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Research Paper

Profiling of Active Compounds of Extract Ethanol, n-Hexane, Ethyl Acetate and Fraction Ethanol of Star Anise (*Illicium verum* Hook. f.) and Determination of Total Flavonoids, Total Phenolics and Their Potential as Antioxidants

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Abstract
Profiling of chemical compounds on star anise extract and fractions showed the content and looked for active compounds. The main compounds in alkaloids, flavonoids, saponins, phenolics, and triterpenoid forms were identified based on phytochemical screening. FTIR and GC-MS analysis were used to purify the extract and fractions' main compounds. After analyzing the main components of extract and fractions, a correlation was made between the total phenolic and flavonoid content. The total phenolic content was determined by adding folincalteau complexing solution converted to gallic acid equivalent (GAE), while the total flavonoid content was determined by $AlCl_3$ complexing solution converted to Quercetin Equivalent (QE). The total phenolic yields for extract ethanol, n-hexane fraction, ethyl acetate fraction, and fraction ethanol were obtained at 106.45, 52.30, 93.46, and 148.97 mgGAE/g, respectively. Then the total flavonoid results were 107.45, 58.94, 148.99, 140.01 mgQE/g. The total content of phenolics and flavonoids illustrated the number of active compounds that have a role as free radical scavengers due to their group. Antioxidant activity was tested with 1,1-diphenyl-2-picrylhydrazyl (DPPH) on extracts and fractions, with the best IC_{50} results obtained in the ethanol fraction 0.416 mg/mL.

Keywords
Star Anise, Antioxidant, Total Flavonoid Content, Total Phenolic Content

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1. INTRODUCTION

Traditional medicine is a significant field of science, so research on natural ingredients is an exciting topic. Traditional herbs, spices, and medicinal plants have been studied to the extent of isolating active compounds from plants with strong potentials, such as antioxidants associated with the content of various phenolic compounds (Sofowora et al., 2013; Mahomoodally, 2013). The primary cause of phenolics' antioxidant activity is their redox capabilities, which allow them to function as reducing agents, hydrogen donors, singlet oxygen molecules, and metal chelators (Wang et al., 2011; Liang et al., 2010). Radicals can be produced in excess as a product of metabolic processes, which can lead to oxidative damage to biomolecules and a variety of chronic illnesses. Numerous studies show a negative correlation between the consumption of foods high in antioxidants and the prevalence of human diseases like diabetes, cancer, atherosclerosis, aging, and other degenerative diseases (Tan et al., 2018).

The star anise (*Illicium verum* Hook. f.) belongs to the Illicium and Magnoliaceae family, an important species in traditional Chinese medicine and used in contemporary medicine in East Asian countries (Sharafan et al., 2022). This plant is widely cultivated in China and Vietnam (Wang et al., 2011). Anise flower is considered food and medicine. The focus of the research on star anise is mostly carried out in food, medicine, and cosmetics (Wei et al., 2014). Trans-anethole is a primary component in star anise essential oil. Due to its sweet flavor and aromatic properties, it is widely utilized in the culinary, fragrance, and pharmaceutical industries (Aprotosoaie et al., 2016). According to recent studies, trans-anethole has potent antioxidant, anti-inflammatory, and anti-obesity properties, which are also important in developing cosmetics and pharmaceuticals (Sharafan et al., 2022).

Extracts of *Illicium verum* just are safe to use in the human body (Valter et al., 2017). Extract *Illicium verum* has a good potential as a source of natural antioxidants. The results of identification and isolation of active compound from ethyl acetate fraction already tested with its cytotoxicity, which shows

low toxicity. It can be used as a preservative in the food or pharmaceutical industries, providing the resulting organoleptic effect is acceptable (Sabry et al., 2021).

The antioxidant properties of extract fractionated ethanol with hexane, ethyl ether, chloroform, ethyl acetate, and plant supercritical CO_2 extracts were evaluated using the inhibitory effect of 1,1-diphenyl-2-picrihydrazyl (DPPH) radical, the total of equivalent antioxidant capacity and reducing power assays. In addition, the total phenolic content and the total flavonoid content were also determined (Yang et al., 2012).

There are many Common flavonols in the *Illicium verum* fruit. They are kaempferol with its glycosides and quercetin with its glycosides also. Extract Dichloromethane of L leaves. Verum produces a seco-cycloartane ring of 3,4-seco(24Z)-cycloart-4(28),24-diene-3,26-dioic acid, a methyl ester which form of gigantic acid. One known alkyl glucoside, (R)-sec-butyl-D-glucopyranoside, was isolated from the fruit of *Illicium verum*.

In this study, profiling of chemical compounds from extract and star anise flower fractions to see the main compounds that make up the extract and each fraction. Tests on a total of phenolics and a total of flavonoids were carried out to determine the potency of the most dominant compounds in 1,1-diphenyl-2-picrylhidrazyl (DPPH) inhibition.

2. EXPERIMENTAL SECTION

2.1 Materials

Materials: Star anise (*Illicium verum*), ethyl acetate, n-hexane, Mg powder, amyl alcohol, potassium hexacyanoferate (III), $FeCl_3$ (1% and 10%), HCl, chloroform, anhydrous CH_3COOH, dragendroff reagent, mayer, wagner, toluene, folin-ciocalteu reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and quercetin purchased from (Sigma Chemicals), NaOH, gallic acid, aluminum (III) chloride ($AlCl_3$), hydrochloric Acid, ethanol, ethanol 70%, distilled water.

Instrumentation: Extraction was carried out with Bransonic help of Ultrasonic Bath series CPX1800H 40 KHz evaporators. The Infrared spectrum was measured by using ATR-FTIR using carry 630 Agilent Technology. Shimadzu UV-1800 UV/Visible Scanning Spectrophotometer. Gas Chromatography-Mass Spectra (GC-MS) Shimadzu QP 2010 SE.

2.2 Methods

2.2.1 Extraction Procedure

One hundred grams of star anise powder was placed into the maceration container. Then 1000 mL of 96% ethanol was added until all samples were submerged, covered, and left for 24 hours. Then maceration was stirred once every 24 hours for three days and filtered using filter paper. The filtrate obtained was evaporated using a rotary evaporator, so a thick extract was obtained.

2.2.2 Fractionation Procedure

The condensed extract of star anise was fractionated by trituration. First, 5 g of the sample was dissolved with 50 mL of n-hexane, stirred with an ultrasonic batch help, filtered to obtain compounds soluble in non-polar solvents, and n-hexane has added to 50 mL again until a clear solution was obtained. Then proceeded with adding 50 mL ethyl acetate, carried out in the same way to obtain the soluble ethyl acetate fraction. The last was dissolved with ethanol to get the ethanol soluble fraction. After obtaining the n-hexane fraction, ethyl acetate fraction, and ethanol fraction, each fraction was concentrated using a rotary evaporator to obtain a viscous fraction.

2.2.3 Phytochemical Screening

The flavonoid test was carried out by adding Mg powder + 1 mL HCl (p) + 1 mL amyl alcohol, producing a red, yellow, or green-brown solution on the amyl alcohol layer. The phenolic test was carried out by adding 1% $FeCl_3$ in produced green, purple, blue, to black colours. The saponin test was carried out by adding 10 mL of hot water + 1 drop of 2 N HCl in produced a stable foam. The alkaloid test was carried out by adding 1 mL of HCl 2 N + 9 mL of heated aquadest + Dragendroft reagent, in produced an orange to be red precipitated, and if added, Mayer's reagent would produce a yellowish-white precipitate and Wagner's reagent would produce a brown precipitated. The steroid/triterpenoid test was carried out by adding 2 mL of chloroform + 0.5 mL of anhydrous acetic acid + 1 drop of H_2SO_4 (p) in produced green steroids and blue/red triterpenoids.

2.2.4 Total of Phenolic

Total phenolic was carried out by making concentration series of gallic acid (4, 5, 6 ,7, 8; 9, and 10 μg/mL) as a standard solution. Folin Calteau reagent was added as much as 1.5 mL (1:10) for every 1 mL of sample (extract, n-hexane fraction, ethyl acetate fraction, and fraction ethanol. Then 1.2 mL of 7.5% Na_2CO_3 was added, and 10 mL distilled water was added (Ghazi et al., 2012). The solution was incubated at the temperature room for 120 minutes. The solution was measured at a wavelength of 775 nm using a UV-Vis spectrophotometer.

2.2.5 Total of Flavanoid

Used 50 mg of quercetin standard and dissolved into 50 mL of ethanol. The solution stock was pipetted in 1 mL and then made to 10 mL with ethanol to obtain a concentration of 100 μg/mL. From the 100 μg/mL quercetin solution standard, several concentrations were prepared, namely 20 μg/mL, 40 μg/mL, 80 μg/mL, and 100 μg/mL. From each concentration of quercetin solution standard, pipetted 4 mL, then added 0.1 mL of 2% $AlCl_3$, 2.8 mL of distilled water, and 0.1 mL of 1 M sodium acetate (Chadchan et al., 2017), added ethanol to see a mark. in 10 mL volumetric flask. Samples were incubated for 30 minutes in a temperature room. The absorbance was determined using the UV-Vis spectrophotometry method at a maximum wavelength of 436.5 nm. Measurement of extract and fraction samples was carried out by weighing a 10 mg sample dissolved in 10 mL of a volumetric flask for produced 10,000 μg/mL.

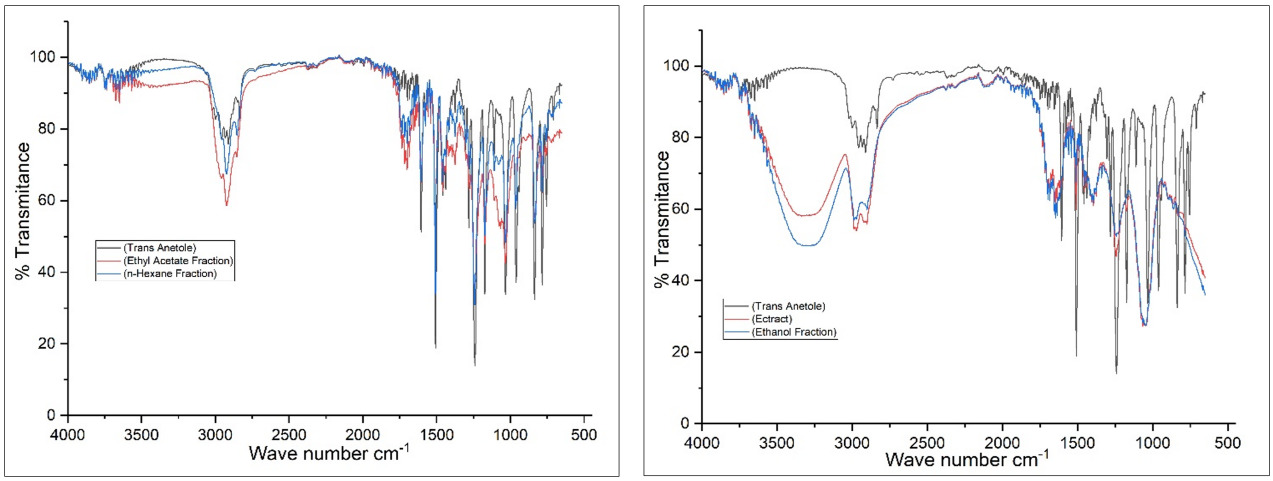

Figure 1. Comparison of FTIR Spectra of Trans Anetol Standards with Extracts, n-Hexane Fractions, Ethyl Acetate Fractions and Ethanol Fractions

2.2.6 Antioxidant Activity Test of Extract, n-Hexane Fraction, Ethyl Acetate Fraction, and Fraction Ethanol of *Illicium verum*

Antioxidant activity was determined according to Syarifah et al. (2022) with a few modifications. The extract and each fraction were prepared into 1000 μg/mL using ethanol by weighing 50 mg extract in 50 mL ethanol. Then several concentrations were made at 200, 300, 400, 500, 600, and 700 μg/mL. Each solution series was on a 0.2 mL pipette, and 3.8 mL of 0.5 mM DPPH solution was added. The mixture's absorbance with 515 nm wavelength was measured after it was incubated for 30 minutes in a dark environment. By leaving out the sample from the measurement, the blank was measured at the same wavelength as the sample. The positive control used was a routine standard solution with 2-10 μg/mL concentration. The absorbance obtained from the measurement analyzed of antioxidant percentage activity was.

$$\%\text{Inhibiton} = \frac{A_{\text{blank standard}} - A_{\text{sample}}}{A_{\text{blank standard}}} \times 100\%$$

3. RESULTS AND DISCUSSION

The research was conducted by extracting the star anise, with five replications of extraction. Extraction was carried out by weighing ±100 mg of star anise Simplicia. It can be seen that the replicated data showed stable data with an average yield of 11.17% shown in Table 1.

After extraction, the next process was the fractionation of 3 types of solvents. Fractionation was carried out with ultrasonic help because of the sticky nature of the extract, and it needed special treatment to remove the compound from the matrix. Based on the data, it can be seen that the largest fraction was in ethanol solvent. The results showed that the n-hexane fraction was dark green, while the ethyl acetate fraction was slightly green, and the ethanol fraction was brown. It was possible that many compounds contained in star anise dissolved in ethanol and had a more complex composition with a large molecular weight. It will be further characterized using GC-MS Shimadzu QP 2010 SE and ATR-FTIR 630 Agilent Technology.

Table 1. Extraction of Star Anise

Extraction	Star Anise Powder (g)	Extract (g)	%Yield
1	100.05	12.8142	12.81
2	100.01	10.6478	10.65
3	100.02	10.7658	10.76
4	100.08	11.1064	11.10
5	100.17	10.5504	10.54
Mean			11.17±0.9394

3.1 Profiling of Chemical Compounds in *Illicium verum*

The first step used to profile compounds in star anise was screening phytochemicals. This identification will give directions on chemical compounds that contain extracts and fractions. Secondary metabolites in star anise have an important role in its activity. Star anise contains flavonoids, alkaloids, saponins, phenolics, and steroids. Phytochemical screening of n-hexane fraction, ethyl acetate fraction and ethanol fraction, and extracts showed that the secondary metabolites are more abundant in extract and ethanol fraction with positive tests for all of almost tests. It showed in Table 2.

Based on Figure 1 FTIR spectral pattern, there was a similarity between the trans-anethole standard and n-hexane fraction, which showed the n-hexane fraction dominated by trans-anethole compounds. Pure anethole showed a sharp peak at 2959 cm^{-1} for aromatic -CH stretching vibration, 1727 cm^{-1} corresponds to the phenyl ring, 1297 cm^{-1} for the ether group (Chaudhari et al., 2020), 1600-1433 cm^{-1} for -OH bending, and 1073 cm^{-1} and 746 cm^{-1} for the C-O vibration of alcohol.

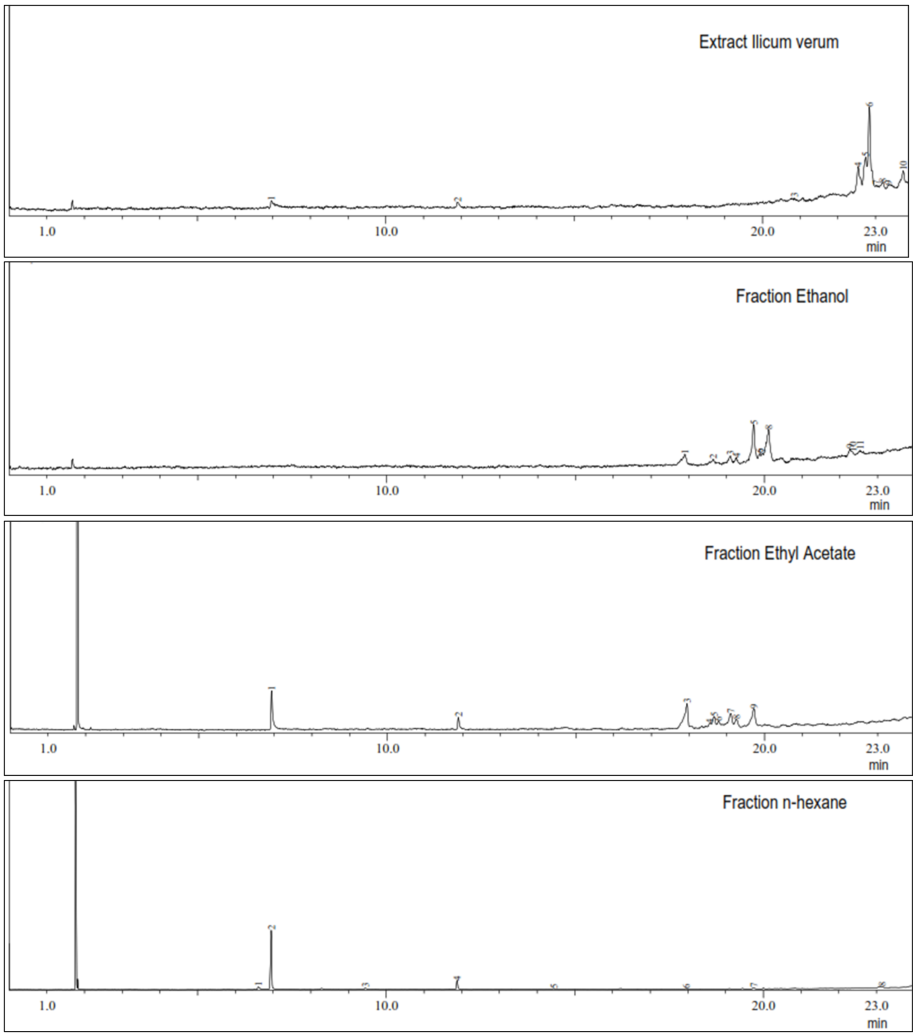

Figure 2. GC Chromatograms of Ethanol Extract, Ethanol Fraction, Ethyl Acetate Fraction and n-Hexane Fraction of Star Anise

At wave numbers 667-957 cm^{-1}, it showed strong aromatic C-H vibrations.

3.2 Profiling GC-MS of *Illicium verum*

Based on Figure 2 and Figure 3, the GC chromatogram followed by an analysis of the main compounds has three main constituents in extract and star anise flower fraction. The extract chromatogram results showed the diversity of compounds indicated by the number of peaks on the chromatogram. The content of trans-anethole was the main component. After fractionation, the content of trans-anethole became very large in the n-hexane fraction, with a 73.6% concentration. Trans-anethole solubility was a significant factor because it dissolved in non-polar solvents. Whereas in ethyl acetate fraction, the main components of the fraction are pheniculin and p-methoxy benzaldehyde. There are various phenolic compounds in methanol fraction, including catechins, chlorogenic, coumarins, and flavonoid groups such as quercetin, rutin, and other large-structured fatty acids (Yu et al., 2021; Zidan et al., 2019). The ethanol fraction in this study was dominated by the fatty acid dodecanoic acid, 1,2,3-propanetriyl ester, and the dominant flavonoid, luteolin. It occurs because of the differences in the way fractions are treated. Luteolin compounds and dodecanoic acid fatty acids, 1,2,3-propanetriyl esters, are more likely to dissolve in the ethanol fraction due to their polar nature (Achi and Ohaeri, 2015).

3.3 The Total Phenolic Content

Folin-Ciocalteu solution was a rough estimate of total phenolics. Additionally, the phenolic compounds have different reactions in various solvents during extraction, which gives affects how well its function is in the DPPH experiment. It depends on the amount of phenolic compound groups that contain in the solvent extract. From the results of total phenolic determination, polyphenolic compounds have a higher affinity towards water molecules. Hence, it dissolved in polar solvents such as water.

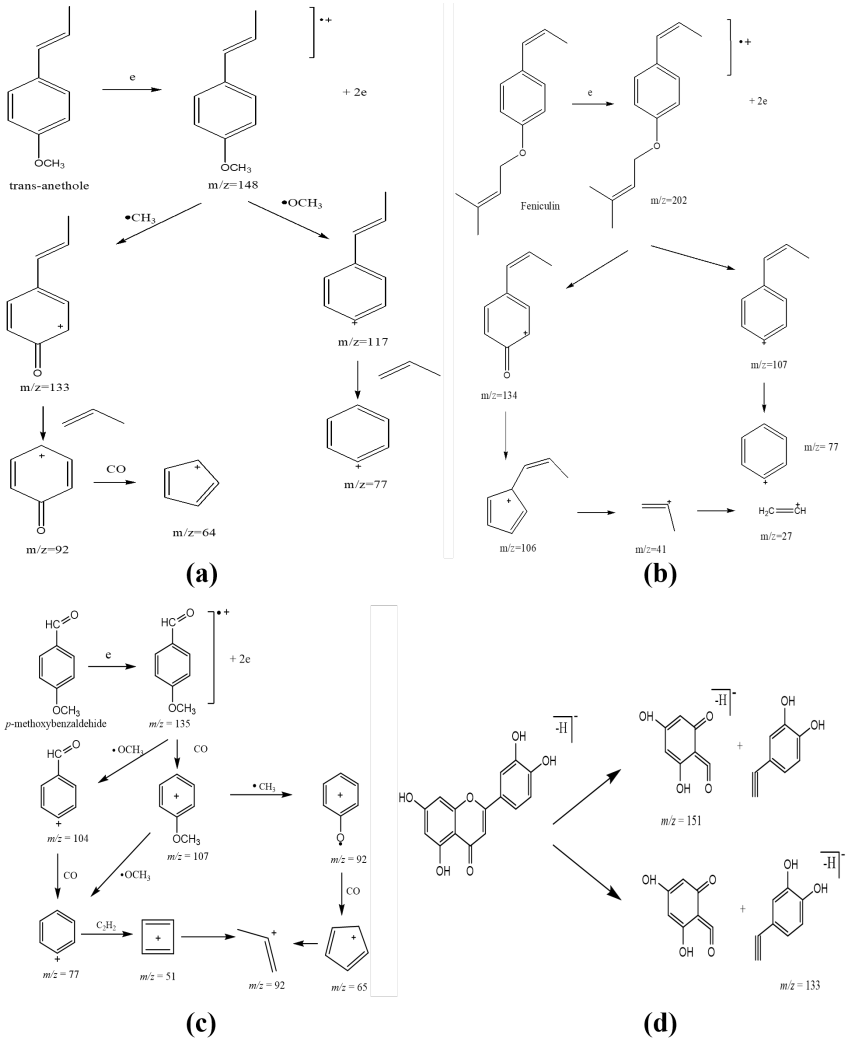

Figure 3. Mechanism of Fragmentation Mind Compound in Star Anise Fraction (a) Trans-Anethole (b) Feniculin (c) p-Methoxy Benzaldehyde (d) Luteolin

Extracts polar have much higher phenolic content than others. For example, extract ethanol and extract methanol, where these solvents can dissolve phenolic compounds derived from secondary plant metabolites. The results based on Table 3 were the measurements of total phenolic in extract and star anise fraction showed the largest content of phenolic compounds was found in ethanol fraction 148.98 mgGAE/g. This is under the solubility of phenolic compounds, where the -OH group possessed by these compounds will make the solubility in polar solvents greater (Li et al., 2018; Babbar et al., 2014). The more hydrogen bonds formed between phenolic compounds and the solvent, the higher its solubility.

3.4 The Total Flavonoids Content

The total measurement of flavonoids using the $AlCl_3$ reagent showed in Table 4. The highest levels of flavonoids were obtained in ethyl acetate fraction with 148.97 concentration of mgQE/g. Flavonoids will dissolve in distilled water, ethanol, methanol, and ethyl acetate. Ethyl acetate has semi-polar characteristics, so it can attract flavonoid types in the form of glycones and aglycones. The levels of flavonoids with ethyl acetate solvent are higher than the levels of flavonoids with other solvents. The degree of polarity of ethyl acetate may be the same as that of flavonoids, according to Gulo et al. (2021). Extraction effectiveness is affected by the solubility level of solvent material. A compound will dissolve in a solvent with the same degree of polarity. The polarity degree of a solvent is expressed by the magnitude of the dielectric constant. The dielectric constant is expressed as the repulsive force between two electrically charged particles in a molecule. The higher the dielectric constant, the more polar solvent will be (Herrera-Pool et al., 2021).

Table 2. Phytochemical Screening Test

Test	Reagent	Ethanol Extract	n-Hexane Fraction	Ethyl Acetate Fraction	Ethanol Fraction
Alkaloids	Mayer	(+)	(-)	(+)	(-)
	Dragendroff	(+)	(-)	(-)	(+)
	Wagner	(+)	(-)	(-)	(+)
Flavonoids	Sample + HCl (p) + Mg powder + Amyl alcohol	(+)	(-)	(-)	(+)
Saponins	Sample + Aquadest + HCl 2 N	(-)	(-)	(-)	(+)
Phenolic	Sample + $FeCl_3$ 1%	(+)	(-)	(+)	(+)
Steroids	Sample + chloroform, heat + acetic acid anhydrous + H_2SO_4	(+)	(+)	(+)	(+)

Table 3. Total Phenolic

Sample	Total Phenolic (mgGAE/g)
Extract Ethanol	106.45
n-Hexane Fraction	52.31
Ethyl Acetate Fraction	93.46
Ethanol Fraction	148.97

Table 4. Total Flavonoids

Sample	Total Flavonoids (mgGAE/g)
Ethanol Extract	107.45
n-Hexane Fraction	58.94
Ethyl Acetate Fraction	148.99
Ethanol Fraction	140.01

3.5 Antioxidant Activity Assays

The DPPH radical is a stable free radical organic with a wavelength maximum of around 515–528 nm. This reagent is useful for measuring antioxidant compounds, both organic and inorganic, dissolved in liquids. In the DPPH test, antioxidants reduce DPPH radical with purple colour to be a faded yellow compound. The ability to ward off DPPH radicals depends on the tanning ability of antioxidants. It has been hidden that cysteine, glutathione, ascorbic acid, and tocopherol, can reduce the concentration of 1, 1-diphenyl-2-picrylhydrazine with their hydrogen storage ability (Rao et al., 2012).

The antioxidant effect on DPPH radical inhibition is based on the ability of extract compounds and fractions to donate their hydrogen or radical scavenging activities. The free radical scavenging properties of extract ethanol and n-hexane, ethyl acetate, and ethanol fractions are present in Table 5. A lower IC_{50} value indicates higher antioxidant activity (Yang et al., 2012). Based on the data above, it can be seen that the extract ethanol and fraction ethanol had the highest antioxidant activity, followed by ethyl acetate fraction with IC_{50} values of 0.548, 0.416, and 1.008 mg/mL, respectively. However, the hexane fraction showed the poor ability of DPPH scavenging. Antioxidant properties can be used as a reference to improve the shelf life of products such as spices. Natural antioxidants are useful to protect cells from damage caused by oxidative stress, which generally considers the cause of premature ageing, degenerative diseases, and cancer. It has been widely reported that many polyphenols contribute significantly to total antioxidant activity in fruits and vegetables (Luo et al., 2002).

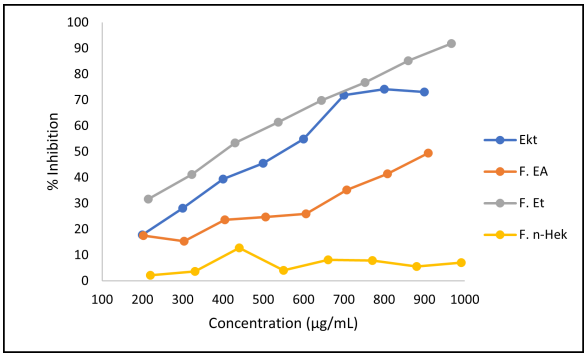

Figure 4. DPPH Radical Scavenging at Various Concentrations of Ekt (extract), F. EA (Ethyl Acetate Fraction), F. Et (Ethanol Fraction), F. n-Hek (n-Hexane Fraction)

Based on Figute 4, it can be seen that extract and star anise extract have the ability to scavenge free radicals. This result is related to the electron-donating ability of the carboxylic acid group. Both effects of conjugation and induction together determine that -COOH is a strong electron-withdrawing group,

Table 5. Antioxidant Activity of *Illicium verum*

Sample	Concentration μg/mL	% Inhibition	Equality y= bx+a	EC_{50} mg/mL
Ethanol Extract	200	17.75	y = 0.0854x + 3.161	0.548
	300	28.07	r^2 = 0.9559	
	400	39.45		
	500	45.48		
	600	54.94		
	700	71.82		
	800	74.20		
	900	73.07		
Ethyl Acetate Fraction	200	17.46	y = 0.046x + 3.5918	1.008
	300	15.41	r^2 = 0.9179	
	400	23.64		
	500	24.69		
	600	25.90		
	700	35.21		
	800	41.48		
	900	49.50		
Ethanol Fraction	200	31.70	y = 0.0797x + 16.851	0.416
	300	41.23	r^2 = 0.9928	
	400	53.43		
	500	61.40		
	600	69.84		
	700	76.82		
	800	85.13		
	900	91.81		
n-Hexane Fraction	200	2.21	y = 0.0035x + 4.3116	13.054
	300	3.72	r^2 = 0.0814	
	400	12.74		
	500	4.14		
	600	8.10		
	700	7.94		
	800	5.56		
	900	7.02		

-CH=CHCOOH is a weak electron-withdrawing group, and $-CH_2COOH$ is a weak electron-donating group. Electron-donating groups can increase the electron cloud density of the benzene ring, lower the dissociation energy of phenolic hydroxyl bonds and then increase the ability to capture free radicals (Chen et al., 2020).

4. CONCLUSION

Star anise extraction can be carried out using ultrasonic maceration, producing a relatively high yield. The trituration technique carried out the separation of compounds based on solubility in solvents with different polarities. The results of compound profiling using FTIR and GC-MS showed that three main compounds were dominant in extracts and fractions. Tests for total phenolic, flavonoid, and antioxidant activity showed a linear relationship where the higher fraction content of phenolic and flavonoid compounds, the higher antioxidant activity indicated by IC_{50} value for DPPH radicals.

5. ACKNOWLEDGMENT

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