of the cytoplasm containing proteins and bacterial cell walls (Lestari, et.al 2019). There are three flavonoid mechanisms as antibacterials among others by inhibiting nucleic acid synthesis, inhibiting cell membrane function, and inhibiting energy metabolism. The antifungal mechanism of action includes causing disruption to the cell membrane. This disruption occurs due to the presence of ergosterol in fungal cells. Ergosterol is a very important sterol component and is very easily attacked by polyene-derived antibiotics. The polyen-ergosterol complex that occurs can form a pore and through the pore the essential constituents of fungal cells such as K ions, inorganic phosphates, carboxylic acids, amino acids, and phosphate esters leak out to cause the death of fungal cells (Sari, et.al 2013). Another mechanism of antifungal action is by inhibiting ergosterol biosynthesis in fungal cells. The mechanism of action of these antifungi is by causing irregularities in the cytoplasmic membrane of the fungus by changing the permeability of the membrane and changing the fungi membrane in the process of transporting essential compounds that can cause metabolic imbalances, thus inhibiting ergosterol biosynthesis from fungal cells.

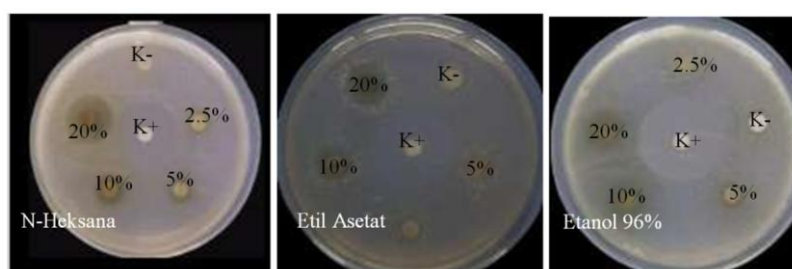


Figure 3: Antimicrobial activity of *P. retrofractum* leaf extract with different solvent type against *S. mutans* bacteria

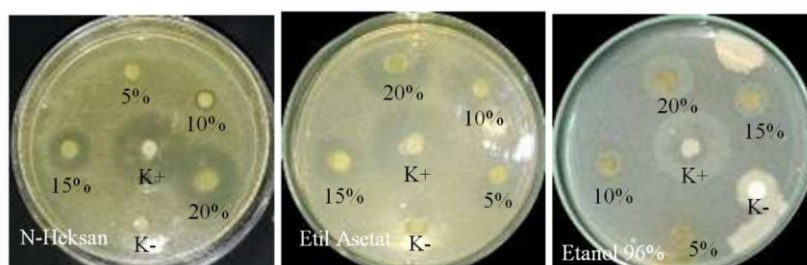


Figure 4: Antimicrobial activity of *P. retrofractum* leaf extract with different types of solvents against *C. albicans* fungus

5. Conclusion

The results of the research that has been done, show that the best flavonoid levels and antioxidant activity are 70% ethanol extract of *P. retrofractum* leaves, namely 1.2702% with antioxidant activity of 40.7475 ppm with a very strong category. The results of the antibacterial test showed that the n-Hexan extract had the highest Diameter of Inhibition Area with Intermediate category for S mutans and C albicans at 20% concentration with an average DDH of S mutans 17.37 mm and DDH of C albicans 17.12 mm. The results of the toxicity test showed that the three leaf extracts were strongly toxic.

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