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Identification of Compounds from the *Illicium* Extract

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ABSTRACT

This study is to highlight the various uses/benefits of star anise (Illicium species). It is regarded as the sole genus from the Illiciaceae, which originated from the Schisandraceae family. This plant is known as "bunga lawang" among the Malays. It has long been used in traditional Chinese medicine. The medicinal properties of the star anise include it being antimicrobial and antiviral. Previous publication mentioned on the anti-cancer attributes of Illicium's essential oils against colon cancer. The star anise also can be used as an antiviral agent that led to the existence of drug, Tamiflu®. The objectives of this research are to review the Illicium genus and to report the separation of the compounds from the star anise seed. Standard analytical chemistry methods and high-field nuclear magnetic resonance spectroscopic techniques were utilized in order to identify the secondary metabolites. From the literatures, the isolation of natural biomolecules from Illicium species could be achieved by using simultaneous distillation-extraction and solid phase microextraction. Furthermore, the determination of volatile compounds could be accomplished via gas chromatography-mass spectrometry. Shikimic acid is also the target compound to be extracted, due to its role in the antiviral synthesis. The non-volatile component could be obtained from liquid chromatography. Finally, it is found that mixtures of trans-anethole, anisaldehyde and a triglyceride could be detected from the seed extract.

Keywords: *chromatography; extraction; Illicium; spectroscopy; star anise*

1. INTRODUCTION

The star anise is a characteristic spice, obtained from the fruits of *Illicium verum* Hook. f. (Illiciaceae) [1]. It is originally distributed in tropical areas of Asia. The star anise is a well-known spice in Nyonya cuisine, among the Chinese Peranakan and the Malays [2]. It is also widely used in household, food and pharmaceutical industry [3]. It contains 5 - 8 % of volatile essential oil and produces star-shaped and reddish-brown fruits, consisting of six to eight follicles arranged in a whorl [1], as shown in Figure 1. Among the Malays, the star anise is named as "bunga lawang" or "bunga bintang" or "bunga pecah lapan" [4], which is derived from its eight-pointed star shaped flower. This herb is embedded in the traditional Malay culture e.g. in designing or weaving special fabric motifs or pattern for songket and its commercialized products, such as "samping" or short sarong [4]. The formulation of star anise in the tea form is available in the international market, however, local tea manufacturers have not yet produced such tea mixtures. It is hoped that an updated review of the *Illicium* genus could be presented

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here. Meanwhile, the experimental part of this research would report the separation of the compounds from the seed.

Figure 1: A self-taken photograph of the star-shaped fruits of *I. verum*, showing their seeds.

1.1. The pharmacological activities of Illicium species

A review on the botany, traditional use, chemistry and pharmacology of *Illicium verum* was published [5]. The potential of *I. verum* as an environment-friendly insect repellent was explored [6]. The star anise was also shown to possess potent antimicrobial properties. Previous chemical studies indicated that this antimicrobial property is due to the presence of anethole in the dried fruit [7]. Both methanol and ethanol extracts of *I. verum* had antibacterial activity [3]. The extractives of *I. verum* was reported to contain rich immunogenetic function components, which had a huge potential in biomedicine [8]. The star anise is generally considered as harmless. Nevertheless, it can sometimes cause a severe intoxication resulting in various neurological and gastrointestinal symptoms in infants [9]. It was stated that the poisoning by star anise herbal product for the infant was probably related to the contamination with *I. anisatum*, and not due to the continuous intake of *I. verum* [10]. *I. anisatum* is another *Illicium* plant similar to the star anise, but is regarded as a highly toxic species.

1.2. The phytochemistry of Illicium species

Shikimic acid (Figure 2) or (3*R*, 4*S*, 5*R*)-3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid ($C_7H_{10}O_5$, MW 174.15 CAS RN 138-59-0), can be isolated from the star anise (*I. verum* Hook. F.) as colourless crystals, mp 185-187 °C, (c = 0.0104 g/mL, water). It is commercially available and serves as a precursor for the synthesis of the medicinal drug Tamiflu®, used in the treatment of both influenza viruses A and B [11].

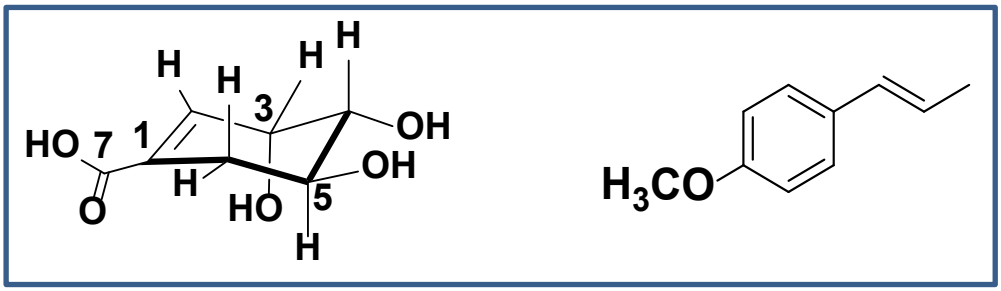

Figure 2: The chemical structure of shikimic acid (left) and *trans*-anethole or *para*-propenylanisole or (E)-1-methoxy-4-(1-propenyl)benzene (right).

Payn and Edmonds [12] outlined the extraction of shikimic acid from star anise, to expose it to the undergraduates in the organic and natural product courses. The authors used Soxhlet extraction, followed by ion exchange chromatography to isolate crude shikimic acid.

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Subsequent charcoal decolorization and recrystallization from toluene and methanol, would afford the pure shikimic acid in 2–7% w/w yield. It was later reported that rapid separation (approximately 5 minutes) of shikimic acid from Chinese star anise, could be accomplished with hot water extraction at the temperature of 120 °C or higher [13]. The determination of this acid could also be achieved via hyphenated techniques such as Liquid Chromatography–Ultra Violet (LC–UV) and Liquid Chromatography–Electron Spray Ionization–Mass Spectrometry (LC–ESI–MS) [14].

A flash column chromatography pre-packed with molecularly-imprinted polymers, was demonstrated as another rapid, feasible and economical method for the extraction of Chinese star anise on a large scale, when compared with HPLC [15]. More advanced extraction protocols such as ultrasound-assisted and microwave-assisted extractions are also considered as two promising technologies for shikimic acid extraction [16]. Later, a newer method for rapid, simple, inexpensive and efficient extraction plus isolation of shikimic acid from star anise was published [17]. The technique involved pressurized hot water extraction (PHWE) of multigram quantities of shikimic acid using an unmodified household espresso machine.

Previous chemical studies indicated that the antimicrobial property of star anise is due to the presence of *trans*-anethole (Figure 2) in the dried fruit [7]. The content of *trans*-anethole was the highest, followed by linalool. Meanwhile, limonene and 4'-methoxypropiophenone were the lowest amount. The aromatic profile of the star anise and the structure-odour relationship of anethole were also investigated [18]. Along *trans*-anethole, anisaldehyde, estragole and farnesol are the aromatic compounds harvested by Simultaneous Distillation-Extraction (SDE) technique [1]. A study on the Soxhlet's extraction of star anise oil and the preliminary investigation of its antibacterial activity were written [19]. Borah [20] summarized various techniques, including the extractions, synthetic methodologies and microbial production, which were employed in order to obtain the shikimic acid.

2. EXPERIMENTAL

2.1. General

The dried sample of star anise was obtained from the retails. The seeds were taken out from the seed pods and placed in the different vial. The chemicals which were analytically graded (from Merck) included toluene as the extraction solvent. Ethyl acetate and toluene were utilized as mobile phases. Meanwhile, anisaldehyde was used as spray reagent in developing spots in thin layer chromatography (TLC). The analytical TLC plate (silica gel 60 F_{254}, alumina coated) was used to detect the presence of compounds in the extracts. Meanwhile, the preparative TLC glass plate was coated with silica gel 60 F_{254} (20 x 20 cm).

2.2. The extraction and purification of the crude herbal drug

The seed and seed pods were crushed by using pestle and mortar into smaller particles, before they were extracted with toluene. The extractives were left for a week, without agitation. They were monitored by using TLC (toluene and ethyl acetate as the mobile phase). The changes of the mobile system were tested until a good separation on the TLC plate was obtained. In the analytical chromatography, the seed and seed pods extracts were drawn up into a capillary tube and were spotted on a dry TLC plate. High concentration of samples was avoided to prevent the formation of tailing and streaking. The different capillary tube was used to withdraw each

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sample to avoid cross contamination of extract with another. The TLC plate was put in the TLC chamber in the stand position for development of the spots of the compounds. Next, the plate was removed from the chamber and the solvent was allowed to evaporate at room temperature or by using a Bosch heat gun. The positions of the spots were observed by placing the plate under short and long wavelength of ultraviolet (UV) light lamp. The plate was sprayed with anisaldehyde to identify the spots that did not appear under UV lamp. The compound that was isolated by using TLC was later purified by using preparative TLC. The plate was covered with aluminium foil in the middle to avoid the contamination during the spraying of anisaldehyde at the right and left sides of the plate. The bands that appeared after spraying of anisaldehyde were scrapped off and collected in the small beakers. In order to achieve high purification, the only part that was not contacted with anisaldehyde, was scrapped off. The collected silica in the beaker then was washed thoroughly with chloroform before filtration, to remove the silica gel. After scrapping and filteration, the compounds were kept in the test tube.

2.3. The identification of the compounds

Bruker 500 MHz Ultrashield^{TM} Nuclear Magnetic Resonance (NMR) spectrometer was utilized to record the ^{1}H NMR spectra for the sample at room temperature, in deuterated solvent ($CDCl_3$). The spectra were presented in parts per million (ppm) downfield from tetramethylsilane (internal standard) e.g. in Figure 3. NMR data were given as multiplicity (d= doublet, dd = doublet of doublets, m = multiplet, s = singlet, t = triplet, e.g. in Table 1) and number of protons. The spectra were analyzed, to elucidate the structure of the compounds.

3. RESULTS AND DISCUSSION

It was found that the best solvent system for TLC development comprised toluene 93% and ethyl acetate 7%, for both seed and seed pods extracts. 9.3 ml of toluene and 0.7 ml of ethyl acetate were prepared and poured into analytical TLC chamber as a mobile phase. The TLC plate showed only one spot for both seed and seed pod of star anise extract, when observed under short wave UV light. A number of spots were revealed from the seed extract, after spraying with anisaldehyde, when compared to the pod extract, as only one spot was observed.

The preparative TLC was conducted for purification of compounds. The toluene extract of star anise seeds was streaked on the plate. The plate was developed in a big TLC tank with the mobile phase of 93% toluene and 7% ethyl acetate. About 46.5 ml of toluene and 3.5 ml of ethyl acetate were mixed in the tank and closed with aluminium foil. Spots were observed under short wave, but there was no spot under the long wave of UV light. After the anisaldehyde spraying and plate heating, 4 bands appeared and each band was scrapped out carefully. Then, they were washed and filtered thoroughly with chloroform. All four bands were subjected to NMR analysis but only Band 1 consisted of pure compound, as compared to the other bands. Pure compounds from Band 1 were analyzed by using ^{1}H-NMR (500 MHz, $CDCl_3$). The result from the NMR analysis was tabulated in Table 1.

Based on ^{1}H-NMR spectral analysis, three compounds were detected. The peak that appeared in the region of δ_H 9.92 ppm (singlet, 1 H), (Figure 3, Table 1) could indicate the presence of an aldehyde. Furthermore, two peaks were observed in the region of δ_H 7.03 and 7.87 ppm (doublets, J =10 Hz, 4 H), which correspond to the *ortho*-coupled aromatic protons, as in 4-methoxybenzaldehyde. The methoxy group was detected when two singlets were seen at two chemical shifts of δ_H 3.92 and δ_H 3.82 ppm (each singlet was equivalent to 3 H). Both

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peaks might appear from two different compounds. Other than these signals, the ^{1}H-NMR spectrum showed two sets of two chemically equivalent aromatic protons in the region of δ_H 6.85 and δ_H 7.26 ppm (doublets, J = 8 Hz, 2 H each). These resonances could indicate the presence of *ortho*-coupling aromatic protons. The two protons, H-2 and H-6, were close to the inductive electron withdrawing effect of the oxygen atom, possibly from the methoxy group. They were assigned to the signals at δ_H 7.26 ppm (2H, d, J = 8, H-2 & H-6, overlapping). Meanwhile, the other two aromatic protons were assigned to the signal at δ_H 6.85 ppm (2H, d, J = 8, H-3 & H-5). In addition, the peaks in the region of δ_H 6.35 and δ_H 6.12 ppm respectively could be assignable to a benzylic proton and a *trans*-coupled olefinic proton. Both sp^2 protons of the propenyl moiety resonated at δ_H 6.35 ppm (1H, doublet, J = 15, H-1') and δ_H 6.12 ppm (1H, multiplet, H-2'). Therefore, these observations could prove the presence of *trans*-anethole in the seed of star anise (Figure 2) [21]. Moreover, the multiplet in the chemical shift of δ_H 5.37 ppm could indicate the fatty acid moiety. Meanwhile, the spectrum showed the presence of a glyceride backbone in the regions of δ_H 5.29, 4.31 and 4.17 ppm. At the same moment, a triplet was seen at δ_H 2.79 ppm, which could indicate the linoleic acid component. The rest of the proton regions at δ_H 2.33, 2.06, 1.63 and 0.93 ppm, were ^{1}H-NMR peaks dedicated for fatty acid component. Lastly, the signal in the region δ_H 0.87 showed the presence of the terminal methyl group.

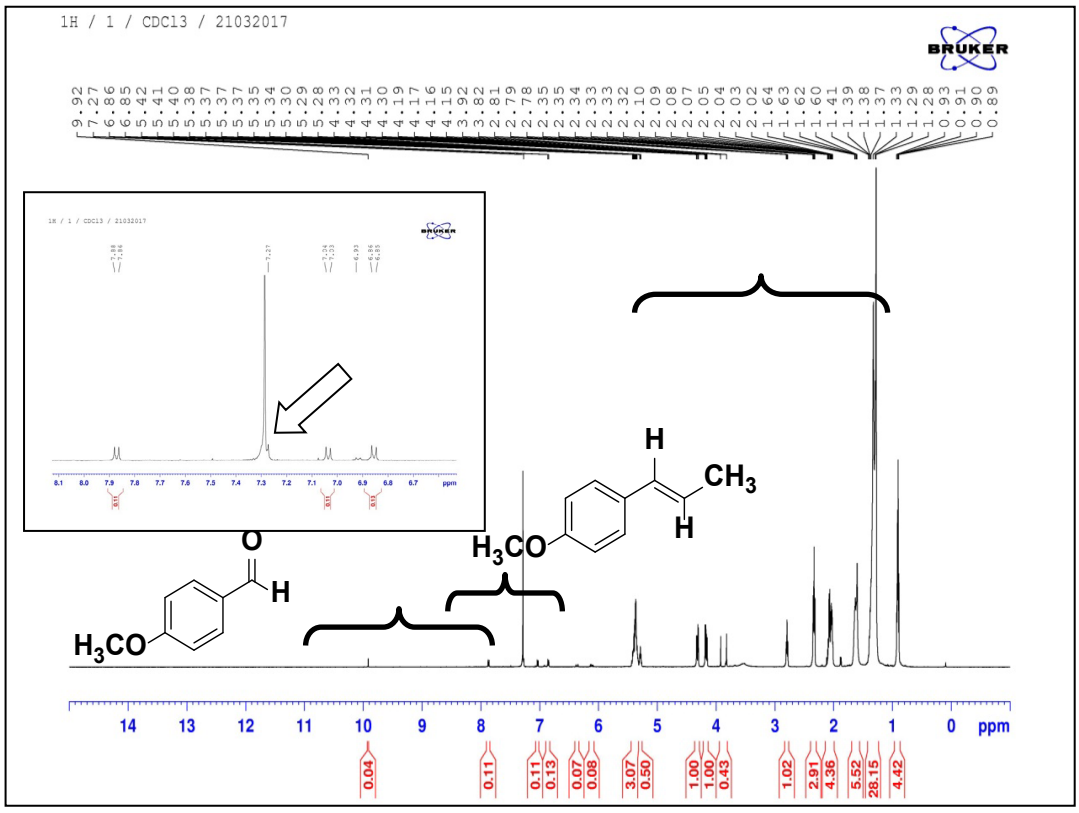

Figure 3: The ^{1}H-NMR (500 MHz) of Band 1 from the star anise's seed extract. The spectrum enlargement (δ_H 6.85 – 7.87 ppm) showed anethole's overlapping doublet near the solvent peak.

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It is noted that the presence of both *cis* and *trans* isomers of anethole in the extract of star anise's seed was previously proven. However, in this study, only trans-anethole was found from the extract (Figure 2, Figure 4). This might be due to the method of detection. The gas chromatography was reported as the most suitable technique to identify both major and minor compounds in star anise oil. *Trans*-anethole was found nearly as 94%, while *cis*-anethole was detected only by 0.14% [22].

Table 1. The ^{1}H-NMR data for Band 1 of the seed extract.

Multiplicity (J, Hz)	δ_H (ppm)	Integration	Interpretations
s	9.92	1 H	Aldehyde group in anisaldehyde
d, J =10 Hz	7.87	2 H	Aromatic protons, *ortho*-coupling in anisaldehyde
d, J =10 Hz	7.03	2 H	Aromatic protons, *ortho*-coupling in anisaldehyde
s	3.92	3 H	Methoxy group in anisaldehyde
d, J = 8 Hz	7.26	2 H	Aromatic protons, *ortho*-coupling in anethole
d, J = 8 Hz	6.85	2 H	Aromatic protons, *ortho*-coupling in anethole
d, J = 15 Hz	6.35	1 H	Benzylic proton in anethole, the *trans*-coupling
m	6.12	1 H	Olefinic proton in anethole, the *trans*-coupling
s	3.82	3 H	Methoxy group in anethole
m	5.37	6 H	Lipid component, 3 chains of fatty acid
m, J = 4.5	5.29	1 H	Glyceride backbone, -OC**H**$(CH_2)_2$
dd, J = 12, 4 Hz	4.31	2 H	Glyceride backbone, -OCH$(C$**H**$_2)_2$
dd, J = 9, 6 Hz	4.17	2 H	Glyceride backbone, -OCH$(C$**H**$_2)_2$
t, J = 6.5 Hz	2.79	2 H	Linoleic acid component, =CH-C**H**$_2$-CH=
t, J = 8 Hz	2.33	6 H	Fatty acid component
m	2.06	8 H	Fatty acid component
s	1.63	56 H	Fatty acid component
s	0.93	2 H	Fatty acid component
t, J = 6.5 Hz	0.87	9 H	Terminal methyl for lipid component

d= doublet, dd = doublet of doublets, m = multiplet, s = singlet, t = triplet

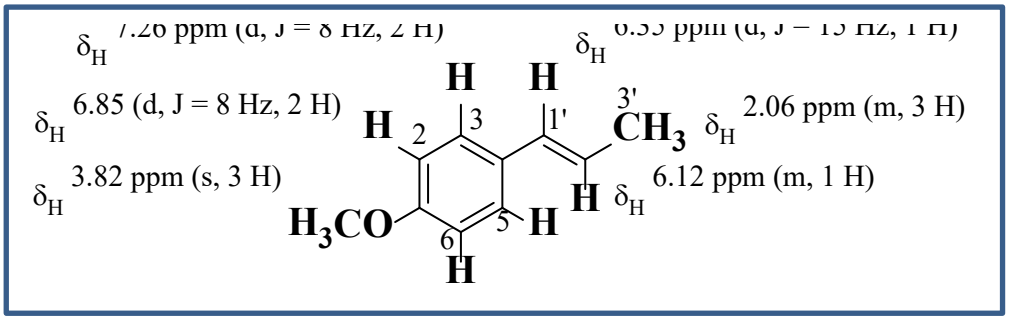

Figure 4: The ^{1}H-NMR chemical shifts of the protons in *trans*-anethole.

In this study, anisaldehyde (Figure 5) was detected in the extract. It is believed that the compound is naturally a minor constituent of the star anise [23]. The presence of this molecule is not due to the utilization of the similar chemical as the staining agent. Nevertheless, it is recommended that future research would acquire another spraying reagent such as vanillin [24].

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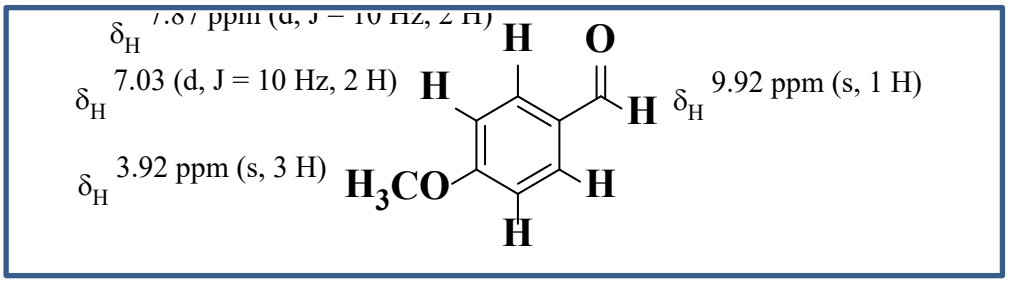

Figure 5: The ^{1}H-NMR chemical shifts of the protons in *para*-anisaldehyde.

There were possibly 3 olefinic moieties (-CH=CH-) of the lipid component of the star anise. The chemical shift of these olefinic protons were seen at δ_H 5.37 ppm (m, 6 H, 3 x HC=CH). Two HC=CH could originate from two linoleic acids and one HC=CH from oleic or petroselinic acid component. The glyceride backbone was observed as δ_H 5.29 ppm [m, J = 4.5 Hz, 1 H, -OCH(CH_2)$_2$], δ_H 4.31 ppm [dd, J =12, 4 Hz, 2 H, -OCH(CH_2)$_2$] and δ_H 4.17 ppm [dd, J = 9, 6 Hz, 2 H, -OCH(CH_2)$_2$]. From Figure 5, the absence of any other ^{1}H-NMR signal in δ_H 3-4 ppm was noted, indicating the absence of methoxy group or any terminal methyl ester – $COOCH_3$, dedicated for the major compound. This is deduced from the integration of the methyl groups, which are only permitted for the minor compounds. Therefore, it was comprehended that Band 1 did not contain fatty acid methyl esters. This lipid might consist of the linoleic acid component, due to the presence of δ_H 2.79 ppm [t, J = 6.5, 2 H, =CH-CH_2-CH=]. In addition, the lipid component could comprise of 3 chains of fatty acid. Therefore, it is possibly a triglyceride. This statement could be supported by the NMR signal at of δ_H 2.33 ppm [t, J = 8, 6 H = 3 x CH_2 of -CH_2COO-].

4. CONCLUSION

From the literatures, the natural biomolecules could be separated from the star anise seed. The determination of volatile compounds could be accomplished via gas chromatography-mass spectrometry. Shikimic acid is also the target compound to be extracted, due to its role in the antiviral synthesis. The non-volatile component was obtained from the liquid chromatography. It is also found that mixtures of three compounds, could be detected in Band 1 from the seed extract, via spectroscopic method. The first compound could be the 4-methoxybenzaldehyde or *para*-anisaldehyde. The second compound, would be *trans*-anethole. Lastly, a triglyceride could be determined, which was suggested by the presence of the 3 hydrocarbon chains of the fatty acids, the glyceride backbone, and the terminal methyl.

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