Extraction of *trans*-Anethole from Star Anise (*Illicium verum*) Using Combination of Microwave, Ultrasonic, and Enzyme Assisted Methods and Evaluation of Their Antibacterial Activity

Elvina Dhiaul Iftitah[1,2*], Warsito Warsito[1,2], Vivi Nurhadianty[2,3], Fitri Ariadna Sodi Miranda[4], and Rafika Nur Hafidah[5]

[1]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, Brawijaya University, Jl. Veteran, Malang 65145, Indonesia*

[2]*Essential Oil's Institute, Brawijaya University (Entrepreuneur Building, 8th floor), Jl. Veteran, Malang 65145, Indonesia*

[3]*Department of Chemical Engineering, Faculty of Engineering, Brawijaya University, Jl. M.T. Haryono 167, Malang 65145, Indonesia*

[4]*Master of Chemistry Study Program, Faculty of Mathematics and Natural Sciences, Brawijaya University, Jl. Veteran, Malang 65145, Indonesia*

[5]*Undergraduate of Chemistry Study Program, Faculty of Mathematics and Natural Sciences, Brawijaya University, Jl. Veteran, Malang 65145, Indonesia*

*** Corresponding author:**

email: vin_iftitah@ub.ac.id

Received: November 22, 2023
Accepted: January 8, 2024

DOI: 10.22146/ijc.79341

Abstract: *This study examines the efficiency of non-conventional extraction methods to obtain* trans-*anethole from* Illicium verum *using single and combination extraction techniques: microwave assisted extraction (MAE), ultrasound-assisted extraction (UAE), and enzyme assisted extraction (EAE) employing lipase from* Aspergillus oryzae. *All extraction methods were conducted using 96% ethanol (1:5 w/v) with varying time durations. The resulting product was an oleoresin, subsequently analyzed and separated using thin liquid chromatography (TLC) and column chromatography employing a solvent mixture of toluene and ethyl acetate in a 9:1 ratio and identified via gas chromatography-mass spectra (GC-MS). The results revealed that trans-anethole yields from a single extraction method were 30.76% (MAE), 41.05% (UAE), and 40.90% (EAE). The combination of extraction methods, such as MAE-UAE, MAE-EAE, and UAE-EAE, produced* trans-*anethole yields of approximately 42.73%, 52.80%, and 45.02% respectively, surpassing the yields of the single extraction method. Notably, the triple extraction method of MAE-UAE-EAE yielded the highest* trans-*anethole content at 56.00%. Antibacterial testing against* Staphylococcus aureus *was performed on all samples. The* trans-*anethole demonstrating the highest inhibitory effect was obtained from the double extraction method, particularly the combination of UAE-EAE. These results underscore the synergistic efficiency of combining microwave, ultrasound, and enzymatic extraction methods, highlighting their superior efficacy in obtaining* trans-*anethole.*

Keywords: *EAE;* Illicium verum*; MAE;* trans-anethole*; UAE*

■ INTRODUCTION

Star anise (*Illicium verum*) or bunga lawang has an essential oil content of about 2.5 to 3.5% fresh and 8.0 to 9.0% dried. The most abundant component in star anise oil is *trans*-anethole, with a percentage reaching 85 to 90% [1]. *Trans*-anethole has water-soluble properties, high volatility, strong odor, and low physichochemical stability [2]. *Trans*-anethole has anti-metastatic activity, anti-oxidative, antimicrobial, antiviral, and anti-inflammatory properties. It has also experimentally

shown that *trans*-anethole is non-genotoxic and non-carcinogenic [3]. Based on the above findings, it is important to enhance the bioavailability of *trans*-anethole, by improving the extraction efficiency and enhancing the bioactivity during the treatment of the raw material.

Trans-anethole can be extracted from star anise using traditional, conventional, or non-conventional methods. Traditional method is mainly based on organic solvent extraction, such as heat reflux and Soxhlet extraction, which usually need large amounts of harmful organic solvent and they are not environmentally friendly [4]. The lab-intensive and time-consuming extraction and isolation process has been the bottleneck of the application of natural products in drug development which is caused by the loss of some active components.

There is an urgent need to develop effective and selective methods for the extraction and isolation of bioactive natural products [5-6]. Recently, some new techniques for non-conventional extraction methods were developed. Extraction with non-conventional methods is an extraction that carries green analytical chemistry, which is more environmentally friendly with the use of minimal energy and solvents compared to traditional methods [4,7]. Among these techniques, great attention was paid to microwave-assisted extraction (MAE), ultrasonication-assisted extraction (UAE), and enzyme-assisted extraction (EAE) [5-9].

MAE (Fig. 1(a)) provides rapid, safe, and cost-effective heating by using microwave energy with a frequency of 2.5 GHz. It can cause an athermal effect by breaking the hydrogen bonds between the extracted molecule and matrix of samples and can significantly retain the biological activity of the target compounds [8,10]. The UAE (Fig. 1(b)) allows greater penetration of solvent into the samples and increases the contact surface area, as well as generates expansion-compressions by ultrasonic waves with a frequency of 20 kHz, which will significantly enhance the extraction efficiency of bioactive molecules [5,11-13]. The EAE is an extraction method that uses enzymes to damage and degrade plant cell walls; thus, the active compounds are easily and quickly released in a relatively short time [9,14]. These advanced combination techniques of enzyme-microwave-ultrasonication assisted have been applied for the curcumin extraction efficiencies from turmeric. Response surface optimization (RSM) was used to select the optimum extraction condition by implementing a Box-Behnken design. The extraction ratio and antioxidant activity of extracted species are 2.89 and 83.95% under the optimal conditions, respectively, which are close to the predicted values and much higher than the single extraction approach [5].

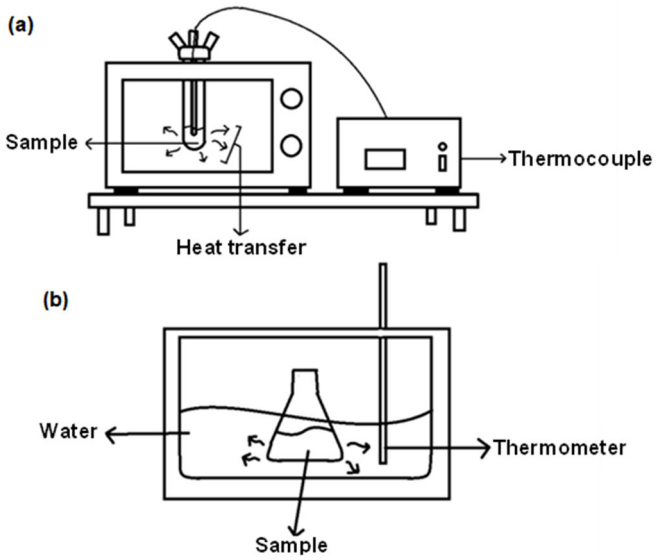


Fig 1. Non-conventional method of extraction: (a) MAE and (b) UAE

As far as we know, there are limited reports about highly efficient extraction technology for *trans*-anethole extraction from *I. verum*. The highly efficient extraction technology is of great significance for the full utilization of *trans*-anethole. Star anise essential oil has been reported to have antibacterial potential, which is effective against Gram-positive bacteria such as *S. aureus* compared to Gram-negative bacteria [15]. *S. aureus* is a bacterium that can cause food poisoning due to enterotoxin contamination, dermatitis, impetigo, respiratory tract abscess, and mastitis [15-18]. Therefore, in this study, different combination methods and extraction times on the total extraction efficiency and antibacterial activity of extracted *trans*-anethole were assessed using GC-MS and inhibitory capacity toward *S. aureus*, respectively.

■ EXPERIMENTAL SECTION

Materials

The well-mashed star anise (*I. verum*) was obtained by electric blender. Ethanol 96% (CV Dunia Kimia Lestari), lipase enzyme from *A. oryzae* which immobilized on immobead 150 with activity 1800 U/g (Sigma Aldrich), toluene (Smart-Lab), ethyl acetate (Supelco Merck), alumina (Merck, Millipore), TLC silica gel 60 F_{254} plate (Merck), commercial silica sand, nutrient agar (Merck), rifampicin, sterile physiological of 0.9% NaCl, and *S. aureus* bacteria (Bacterial cultures from Microbiology Laboratory of UNS) were used in this work.

Instrumentation

A set of modified microwave oven (Electrolux microwave oven with thunder IC thermocouple regulated DC power supply), ultrasonic cleaner Clan and JT CL5120-1, Heidolph hot plate stirrer, a set of digital IKA HB 10 rotary evaporators, GC-MS Shimadzu QP2010 Ultra, autoclave, biosafety cabinet, destruction stove, incubator, Vernier calipers, and UV lamp were used in this work.

Procedure

Single extraction method of MAE, UAE, and EAE

An amount of 20 g of grounded star anise was mixed well with 100 mL ethanol solvent (1:5 w/v). These mixtures were prepared for each single extraction method. MAE proceeded by putting the mixtures into the modified microwave oven and treated at 40 °C under the power of 800 W (high) for different times (15 and 30 min). UAE proceeded by putting the mixtures into the sonicator batch and ultrasonicated them at 40 °C for different times (30 and 45 min). EAE was proceeded by adding 2 g of immobilized enzyme of lipase from *A. oryzae* into the mixture (1:50 w/v) and treated for different times (1 and 2 h) at 40–50 °C under pH 7. Then, each extracted oil was evaporated to remove the ethanol solvent until the oleoresin products were obtained in a dark brown color. Specifically, for extracted oil from EAE method, the immobilized enzyme was filtered first before evaporating the ethanol solvent. Next, we provided sample codes for each oleoresin product at a specific time on MAE, UAE and EAE as the A1-A2, B1-B2, and C1-C2, respectively.

Combination extraction method (double and triple extraction) of MAE, UAE and EAE

The double and triple extraction methods were carried out in the order of MAE-UAE, MAE-EAE, UAE-EAE, and MAE-UAE-EAE, respectively. The general procedure for the combination method was developed based on the best condition of the highest concentration of *trans*-anethole among A1-A2, B1-B2, and C1-C2, which was obtained from GC-MS analysis. Double extraction of MAE-UAE was proceeded by putting the mixture of 20 g grounded star anise and 100 mL ethanol into the microwave oven. Then, after being treated by microwave irradiation, the mixture was ultrasonicated into a sonicator batch. Double extraction of MAE-EAE was proceeded by putting the mixture of 20 g grounded star anise and 100 mL ethanol into the microwave oven. Then, after being treated by microwave irradiation, 2 g of immobilized enzyme of lipase from *A. oryzae* was added to this solution. The double extraction of UAE-EAE was proceeded by putting the mixture of 20 g grounded star anise and 100 mL ethanol into the sonicator batch. Then, after ultrasonicated, 2 g of immobilized enzyme of lipase from *A. oryzae* was added into this solution. Triple extraction of MAE-UAE-EAE was proceeded by putting the mixture of 20 g grounded star anise and 100 mL ethanol into the microwave oven. Then, after being treated by microwave irradiation, the mixture was ultrasonicated, and finally 2 g of immobilized enzyme of lipase from *A. oryzae* was added to this solution. Meanwhile, we also provided sample codes for each oleoresin product from double and triple extraction of MAE-UAE, MAE-EAE, UAE-EAE, and MAE-UAE-EAE as the D, E, F, and G, respectively.

Analysis and purification of oleoresin products

Each of the obtained oleoresin products, A1-A2, B1-B2, C1-C2, D, E, F, and G, were analyzed and purified by using TLC and column chromatography. The solvent mixtures of toluene:ethyl acetate (9:1) were chosen as an eluent solvent. Meanwhile, the *trans*-anethole standard solution was also prepared using ethanol as the solvent and analyzed using TLC with a similar eluent solvent mixture. Then, fractions or eluate from column chromatography, which have the same

spot of *trans*-anethole standard under TLC analysis, were combined, evaporated, and analyzed further using GC-MS.

Antibacterial activity assay of trans-*anethole extract*

All *trans*-anethole extracted from samples with codes A1-A2, B1-B2, C1-C2, D, E, F, and G were assessed for antibacterial activity assay. The agar well diffusion method was used for the antimicrobial activity assay. Firstly, bacterial culture for *S. aureus* (ATCC 25923) was prepared by dissolving 11.23 g of nutrient agar (NA) in 400 mL distilled water and added to a petri dish then cooling until solid. Next, NA media, sterile physiological NaCl solution, and all the equipment were sterilized to avoid contamination using an autoclave at 121 °C for 2 h. Meanwhile, the bacterial inoculum was carried out by mixing 100 µL of rejuvenated bacteria of *S. aureus* into 5 mL NaCl and homogenized. In the next step, the agar surface plate is inoculated by spreading 100 µL of bacterial inoculum over the entire agar surface and making the wells in the agar around 6–8 mm in diameter. The wells are punctured with a sterile cork borer or with the backside of a sterile blue micropipette tip. The petri dish was divided into three parts (positive control, negative control, and extracted sample). The positive control hole was made by adding 3 mg of rifampin in 100 mL of distilled water (30 ppm). The sample hole was added with paper discs that had been soaked in the sample. Petri dishes were covered and wrapped with plastic wrap. Then, the petri dish was put in an incubator for 24 h at 37 °C. After the incubation period, the diameter of the inhibition zone of bacteria was calculated by visualizing the blank space around the paper disc to evaluate the antibacterial performance of the A1-A2, B1-B2, C1-C2, D, E, F, and G samples.

■ RESULTS AND DISCUSSION

Single Extraction Method of MAE, UAE, and EAE

The solvent used to extract is 96% ethanol, which is a polar compound because it can avoid enzyme deposition, maintain the pH at a constant value and eliminate the influence of pH on the extraction process [9]. Essential oil's compound in star anise is also easily extracted using ethanol, which can adjust its antimicrobial activity [1].

The obtained oleoresin was observed to determine whether it had specific characteristics like the odor of star anise, dark brown in color, and thick in texture. Oleoresin is an extract consisting of volatile (essential oil) and non-volatile (resin and gum) components [2]. Then, the obtained oleoresin was purified by column chromatography using toluene:ethyl acetate (9:1) as eluent. The type and composition of eluent were chosen based on their capability to give the best resolution when separated by the TLC system. Each fraction from column chromatography was then analyzed again with TLC, and finally, analysis was carried out using GC-MS to determine the percentage of *trans*-anethole. The results of oleoresin and *trans*-anethole concentrations in a single method are presented in Table 1.

Based on the result of a single method, it has shown that all samples showed the presence of a *trans*-anethole target compound. Increasing the treating time, the extraction process was also enhanced (MAE and UAE), but further increasing the treating time will result in a decrease in the extracted *trans*-anethole (EAE). Increasing the treating time gives higher penetration of the solvent and a higher local temperature of treated samples, which may increase the molecule's diffusion rate in the system. Specific in EAE, increasing the time of

Table 1. Oleoresin (%yield) and *trans*-anethole (%) obtained by a single extraction method

Extraction type	Time (min)	Sample code	Oleoresin (g)	%Yield	*trans*-anethole (%)
MAE	15	A1	1.14	5.70	13.98
	30	A2	1.24	6.20	30.76
UAE	30	B1	1.24	6.20	18.10
	45	B2	1.24	6.20	41.05
EAE	60	C1	1.04	5.20	40.90
	120	C2	1.00	5.00	31.47

treatment could not increase the *trans*-anethole because there are more compounds that have been extracted as by-products and may even induce the denaturation or oxidation of the extracted samples.

In the MAE process, waves or bubbles are formed during extraction due to the emission of microwaves as a temperature control. This causes the compound to be easily extracted from the matrix due to the swelling process. The addition of time allows an increase in the possibility of penetration of the sample into the solvent but also allows the denaturation of the compound to be extracted due to the local temperature in the system. In the UAE process, ultrasonic waves (acoustic cavitation) which are formed due to changes in mechanical energy, increase the mechanical effect that can facilitate the penetration and mass transfer process. This acoustic effect also causes conduction due to the energy distribution process in the extract media. This is an evident from the longer the extraction time, the higher the *trans*-anethole percentage. In the EAE process, the enzymes used can catalyze the hydrolysis of 1,4-glycosidic bonds involved in the degradation of cellulose [19]. The destruction of cellulose as the main constituent of polysaccharide cell walls causes *trans*-anethole compounds to be easily extracted (Fig. 2).

The single method produces compounds that always appear in addition to *trans*-anethole, namely ethylbenzene, 1,2-dimethylbenzene, and benzaldehyde. These compounds are the result of oxidation of *trans*-anethole, namely the aldehyde group and minor alcohol compounds. Based on the exposure, the method conditions that produced the highest *trans*-anethole were used for the simultaneous extraction method.

Combination Extraction Method (Double and Triple Extraction) of MAE, UAE and EAE

The order of combination extraction for double and triple extraction is carried out based on the method of highest energy first then continued with the other. This was done because *trans*-anethole has low physicochemical stability [2]. This minimizes the occurrence of breaking the bonds of the target compound into other compounds due to exposure to higher energy sources. The double extraction methods were labelled as MAE-UAE, MAE-EAE, and UAE-EAE and triple extraction method was labeled as MAE-UAE-EAE. The results of oleoresin and *trans*-anethole compounds are presented in Table 2.

The results of the analysis showed that all samples contained *trans*-anethole with a higher value than the concentration of *trans*-anethole in each of the single method. On the other hand, the average amount of oleoresin and %yield was lower than the previous method. Significantly, the G sample obtained from the triple extraction method has the highest concentration of

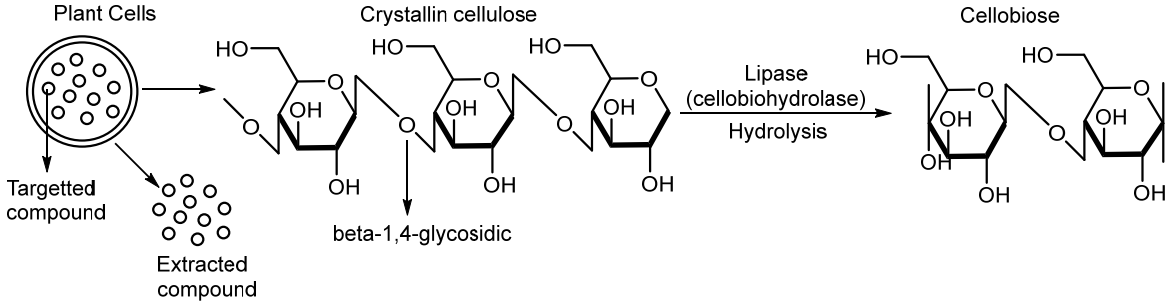


Fig 2. Scheme of cellulose destruction by lipase (cellobiohydrolase) [19]

Table 2. Oleoresin and *trans*-anethole obtained by double and triple extraction methods

Extraction type	Sample code	Oleoresin (g)	%Yield	*trans*-anethole (%)
MAE-UAE	D	0.83	4.15	42.73
MAE-EAE	E	0.80	4.00	49.19
UAE-EAE	F	1.36	6.80	45.02
MAE-UAE-EAE	G	1.44	7.20	56.54

Table 3. Compounds obtained from each extraction method were analyzed by GC-MS

Compounds	Extraction method			
	MAE-UAE (%)	MAE-EAE (%)	UAE-EAE (%)	MAE-UAE-EAE (%)
Ethylbenzene	14.93	18.60	22.04	17.07
1,2-Dimethylbenzene	17.60	21.75	25.92	19.98
2-Butoxyethanol	9.58	4.50	0.27	3.53
Benzaldehyde	2.80	3.60	4.82	3.34
1-Methoxy-4-(2-propenyl)benzene	0.67	-	-	-
4-Methoxy-benzaldehyde	1.73	0.68	4.15	0.78
Trans-anethole	42.73	49.19	45.02	56.54

trans-anethole compared to other combination methods. It assumed that the triple extraction method was quite effective in extracting the *trans*-anethole compound. In addition, there were a few compounds that existed as by-products that formed during the extraction process. We predicted that these compounds are possible intermediates in the *trans*-anethole biodegradation pathway which is produced through oxidation followed by demethylation and hydroxylation reaction and gives the products like aldehydes and alcohol compounds. Table 3 shows in detail some of the compounds obtained from the combination extracted method (double and triple extraction), which were analyzed by GC-MS.

Analysis of the Existence of *trans*-Anethole Compounds for Each Extraction Method

The existence of *trans*-anethole compounds obtained for each of extraction method is presented in the following graph (Fig. 3). Based on Fig. 3, combination of double and triple methods of extraction even the period of treatment will increase the amount of *trans*-anethole. For instance, when we focused on MAE, there was a significant difference of the *trans*-anethole amount within the method which involving the MAE (sample: A1, A2, D, E, and G). This is related to the optimization of *trans*-anethole compounds that have not had enough time to extract in the initial extraction method. Fig. 4 shows the graphic of the increasing amount (in percentage of concentration) of *trans*-anethol for MAE and UAE methods. The comparison included single, double, and triple extraction with a focused-on MAE and UAE methods.

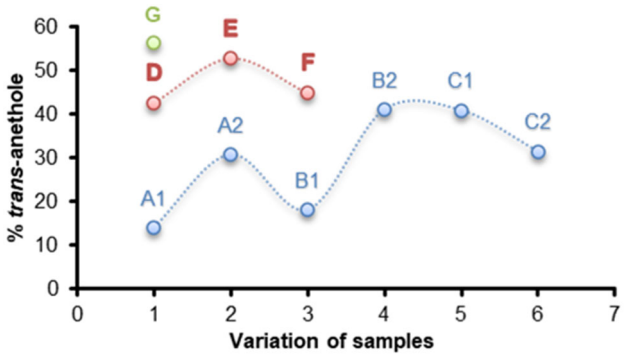


Fig 3. Correlations the type of extraction method *trans*-anethole compounds (%)

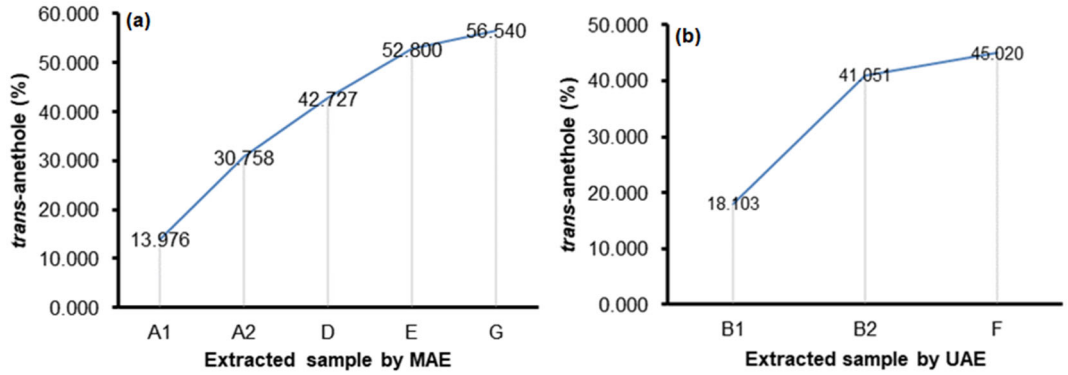


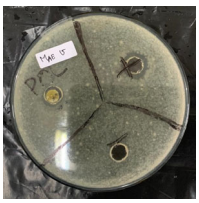
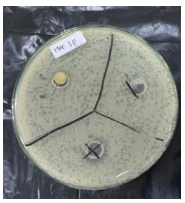
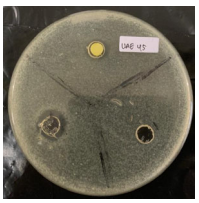
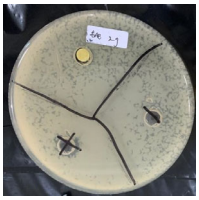
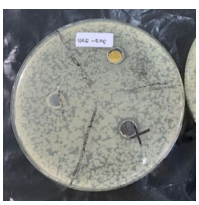
Fig 4. Comparison amount of *trans*-anethole compound extracted by (a) MAE and (b) UAE

Some of by-product compounds that formed during the treatment were obtained because of the oxidation process of *trans*-anethole compounds. Subsequently, it can support the benefits of star anise's essential oil. For instance, 4-methoxy-benzaldehyde compounds contribute to antimicrobial activities and other phenolic compounds to antioxidant activities [16]. *Trans*-anethole from star anise has also been reported to it can give extra benefit when it is synergic with its derivatives such as *p*-anisic acid, *p*-anisyl alcohol and ethyl *p*-methoxycinnamate [19]. Other compounds that can exist as *trans*-anethole derivatives are linalool and terpineol which can be obtained during purification.

Antibacterial Activity Assay of *trans*-Anethole Extract

The bacteria used are *S. aureus* bacteria. Observations made for 24 h carried out in duplicate showed various inhibition zones seen in the purified *trans*-anethole. The diameter of the inhibition zone is presented in Table 4. The highest inhibition zone was about 12.96 mm and the lowest was about 7.55 mm. Based on the observation of inhibition, the sample that has the highest inhibition is the F sample. The diameter of the inhibition is used as a measure of bacterial activity where the diameter of the inhibition zone is directly proportional to the antibacterial activity [5]. This happens because the compound formed is a *trans*-anethole derivative compound that acts as an antibacterial. All components categorized as essential oils can act as antibacterial [20]. In addition, the antibacterial activity of F sample (UAE-EAE) had lower radiation energy exposure compared to D, E, and G samples, but it could be increased from single-method extraction. When compared with the Soxhlet method which was carried out for 2 h, the antibacterial activity of the purified results did not show significant results compared to the F sample. This is caused by direct heating at the time of extraction. In other methods, extraction

Table 4. Inhibition diameter zones for *trans*-anethole from different extraction methods against *S. aureus* after incubation of the plate

Inhibition Zone (mm)			
A1	A2	B1	B2
			
10.50	8.80	7.71	10.91
C1	C2	D	E
			
10.24	8.67	7.98	12.06
	F	G	
			
	12.96	11.08	

temperature, evaporation, and radiation exposure can cause antibacterial components to lose their stability (thermolabile) due to degraded components so that antimicrobial activity is reduced [7].

S. aureus bacteria are Gram-positive bacteria that do not have a cell membrane but are composed of thick peptidoglycan, so antimicrobial compounds are more difficult to penetrate [9]. Compounds in the purified oleoresin extract of star anise could attack polypeptides and cause osmotic pressure so that bacterial cells are lysis. The use of ethanol as a solvent also supports the effectiveness of inhibiting bacterial growth because it is a polar compound.

CONCLUSION

In this study, MAE, UAE and EAE were used for *trans*-anethole extracting from oleoresin of *I. verum* oil and improving the antibacterial activity against *S. aureus*. The effect of a single and different combination of double and triple methods and time consuming on the *trans*-anethole extraction were investigated. Extraction was run effectively for 30, 45, and 60 min for MAE, UAE, and EAE, respectively. Our results demonstrated that under double and triple combination extraction methods, MAE-UAE, MAE-EAE, UAE-EAE, and MAE-UAE-EAE, the maximum extraction efficiency of *trans*-anethole is 42.73, 49.19, 45.02, and 56.54% respectively. Antibacterial activity tests were conducted against *S. aureus* bacteria. For UAE-EAE, with the highest inhibition diameter zone of 12.96 mm. This study provided a new technology through the combination extraction method, which could improve the extraction efficiency of *trans*-anethole from oleoresin of oil.

ACKNOWLEDGMENTS

In sincere appreciation, we would like to convey our thanks to Universitas Brawijaya for the research funding provided through the Doctoral Research Grant program, Senior Lecturer, at the Faculty of Mathematics and Natural Sciences, for the fiscal year 2022, under contract number: 3110.9/UN10.F09/PN/2022.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

AUTHOR CONTRIBUTIONS

Elvina Dhiaul Iftitah, the head of the research team, coordinated all research activities, from proposing the proposal, conducting the research, and the publication process, both in the form of journal articles and intellectual property rights (IPR). Warsito, assisted in the selection of raw materials and the selection of the best trans anethole products from the extraction methods of single and simultaneous MAE, UAE, and EAE. Vivi Nurhadianty, assisted and guided students from the preparation of bacteria, creation of starters, to the conventional biotransformation process (fermentation). Fitri Ariadna Sodi Miranda, conducted conventional extraction (Soxhlet extraction) of *trans*-anethole compounds from clove flowers and compared the results with single methods of MAE, UAE, and EAE. Rafika Nur Rofidah, conducted extraction processes using simultaneous methods of MAE-UAE, MAE-EAE, and UAE-EAE, as well as the simultaneous method of MAE-UAE-EAE.

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