BIOVALENTIA: BIOLOGICAL RESEARCH JOURNAL
***e*-ISSN: 2477-1392**
Vol. 8 No. 2, Nov 2022

Antioxidant Activity of Tembesu (*Fagraea fragrans* Roxb.) Leaf

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Peer review under responsibility of Biology Department Sriwijaya University

Abstract

Herbal medicine is becoming a trend in curing several diseases especially degenerative because of the bad effects of synthetic drugs. Tembesu plant (Fagraea fragrans Roxb.) is traditionally used for degenerative diseases. This study aims to separate bioactive compounds (active fraction), pure isolate compounds from tembesu leaf, then observe antioxidant activity. The methods used are maceration extraction, liquid-liquid fractionation, purification of compounds by gravity chromatography column and antioxidant activity by scavenging DPPH radicals. The results showed that the active antioxidant fractions were n-hexane and ethyl acetate fractions. The results of purification of antioxidant compounds, obtained 6 isolates N1, N2, N4, N5, E8 and E11. Antioxidant activity (IC50) of pure isolates N1 (13.72 ppm), N2 (28.93 ppm), N4 (110.44 ppm), N5 (177.23 ppm), E8 (82.50 ppm) and E11 (12 ,86 ppm). The antioxidant compounds that have been isolated are terpenes (N1, N2, N4, E8), phenols (N5) and flavonoids (E11). This study concluded that tembesu leaf have antioxidant potential. Antioxidant bioactive compounds in tembesu leaf have the potential to be developed into standardized herbs and phytopharmaceuticals.

***Keywords**: Tembesu; Free Radicals; Antioxidants;*

Received: April 4, 2022, Accepted: June 12, 2022

1. Introduction

Diabetes mellitus (DM), heart disease, respiratory disease, rheumatoid arthritis and cancer are known as degenerative diseases that are a problem for most people. This degenerative disease is caused by free radicals, both from the environment and within the body itself. Free radicals can induce cancer in tissues or organs of the body [1]. In addition, free radicals can trigger oxidative stress that damages pancreatic β-cells and causes DM [2].

Free radicals can be defined as atoms or molecules that contain one or more unpaired electrons in the valence shell or outer orbit and are able to stand alone. The number of unpaired electrons of free radicals makes them unstable, short-lived and highly reactive [3].

The high reactivity of free radicals can be neutralized by antioxidants. Antioxidants neutralize excess free radicals, protect cells from their toxic effects and prevent diseases caused by oxidative damage. Antioxidants neutralize free radicals by donating one electron from the hydroxyl group to compounds that are oxidants [4].

The use of synthetic antioxidants can be an adverse effect. For example, the use of *Butylated Hydroxytoluene* (BHT) at dose of 150 mg/kgbb was able to initiate the formation of cancer in the lungs of rats [5]. The side effects of synthetic antioxidants are very dangerous, especially if used for a long time. Therefore, it is necessary to explore drugs that have antioxidant properties without side effects. Traditional or herbal medicine can be an option because of its properties in curing disease. Traditional medicine has natural compounds that are able to scavenge free radicals without side effects. These natural compounds can be obtained from the tembesu plant (*Fagraea fragrans* Roxb.)

Tembesu plant is known as a traditional medicine

which has flavonoid compounds, alkaloids, steroids, saponins, tannins, and quinones[6]. One of the compounds that have the potential to be antioxidants are flavonoids. For example, the flavonoids luteolin and quercetin isolated from the plant *Baccharis illinita* have antioxidant activity with IC_{50} values of 4.5 ppm and 2.1 ppm, respectively. The antioxidant activity of these two compounds is very strong [7]. According to the research about flavonoid compound from Katimaha leaves have strong antioxidant activity with IC_{50} values of 52.51 ppm (Ascorbic acid as standard with IC_{50} values 16,11 ppm). The flavonoid compound is potential antioxidant because increase the work of cellular antioxidants by protecting and increasing the work of lipophilic antioxidant [8]

The presence of flavonoid compounds in the tembesu plant causes a strong suspicion that the tembesu plant is efficacious as a natural antioxidant. Tembesu plant has potential as an antioxidant. This study aims to obtain antioxidant bioactive compounds from the most active fraction, test their free radical scavenging activity and isolate pure antioxidant compounds. Antioxidant testing was carried out using thin layer chromatography (TLC) and spectrophotometry methods. It is hoped that pure compounds obtained from tembesu leaf can be developed into standardized herbal medicines to phytopharmaceuticals for healing degenerative diseases.

2. Materials and Methods

The material used is tembesu leaves. Simplicia was obtained from the Botanical Gardens, Faculty of Teacher Training and Education, Sriwijaya University, South Sumatra and has been determined at the Herbarium of Andalas University, West Sumatra. The chemicals used were methanol, n-hexane, ethyl acetate, distilled water, amoxicillin, 6 mm disc paper, Silica Gel 60 (0.063 – 0.200 mm), Silica Gel 60 G for TLC, TLC plate Silica Gel 60 F_{254}, Nutrient Agar (NA), Nutrient Broth (NB), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Dimethyl sulfoxide (DMSO).

2.1 Tembesu Leaf Extract

1000 g of dried simplicia, mashed, and macerated with 4 L of methanol for 24 hours. Maceration was repeated 3 times. The liquid extract was evaporated with a rotary evaporator and the yield was calculated as follow [9] :

$$\text{Precentage yield (\%)} = \frac{\text{Weight of extract}}{\text{Weight of sample}} \text{ x } 100$$

2.2 Liquid-Liquid Fractionation

Liquid-liquid fractionation in this research used n-hexane, ethyl acetate and methanol-water as solvents. 20 mg of extract dissolved in 250 ml of methanol: distilled water = 2:3, then put into a separating funnel. 250 ml of n-hexane was added, shaken until homogeneous and allowed to stand until 2 layers of solvent were formed. The results of the separation are put in different bottles. The same procedure was carried out with ethyl acetate solvent. All fractions were evaporated using a *rotary evaporator* [10].

2.3. Antioxidant Activity of Tembesu Leaf Fraction

Antioxidant activity was determined by TLC. Silica Gel 60 F_{254} plate, size 1x5 cm as stationary phase, eluent (n-hexane, ethyl acetate and methanol) as mobile phase and 0.05% DPPH as spray for spotting. Each fraction was spotted on the chromatogram and eluted using the mobile phase. The chromatogram was sprayed with DPPH. Yellow spots on a purple background on TLC plates that have been sprayed with DPPH indicate the presence of antioxidant active compounds [11]. The yellow spots were measured for their Rf values as follow [12]:

$$\text{Rf} = \frac{\text{Distance traveled by compound}}{\text{Distance traveled by solvent}}$$

2.4 Purification of Compounds by Vacuum Liquid Chromatography (VLC) and Gravity Column Chromatography

VLC used Silica Gel 60 G for TLC as stationary phase and gradient solvent mobile phase n-hexane, ethyl acetate and methanol. The results of vacuum liquid chromatography were tested by thin layer chromatography (TLC) to determine the location of antioxidant compounds. Subfractions containing antioxidant active compounds were purified by gravity chromatography column. The gravity chromatography column used Silica Gel 60 stationary phase (0.063 – 0.200 mm) and mobile phase as solvent n-hexane:ethyl acetate (9:1; 5:5), ethyl acetate and methanol.

2.5 DPPH Free Radical Scavenging Activity of Pure Isolates

Free radical scavenging activity is expressed in IC_{50}, which is the concentration of compounds that can scavenge 50% of free radicals. The method used is the DPPH method

with a DPPH concentration of 40 ppm in methanol solvent. Pure isolate and ascorbic acid were prepared with concentrations of 1000 ppm, 500 ppm, 250 ppm, 125 ppm, and 62.5 ppm respectively in methanol solvent. The test was carried out by mixing 4 ml of the test solution and 1 ml of DPPH. The control used 2 ml of DPPH mixed with 3 ml of methanol. All of mixture was incubated in the dark for 20 min [16] . The absorbance was measured at a wavelength 517 nm for two times. The percentage of inhibition is calculated by the formula [14]:

$$\% \text{ inhibition} = \frac{\text{A control} - \text{A sample}}{\text{A control}} \text{x } 100\%$$

The percentage of inhibition is entered into the graph with the sample concentration as the abscissa (x-axis) and the percentage of inhibition as the ordinate (y-axis). Furthermore, the regression equation is obtained:

$$y = ax + b$$

y = scavenger (5)
a = gradient
x = concentration (ppm)
b = constant

The $IC_{50\ value}$ is obtained by following the formula:
IC_{50} = Antilog 10; x

2.6 Statistical Analysis

Data were analyzed using Microsoft Office Excel. The data displayed is the mean ± standard deviation (SD) of 2 repetitions (duplo).

2.7 Determination of Compound Type

Pure isolates were identified by TLC. The spots formed on the chromatogram were sprayed with 3% H2SO4 and heated on a hotplate. Color change on the chromatogram representation of the compound. Red orange for alkaloids, yellow-brown for flavonoids, yellow for phenols, brown for tannins, purple or blue for terpenoids and green for chlorophyll.

3. Results and Discussion

3.1 Tembesu Leaf Extract

The liquid extract was evaporated with a rotary evaporator to obtain a solid extract. Extract weight was calculated by yield (Table 1).

Table 1. Tembesu Leaf Extract Weight and Yield

Simplicia Weight (g)	Extract Weight (g)	Yield (%)
1000	300	30%

The yield of natural ingredient extracts shows the ability of the solvent to dissolve the active compound. Methanol solvent has the ability to dissolve non-polar, semi-polar and polar compounds. This is similar to the research of *Severinia buxifolia*. Extraction using methanol, distilled water, ethanol, acetone, chloroform and dichloromethane. The result is that methanol extract is the best antioxidant with IC_{50} value at 16.99 µg/mL and ascorbic acid at 50.94 g/mL [15]. Because it is considered quite effective in dissolving active compounds in plants, methanol is the solvent that is most often used in extracting nature.

3.2 Tembesu Leaf Fraction

Fractionation using n-hexane, ethyl acetate and methanol-water. The weight and yield of fractions are shown in Table 2.

Table 2. Tembesu Leaf Fraction Weight and Yield

Solvent	Weight Fraction (g)	Yield (%)
N-hexane	25	10
Ethyl acetate	50	20
Methanol	160	64

The smallest yield was in the n-hexane fraction, while the largest yield was in the methanol fraction. This indicates that the tembesu leaf extract contains a lot of polar compounds.

3.3 Antioxidant Activity of Tembesu Leaf Fraction

Tembesu leaf fraction was spotted on the TLC plate and eluted with the solvent. The chromatogram was sprayed with 0.05% DPPH. The results of the spray formed spots of yellow antioxidant compounds on the chromatogram (Figure 1). Furthermore, the spots of these compounds were measured for their Rf values (Table 3).

Table 3. Rf Value and Antioxidant Activity Tembesu Leaf Faction

Fraction	Number	Rf value (cm)	Antioxidant Activity	Desc
N-hexane	1	0.95	+++	Strong
	2	0.75	+++	Strong
	3	0.50	+++	Strong
Ethyl acetate	1	0.28	+++	Strong
Methanol	1	0.38	++	Medium

The antioxidant activity is based on the intensity of the yellow color on the chromatogram. Strong yellow color intensity (+++) indicates strong antioxidant activity, moderate intensity (++) for moderate antioxidant, weak intensity (+) for weak antioxidant, and no yellow color (-) or the chromatogram remains purple for no activity antioxidant [16]. The n-hexane fraction using n-hexane eluent: ethyl acetate (8:2), had 3 spots of antioxidant compounds with Rf values of 0.95; 0.75 and 0.50. The ethyl acetate fraction using ethyl acetate eluent: methanol (9:1), has 1 spot of antioxidant compound with an Rf value of 0.28. The methanol fraction using methanol as eluent has 1 spot of antioxidant compound with an Rf value of 0.38. The Rf value of the compound in the n-hexane fraction tends to be higher than in the ethyl acetate or methanol fraction.

The physical properties of compounds that are similar to the mobile phase, will be bound in the mobile phase. The mobile phase brings the most soluble compound to the upper boundary of the TLC plate. The compound is less soluble in the mobile phase and has a high affinity for the plate particles, left behind. Non-polar solvents will force non-polar compounds to the upper boundary of the plate, because the compounds dissolve and there is no reaction to the polar stationary phase [17].

A high Rf indicates a non-polar compound and a lower Rf value indicates a semi-polar or polar compound [12].

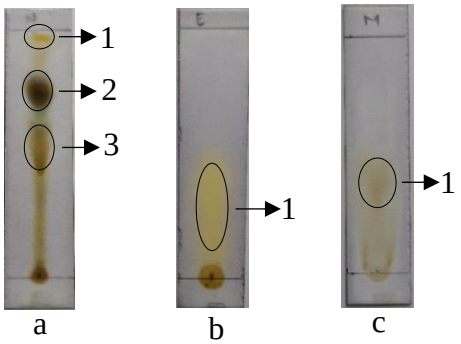

Figure 1. Chromatogram Profile of Tembesu Leaf Fraction. a) n-hexane, b) ethyl acetate, c) methanol

Compounds with strong antioxidants were the n-hexane and ethyl acetate fractions marked with bright yellow spots, while moderate antioxidants in the methanol fraction were marked with yellow spots (Figure 1). The yellow color on the chromatogram is the reaction of antioxidant compounds with DPPH radicals. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) is a stable free radical which has an unpaired valence electron on one nitrogen bridge atom [18]. When free radicals react with antioxidant compounds as electron donors, DPPH will be reduced. This causes the purple color to turn yellow from the picryl group [23] . The dark purple is the color of DPPH with nitrogen at the center of constituent structure. Then due to the reaction with antioxidant compounds, DPPH is reduced to a yellow non-radical compound. If the reaction is continued, the yellow color becomes colorless [20].

Figure 1 shows antioxidant compounds in the n-hexane fraction with 3 spots, then 1 spot in the ethyl acetate and methanol fractions. Spots measured Rf value (Table 3). Antioxidant activity is thought to be due to flavonoid compounds, alkaloids, steroids, saponins, tannins, or quinones contained in tembesu leaf[6].Polyphenolic compounds, flavonoids and anthocyanins isolated from *Thymus kotschyanus* leafhave antioxidant activity [21]. Furthermore, the alkaloid compounds from the *Tinospora crispa plant* also have antioxidant activity [22].

This research showed the fraction that had the strongest antioxidant activity was the n-hexane and ethyl acetate fraction. Both of these fractions were purified by VLC and gravity chromatography column to obtain pure isolates.

3.4 Purification by Vacuum Liquid Chromatography and Gravity Chromatography Column

VLC on the n-hexane and ethyl acetate fractions resulted in 12 subfractions. Each subfraction was tested for antioxidant and antibacterial activity.

The n-hexane sub-fractions that have antioxidant activity are the N1, N2, N4 and N5. Sub fractions were purified by gravity column chromatography and pure isolates were obtained.

The ethyl acetate subfractions that have antioxidant activity are E8, E9, E11 and E12. Mixtures E8 and E9 are labeled E8, and E11 and E12 are labeled E11. This mixing occurs because of the similarity of the chromatogram spots and the Rf value. Purification of the compound was continued by gravity column chromatography and pure isolates were obtained.

3.5 DPPH Free Radical Scavenging Activities

The results of free radical scavenging activity (Table 4) are expressed by the IC_{50} value.

Table 4. Free Radical Scavenging Activities

Sample	Regression	IC_{50} (ppm)	Antioxidant Category
Asc. Acid	y= 0.5348x + 4.6575	4.37	Very strong
N1	y= 0.5016x + 4.8692	13.72	Strong
N2	y= 0.4485x + 4.3446	28.93	Strong
N4	y= 0.4451x + 4.0906	110.44	Weak
N5	y= 0.3754x + 4.1559	177.23	Weak
E8	y= 0.3986x + 4.2361	82.50	Medium
E11	y= 0.2292x + 5.9984	12.86	Strong

The strongest free radical scavenging activity was isolate E11 with IC_{50} of 12.86 ppm. Then the weakest isolate was N5 with IC_{50} 177.23 ppm.

Antioxidant compound with IC_{50} value <10 ppm indicates very strong antioxidant activity. IC_{50} value 10 – 50 ppm, indicates strong activity. IC_{50} value 50 – 100 ppm, is moderately activity. IC_{50} value 100 – 250 ppm indicates weakly activity. And IC_{50} value > 250 ppm indicates inactive antioxidant activity [23].

3.6 Determination of Antioxidant Active Compounds

Determination using the TLC method with n-hexane:ethyl acetate as eluent (Table 5). The chromatogram was sprayed with 3% H_2SO_4 to display the compound spots.

Table 5. Determination of Antioxidant Compounds

Isolate	Eluent (N:E)	Rf Nilai Value	Color	Compound Group
N1	8:2	0.93	Purple	Terpenes
N2	8:2	0.93	Purple	Terpenes
N4	8:2	0.88	Purple	Terpenes
N5	8:2	0.68	Yellow	Phenol
E8	5:5	0.80	Purple	Terpenes
E11	Ethyl acetate	0.25	Yellow brown	Flavonoids

Isolates N1, N2, N4, E8, had purple spots which are a group of terpene compounds. The N5 isolate had yellow spots which belonged to a group of phenolic compounds. The E11 isolate had yellow-brown spots which belonged to a group of flavonoid compounds.

Terpenes are the main components in essential oils in medicinal plants. Terpenes have high potential as natural antioxidants that can prevent oxidative stress that causes degenerative diseases [24]. Thymol from the terpene group, works to donate one H atom from the hydroxyl group to highly reactive free radicals. The result is unreactive free radicals and thymol radicals [25]. The more the number of H atoms in a compound, the higher the antioxidant power [26].

Phenol is a common compound in plants, acting as a natural protector from biotic and abiotic disturbances. Several phenolic compounds including phenolic acids, flavonoids and proanthocyanidins are known as natural antioxidants that can reduce the risk and prevent degenerative diseases including cancer, cardiovascular or metabolic disorders [27].

The antioxidant activity of phenolic compounds is due to the presence of a benzene ring and the number and position of the OH group. The benzene ring is responsible for the stabilization of antioxidant molecules upon reaction with free radicals. One example of phenol is gallic acid. Gallic acid is a phenolic acid containing three hydroxyl groups and one carboxylic acid group [28].

Gallic acid reduces free radicals by transferring H atoms (from the OH group) to LOO^- radical chain carriers [29]. LOO^- free radicals are converted to LOOH radicals, while gallic acid is converted to gallic acid radicals. The LOOH radicals and gallic acid radicals formed are stable compounds and are not harmful to important compounds [28]. Gallic acid and flavonoids belong to the group of phenolic compounds. Both have antioxidant activity [27]. In this study isolate E11 is a flavonoid compound with IC_{50} of 12.86 and is included in the category of strong antioxidants.

Flavonoids are secondary metabolites of plants composed of polyphenols. Flavonoids have biochemical and antioxidant effects that are very helpful in the healing process of several diseases such as cancer, Alzheimer's, atherosclerosis, and other diseases caused by oxidative stress in the body. The basic structure of flavonoids is composed of ring A, ring B, and ring C. Ring A and ring C are always bonded, while ring B is bound to ring C at different positions. Flavonoids are distinguished based on the carbon ring C to which ring B is attached and the degree of unsaturation and oxidation of ring C. Flavonoids in which ring B is attached to position 3 of ring C are called isoflavones. Flavonoids whose B ring is bound at position 4 of the C ring are called neoflavonoids. The B ring bound at position 2 of the C ring can be further divided into several subgroups based on the structure of the C ring. These subgroups are: flavones, flavonols, flavanones, flavanols, flavanols or catechins, anthocyanins and chalcones [30].

Flavonoids work as antioxidants similar to the way terpenes work. The typical chemical structure of flavonoids related to antioxidant activity is the hydroxyl group, the ortho-dihydroxy arrangement in ring B, and the C2-C3

unsaturated bond combined with the C-4 carbonyl group in the C framework [31].

The hydroxyl structure of ring B is the most important part in scavenging free radicals centered on oxygen and hydrogen [32]. The high level of antioxidant activity is directly proportional to the high number of hydroxyl groups in ring B and also the total number of hydroxyl groups in all parts of the compound structure [32]. The free hydroxyl group will donate its hydrogen atom to the radical molecule, so that the radical molecule is stable and produces stable flavonoid phenoxyl radicals [14].

4. Conclusion

The results showed that Tembesu leaf have antioxidant activity. The strongest antioxidant active fractions were the n-hexane (N) and ethyl acetate (E) fractions, while the methanol (M) fraction was categorized as moderate antioxidant. Pure bioactive compounds from the n-hexane fraction that were successfully isolated were terpenes (N1, N2, N4 isolates) and phenols (isolate N5). Then the pure bioactive compounds from the ethyl acetate fraction that were successfully isolated were terpenes (isolate E8) and flavonoids (isolate E11). The strongest free radical scavenging activity was E11 isolate with an IC_{50} value of 12.86 ppm. The presence of these antioxidant bioactive compounds makes tembesu leaf have the potential to be developed into standardized herbs and phytopharmaceuticals.

5. Acknowledgements

Thank you very much to the lecturers who have provided knowledge during lectures, especially to the thesis supervisor who has provided enlightenment during the research process to writing the thesis.

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BIOVALENTIA: BIOLOGICAL RESEARCH JOURNAL
A Sriwijaya Journal For International Biologists
E-ISSN : 2477-1392
Published Since November 2015
by Biology Department, Faculty of Mathematics and Natural Sciences
Sriwijaya University

Vol. 8 No. 2 (2022)

Published: 01-07-2022

Vol 8, No 2 (2022): Nov 2022

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BIOVALENTIA: BIOLOGICAL RESEARCH JOURNAL
A Sriwijaya Journal For International Biologists
E-ISSN : 2477-1392
Published Since November 2015
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Kutipan dari Keputusan Direktur Jenderal Penguatan Riset dan Pengembangan,
Kementerian Riset, Teknologi, dan Pendidikan Tinggi Republik Indonesia
Nomor: 10/E/KPT/2019
Tentang Hasil Akreditasi Jurnal Ilmiah Periode 2 Tahun 2019

BIOVALENTIA: Biological Research Journal

E-ISSN: 24771392
Penerbit: Biology Department, Faculty of Mathematics and Natural Sciences, Sriwijaya University

Ditetapkan sebagai Jurnal Ilmiah

TERAKREDITASI PERINGKAT 3

Akreditasi berlaku selama 5 (lima) tahun, yaitu
Volume 4 Nomor 2 Tahun 2018 sampai Volume 9 Nomor 1 Tahun 2023

Jakarta, 4 April 2019
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