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Effect of Pre-Treatment Methods on the Extractability of *Christia vespertilionis* by Supercritical Carbon Dioxide

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ABSTRACT

Christia vespertilionis is a medicinal herb traditionally used as a complementary and alternative medicine to treat cancer and malaria. This study investigated the effect of pre-treatments of the *Christia vespertilionis* plant on supercritical CO_2 extraction yield and solubility. Four pre-treatments were studied: drying and grinding, doping with absolute ethanol (99%) and 80% (v/v) of ethanol/water, and microwave pre-treatment. The supercritical CO_2 extraction was conducted at a constant 13.8 MPa, 40°C with 24 mL/min flow rate in 40 min of extraction time. It was found that the dried sample after drying and grinding pre-treatment produced the highest yield of 4.56 mg/g, whereas the lowest yield was obtained for the fresh leaves' samples treated with microwave irradiation (1.26 mg/g). Doping techniques with absolute ethanol and 80% (v/v) were comparable in the 2.64 to 2.94 mg/g. GCMS results revealed that *Christia vespertilionis* extract comprises antioxidants, mainly phytol, limonene, and other medicinal compounds such as α-monolaurin and l-ascorbyl 2,6-dipalmitate. This study indicates that adding co-solvent was not the primary technique in supercritical CO_2 extraction to increase the extractability of compounds of interest from plant matrices.

Keywords: Carbon dioxide, *Christia vespertilionis*, co-solvent, medicinal compounds, pre-treatment, supercritical extraction

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INTRODUCTION

The red butterfly wing plant has received increasing public attention due to its medicinal properties in treating several critical diseases. The plant is scientifically known as *Christia vespertilionis* (L.) Bakh. from the Fabaceae family. It was reported that the plant could treat cancer (Wu et al., 2012) and malaria diseases (Upadhyay et al., 2013). Numerous studies on the traditional applications of *C. vespertilionis*, such as healing tuberculosis, bronchitis, colds, muscle weakness, and poor blood circulation (Whiting, 2007), fever treatment (Chassagne et al., 2016), and healing scabies disease (Cambie & Ash, 1994) were done and shown potential medicinal benefits. The plant extract was also reported to possess anti-proliferative potential due to the high content of bioactive compounds, mainly triterpenes, alkaloids, fatty acids, phenol, and long-chained alcohols (Hofer et al., 2013). In another aspect, the alkaloids from the *C. vespertilionis* plant exhibited as an anti-cancer agent for neuroendocrine tumors (Lu et al., 2012) and as a tumor inhibitor when tested on a mouse (Wu et al., 2012). The extract was found could prolong the life span of the tumor-bearing mice. In addition, the plant also possesses strong antiplasmodial activity, where it can act as an antidote against diseases caused by the Plasmodium genus, such as malaria (Dash, 2016). The noble compound responsible for the anti-plasmodial agent found in *C. vespertilionis* extract was named christene with the IUPAC name of 7-isopropylidene-1-methyl-1,2,6,7,8,9- hexahydronaphthalene (Nguyen-Pouplin et al., 2007; Upadhyay et al., 2013). These findings indicate the high potential of the medicinal plant as a complementary and alternative natural medicine for the pharmaceutical industry. Most recent studies have discovered that the root *C. vespertilionis* extracts have shown remarkable anti-breast cancer activity (Ismail et al., 2021; Lee et al., 2020).

However, all the studies on the *C. vespertilionis* plant were based on conventional extraction methods, i.e., organic solvent extraction to extract the bioactive compounds. The technique is not suitable and unsafe for edible products due to the use of toxic chemicals as solvent extraction. The major drawbacks of solvent extraction include compound degradation (due to the elevated temperature), product impurities, extensive extraction duration, and exposure to toxic fumes in surroundings, which will cause health problems and create dirty environments (Rombaut et al., 2014).

The extraction of bioactive compounds from *C. vespertilionis* using supercritical carbon dioxide (SC-CO_2) is scarcely available in the open literature. SC-CO_2 extraction was recognized as a green method, employing high-purity carbon dioxide (CO_2), i.e., 99.99%, as its solvent extraction. CO_2 is an ideal solvent for extracting functional foods and medicines since it is non-toxic, non-corrosive, inert (where it would not affect the extraction output), and safer for consumers and the environment (Mukhopadhyay, 2000). It is due to the advantages owned by SC-CO_2, such as faster extraction time (where the extraction could be completed within 1 to 2h compared to 8 to 12h of solvent extraction method),

the mild operating temperature used (ability to extract, and preserve thermosensitive and volatile compounds), organic solvent-free (no chemical solvent involves except for a certain extraction condition such as additional of co-solvent with a very minimum amount of possible) and clean extract produced where CO_2 can easily be removed by releasing the pressure at the end of the extraction process.

C. vespertilionis, which belongs to the Fabaceae family, is categorized as a plant containing essential oil. In principle, essential oil from plant matrices is difficult to be extracted. According to the plant anatomy of leaves, glandular trichomes (i.e., glandular hairs on the leaf's surface) contain volatile oils and other plant secretions (Beck, 2010). Therefore, breaking the plant sample's glandular cells and cell walls is crucial for easy access to the oil to the extraction solvent before the extraction process.

Numerous studies were reported on enhancing oil extractability in obtaining maximum yield. The method is called sample pre-treatment, such as drying, grinding, microwave, ultrasound, and high-pressure pre-treatments. Drying pre-treatment is necessary to remove the moisture content from the plant's material since fresh plant contains high moisture that will compete with the solvent during the extraction process. Many authors agreed that the ideal moisture content for plant material should be between 3 to 12% (wet basis moisture) (Ivanovic et al., 2014). Reducing sample size is also important for successfully applying SC-CO2, which could be obtained by grinding, chopping, or flaking the sample material (Mustapa et al., 2009). They found that the grinding pre-treatment technique could rupture the glandular cells and wall cells. In addition, a larger surface area can be obtained by decreasing the particle size, increasing the extraction yield due to the increasing solvent-oil contact and accessibility. The distance between solute-solvent contact was reduced to fasten the extraction process. Another technique to improve the extraction efficiency is microwave pre-treatment before being subjected to SC-CO_2 extraction. Theoretically, by exposing plant materials to microwave radiation, a greater extraction yield could be obtained (Uquiche et al., 2008). This phenomenon occurred due to the breaking of plant cell walls by irradiation, generating permanent pores in the sample. Thus, enabling the oil to move out through the permeable cell walls makes it easier to be extracted using the SC-CO_2 method.

For the sample treated using the ultrasound method, many studies had shown that the quality of the extract improved when the concentration of the compound increased through the cavitation phenomenon. Cavitation occurs when the ultrasonic wave passes through the sample degrading the plant cell walls (Herrero et al., 2015). As for the high-pressure pre-treatment method, the exposure of the plant sample to high pressure can lead to higher solvent permeation in the inner cells, and therefore higher and faster extraction will be achieved (Vidović et al., 2014). Overall, the purpose of sample pre-treatments above is to destroy the cell walls and disrupt the plant structures since the methods will be assisted in releasing the oil from cells, leading to the higher recovery of oil from SC-CO_2 extraction.

Another technique to enhance extraction efficiency is adding a small amount of polar co-solvent to the system. This technique is expected could increase the solvent power of supercritical fluid, thus enhancing the ability of CO_2 to dissolve compounds. In this study, several techniques of sample pre-treatments: (1) drying and grinding, (2) microwave pre-treatment on fresh leaves, (3) doping with absolute ethanol (99%), and (4) doping with 80% (v/v) of ethanol/water was employed to the *C. vespertilionis* plant prior to the SC-CO_2 extraction at 13.8 MPa and 40°C for 40 min. The study investigates the effect of different sample pre-treatments on the SC-CO_2 extraction of *C. vespertilionis* yield, solubility, and extracted phytocompounds.

MATERIAL AND METHODS

Materials

C. vespertilionis plant samples containing only leaves were purchased from Pure Rerama Leaf in Bukit Subang, Shah Alam, Malaysia. Absolute ethanol of 99% was purchased from Merck, and carbon dioxide (CO_2) of supercritical fluid grade with a purity of 99.99% used in supercritical fluid extraction was purchased from Air Liquide in Singapore.

Sample Pre-Treatment and Preparation

Samples were prepared accordingly for different pre-treatments.

Drying and Grinding. Fresh leaves were oven-dried at 40°C for 24h to reduce their moisture content from 59% to 8% wet basis. The dried leaves were ground using Waring Laboratory Blender and sieved through Endecotts Octagon 2000 Digital Sieve Shaker. Samples with 0.3 mm of particle size were then kept in the air-tight bag until used for SC-CO_2 extraction. The dried samples prepared by this treatment are labeled as D.

Microwave Pre-Treatment. Fresh leaves with 59 *wt%* moisture content were cut into small pieces (2×2 cm) and placed in a microwave (SHARP, Model R202ZS) to disrupt the plant cell wall over 320 W in the 50s and without any addition of solvent or water. The parameter condition of the microwave used was selected according to the available literature (Yu et al., 2016). After the microwave pre-treatment, the sample was placed in the cotton cloth to extract oil using SC-CO_2. The samples treated with this preparation are classed as M.

Doping with Absolute Ethanol (99%). The samples prepared from the D treatment (i.e., drying and grinding) were doped with a 1:2 weight ratio of absolute ethanol (99%) to samples and mixed before the SC-CO_2 extraction. This sample is labeled as A.

Doping with 80% (v/v) of Ethanol/Water. A similar procedure to sample A was applied for this sample pre-treatment. About 1:2 weight ratio of 80% (v/v) ethanol/water was doped to the dried samples prepared and labeled as W samples.

Supercritical Carbon Dioxide Extraction

SC-CO_2 extraction of *C. vespertilionis* was performed using SFT-100 from Supercritical Fluid Technologies, Inc. (USA) with the maximum pressure and temperature are 68.9 MPa and 150°C, respectively. Figure 1 shows the result of extraction yield for different pre-treatment methods. The CO_2 liquid with 99.99% of purity was used for SFT-100 with a long dip tube and 5.5–6.2 MPa of tank pressure. From each pre-treatment sample, about 5 g of *C. vespertilionis* sample was inserted into an extraction bag (i.e., cotton cloth) and placed in a 25 mL pressurized extraction vessel. The sample was put in the cotton cloth to prevent pressure channeling when the SC-CO_2 fluid passed through the sample. High pressure of liquid CO_2 was pumped into the extractor and regulated by a back-pressure regulator unit. Once the system has equilibrated for a certain set time and at the set temperature and pressure, a dynamic valve was gently opened to allow a continuous flow of SC-CO_2 fluid for 10 minutes. The samples were carefully collected by placing the tubing collection inside the test tube and designing it to pass through the test tube cap. This technique was done for all sample types of pre-treatment. This work conducted the extraction process within 40 min extraction time, 24 mL/min of CO_2 flowrate, 13.8 MPa, and 40°C. The extraction temperature and pressure used in this study were chosen according to the previous history available in the literature (Almeida et al., 2013; Cargnin et al., 2010).

The desired flow rate of SC-CO_2 through the sample was achieved by opening the restrictor valve slowly at 5 mL/min. The extracted oil was collected gravimetrically and measured every 10 min at time intervals by placing a glass vial at the outlet of the restrictor. The CO_2 was decompressed into atmospheric pressure. The amount of extracted oil was weighed using the analytical balance Model Mettler Toledo AB204-S with an accuracy of 0.0001 g. The extraction was repeated three times at identical operating conditions, and the average value with the standard error was

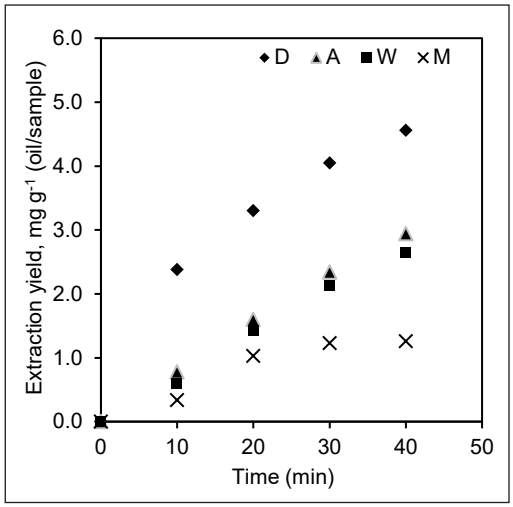


Figure 1. Extraction yield of different sample pre-treatments: dried and ground sample (D), doping with 80% (v/v) ethanol/water (W), doping with absolute ethanol sample (A), and microwave sample (M) at constant SC-CO_2 conditions; 13.8 MPa, 40°C, CO_2 flowrate of 24 mL/min and 40 min extraction time

used for extraction yield determination. The extraction yield was reported as the oil weight in mg per feed sample weight in grams (g). The solubility of oil in SC-CO_2 in each experiment was calculated as a ratio of the mass of extracted oil in mg to the mass of CO_2 consumed in grams (g). Experiments were repeated three times for each sample pre-treatment and calculated as an average and standard deviation.

Scanning Electron Microscopy (SEM)

The surface morphology of the non-processed and SC-CO_2 extraction processed samples (at 40°C, 13.8 MPa, and 40 min) of the dry and microwave pre-treatments samples (i.e., samples D and M) were analyzed by scanning electron microscopy (SEM) model Hitachi S-3400N. The purpose of this method is to observe the morphological changes of the vegetal structures before and after the extraction process. Samples were sputter-coated with gold and examined at 1000 to 2500× magnifications. An acceleration potential of 15 kV was used during the micrograph.

GCMS Analysis

The *C. vespertilionis* extracts were analyzed by gas chromatography-mass spectrometer (GCMS) Model Varian. The qualitative analyses of the extracts were performed using a stationary phase BP-5MS non-polar column with 30 m length × 0.25 mm internal diameter and 0.25 µm thickness from General Separation Technologies Inc. USA. The gas chromatography settings were as follows: 2 min at 60°C, then a progressive increase to 150°C at a rate of 10 °C/min, a further increase to 250°C at a rate of 3°C/min, and finally holds for 1 min. A 1 µL aliquot sample in methanol dilution was injected into the column with a split ratio of 1:10. Helium was used as a carrier gas with a 1 mL/min flow rate in the column. The injector and detector temperatures were 260 and 270°C, respectively. The mass spectrometer was operated in the electron-impact ionization mode at an energy level of 70 eV with a scanning range of 50–500 amu. The components of the extracted oil were identified by comparing their mass spectra (MS) with those available in the NIST (National Institute of Standards and Technologies) MS library and with blank methanol. The analyses were repeated twice for each extract of the pre-treatment sample.

RESULTS AND DISCUSSION

Extraction Yield and Solubility

Figure 1 shows the extraction yield of *C. vespertilionis* oil recovered by SC-CO_2 extraction as a function of the mass of extracted oil per mass of sample used (mg/g). Four different pre-treatments were studied to the sample before introducing it to the SC-CO_2 extraction at 13.8 MPa, 40°C, 24 mL/min CO_2 flowrate, and 40 minutes of extraction time. The sample

pre-treatments used are D: drying and grinding, M: fresh leaves with microwave pre-treatment, A: doping with absolute ethanol, and W: doping with 80% (v/v) ethanol/water.

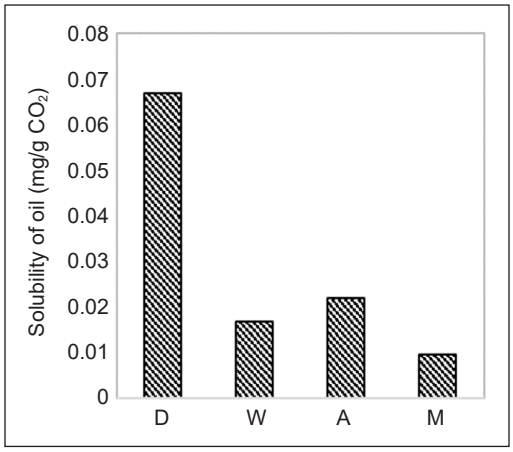


Figure 2. Solubility of *Christia vespertilionis* oil in SC-CO_2 of different sample pre-treatments: dried and ground sample (D), doping with 80% (v/v) ethanol/water (W), doping with absolute ethanol sample (A), and microwave sample (M) at constant SC-CO_2 conditions; 13.8 MPa, 40°C, CO_2 flowrate of 24 mL/min and 40 min extraction time

According to Figure 1, the result revealed that the extraction using the dried sample (D) method (i.e., drying and grinding) produced the highest yield of 4.56 mg/g, followed by the A samples (doping with absolute ethanol) with 2.94 mg/g, W samples (doping with 80% (v/v) ethanol/water) with 2.64 mg/g and the microwave pre-treatment sample (M) of 1.26 mg/g. Nevertheless, the extraction yield obtained from samples A and W were comparable and did not significantly enhance the extractability of essential oil from the plant compared to the D sample treated with drying and grinding. The lowest yield obtained after the microwave pre-treatment (M) was opposite to the finding reported by Mustapa et al. (2015), who found that the highest yield was obtained with an improved extraction rate when the medicinal plant was extracted by microwave technique.

In principle, adding polar co-solvent to the SC-CO_2 can increase the solvent power of the SC-CO_2 by enhancing the polarity of the fluid, hence increasing the extraction yield of the essential oil. However, contradictory results are shown in this study. The A sample doping with absolute ethanol yielded less than the D sample. Several studies demonstrated that adding ethanol increased oil extraction from its plants (Vidović et al., 2014). It could be due to the variation of phytocompounds compositions consisting of the high amount of non-polar compounds in the *C. vespertilionis* leaves extracts. Until today, there are no research reports on the thorough phytocompounds compositions of the *C. vespertilionis* plant. The previous study by Upadhyay et al. (2013) stated that the major components in the *C. vespertilionis* plant are comprised of triterpenes, alkaloids, fatty acids, phenols, alkanes, and long-chained alcohols. However, the composition of the polar and non-polar compounds is unavailable. Therefore, based on the result presented in this work, we hypothesize that the non-polar compounds present in the samples are higher than the amount of the polar compounds. Thus, the addition of polar co-solvent did not improve the extraction yield of the essential oil. Furthermore, the use of co-solvent was found cannot significantly increase the extraction yield. A similar result was obtained by Calvo et al.

(2017), where adding ethanol reduced the selectivity of SC-CO_2. As studied by Reverchon and De Marco (2006), the co-solvent could increase the SC-CO_2 solvent power towards polar compounds. Nevertheless, it also could lower the fluid selectivity.

We divided dried samples into two groups to investigate how moisture content affects the supercritical extraction of *C. vespertilionis* oil. One group was treated with 80% (v/v) ethanol/water and labeled as the "W sample," while the other group was treated with absolute ethanol and labeled as the "A sample." The extraction yield of W and A *C. vespertilionis* samples was comparable, with 2.94 and 2.64 mg/g yield, respectively. The slight variation could be explained by the moisture content of the samples, as indicated in Table 1. Adding 80% (v/v) ethanol/water to the dried sample has increased the water content to 9% wet from the dried sample's original moisture content (8% wet basis). Consequently, the yield of the W sample decreased to 2.64 mg/g due to the higher amount of water that hindered the extraction process. In this case, adding water to the sample increased the mass transfer resistance of the supercritical fluid to penetrate the solid particles to extract the oil. The water acts as a solvent that competes with SC-CO_2, reducing the extraction yield (Liteanu et al., 2013).

Table 1
The moisture content of samples after each pre-treatment

Types of sample pre-treatment	Moisture content (%) Wet Basis	Extraction yield (mg/g)
D	8	4.56 ± 0.52
A	8.25	2.94 ± 0.13
W	9	2.64 ± 0.08
M	59	1.26 ± 0.30

Note. D = dried and ground sample, A = doping with absolute ethanol, W = doping with 80% (v/v) ethanol/water and M = microwave pre-treatment sample

In this work, using absolute ethanol as the co-solvent in the A sample could not improve the solute solubility in SC-CO_2. It can be seen in Figure 2, which shows the solubility of *C. vespertilionis* oil in SC-CO_2 extraction for all types of sample pre-treatments. The solubility of oil from the A sample of 0.022 mg oil/g CO_2 was lower than that of the D sample (0.067 mg oil/g CO_2) when ethanol was added. The finding was similar to the work done by Calvo et al. (2017), where adding 5% (v/v) ethanol co-solvent in the SC-CO_2 extraction process did not improve the yield. It indicates that the 8% wet basis of initial moisture content of the D sample was sufficient to enhance the solubility of *C. vespertilionis* oil into supercritical fluids and facilitate the resistance of solid-fluid mass transfer (Balachandran et al., 2006). On the other hand, between A and W samples, the solubility of extract in the A sample (i.e., 0.022 mg oil/g CO_2 used) was found to be comparable to the solubility of extract in the W sample (i.e., 0.017 mg oil/g CO_2 used) due to the close moisture content owned by each other.

Among all the pre-treatment samples studied in this work, the *C. vespertilionis* sample that was treated with microwave (M) before the SC-CO_2 extraction exhibited the lowest yield of 1.26 mg/g in comparison to other pre-treatment samples (Table 1). Note that the M sample used in this study was fresh leaves with moisture content as high as 59 *wt*%. The leaves were used as it was collected without reducing their moisture content before being treated with microwave irradiation. The purpose of using fresh leaves without any water reduction is to promote high microwave energy absorption by the water, increasing the temperature inside the sample and leading to the expansion and rupture of the cell walls. This phenomenon is called superheating, where the water within the plant matrix facilitates the extraction via energy absorption and cell rupture (Wang & Weller, 2006). However, the lowest extraction yield obtained by the M sample showed the opposite behavior. It could be due to the substantially high-water content in the M sample acting as a barrier and increasing the mass transfer resistance for the SC-CO_2 to extract the essential oil (Pourmortazavi & Hajimirsadeghi, 2007). Furthermore, a bigger sample size of 5 mm diameter on average may also result in low yield compared to other samples. The reduction of sample particle size will shorten the intermolecular distance and increase the mass transfer rate of solutes to the solvent. Shorter distances between particles have made the diffusion process of solutes reach the solid surface higher, thus improving the yield (Santos et al., 2015).

Evaluation of SEM Images

The scanning electron microscopy (SEM) images of the dried sample (D) and microwave pre-treatment sample (M) before and after SC-CO_2 extraction are shown in Figures 3(a)–(d). Figures 3(a) and (b) show the SEM images of sample D (which was prepared by drying and grinding processes) before and after SC-CO_2 extraction. As shown in Figure 3(a), the micrograph of the non-treated sample shows a cloudy and rough surface covered with an oil layer. On the other hand, Figure 3(b) presents sample D after SC-CO_2 extraction. It is observed that the structure seems to be more porous with the deflated surfaces due to the losses of oil during the SC-CO_2 process. Grinding pre-treatment seems to destroy the epidermis of *C. vespertilionis* plant cells revealing accessible solutes to be extracted by SC-CO_2 fluid (Yahya et al., 2010).

As for the sample treated under microwave radiation (sample M) before being submitted to the SC-CO_2 extraction process, the SEM image is shown in Figure 3(c). The magnification of 2500× was used to observe the effect of irradiation on the sample. There was no difference in the structure of the sample. However, for sample M after SC-CO_2 extraction, the SEM image showed a cracked image on the leaf's surface, as shown in Figure 3(d). The extraction pressure was believed to have broken the plant's cell walls, which helped release solutes during SC-CO_2 extraction. As studied by Čolnik et al. (2016),

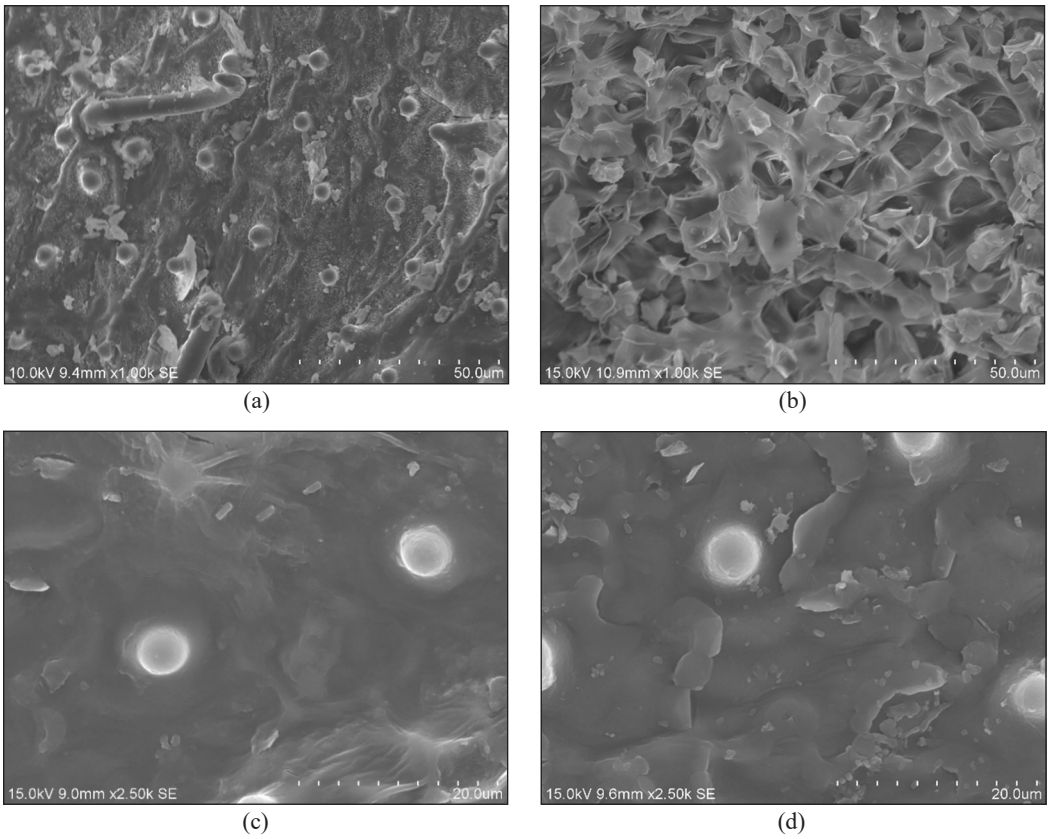


Figure 3. SEM images of (a) sample D before SC-CO_2 extraction, (b) sample D after SC-CO_2 extraction, (c) sample M before SC-CO_2 extraction, and (d) sample M after SC-CO_2 extraction *Note*. Sample D: dry sample which undergoes drying and grinding processes; sample M: microwave pre-treatment sample using fresh leaves without drying and grinding processes

the SC-CO_2 extraction allows the fluid to penetrate and damage the cell walls, releasing the active compounds. However, the depletion of oil in sample M was not much occurred as compared to sample D. This result relates to the extraction yield obtained by sample M, i.e., 1.26 mg/g, which was lower than sample D (4.56 mg/g). It was assumed that the high-water content in the fresh sample had lowered the yield. A similar result was obtained by Viguera et al. (2016), where the microwave pre-treatment was not useful for the wet sample since the water created a barrier during the extraction process.

Phytocompounds Characterization Analysis

All extracts obtained from the D, A, W, and M of *C. vespertilionis* samples were analyzed by GCMS to determine the major compounds present in the essential oil and to determine the effect of sample pre-treatment on the phytocompounds extracted. The GCMS analysis revealed six major compounds in the *C. vespertilionis* extracted under SC-CO_2 extraction

at 13.8 MPa, 40°C, 24 mL/min, and 40 min extraction time. Phytochemical compounds are identified based on the peak area, retention time, and molecular formula using methanol as a blank injection sample.

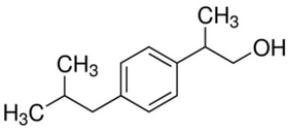
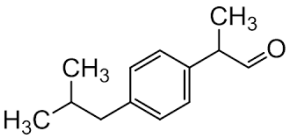
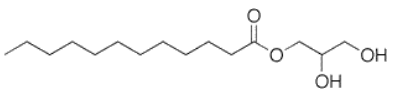
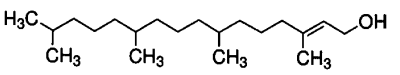
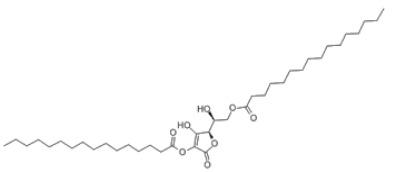
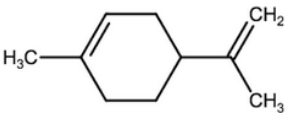
The components' names, percentage peak area, chemical structure, retention time, and component biological activity were tabulated in Table 2. The major constituents in the *C. vespertilionis* extracts were ibuprofen alcohol, 2-(4-isobutylphenyl) propanal, cis-phytol, α-monolaurin, phytol, l-ascorbyl 2,6-dipalmitate, and limonene. Similar phytocompounds were reported elsewhere from *C. vespertilionis* extracted by Soxhlet and maceration techniques (Zambari et al., 2021).

Note that three components, i.e., ibuprofen alcohol, 2-(4-isobutylphenyl) propanal, and phytol, were identified in all samples except the sample from the microwave pre-treatment (M). One component detected from the chromatography analysis of the M sample and extracted by SC-CO_2 extraction was l-ascorbyl 2,6-dipalmitate. It can be explained by the substantially low yield obtained due to its high moisture content. The water may hinder the flow of SC-CO_2 fluid through the sample, decreasing the contact surface between solutes and solvent and making it difficult to extract other compounds. Too high water content in the sample reduced the extraction efficiency and thus lowered the yield. The result was supported by the solubility data of the microwave sample, as shown in Figure 2, where it is the lowest solubility among all samples.

On the other hand, 2-(4-isobutylphenyl) propanol and 2-(4-isobutylphenyl) propanal, which are the impurities of ibuprofen, were found in all *C. vespertilionis* samples except the microwave pre-treatment technique (M sample). The 2-(4-isobutylphenyl) propanol is also known as ibuprofen alcohol or ibuprofenol (Chen & Rosazza, 1994). There are many types of ibuprofen impurities. The one discovered in this work is ibuprofen impurities which is the ibuprofen alcohol, and another is an in-house ibuprofen impurity known as 2-(4-isobutylphenyl) propanal. The compounds were commonly used as reference standards to study the separation and synthesis of ibuprofen drugs and were mainly applied in the pharmaceutical field (Giacomini et al., 2007). Ibuprofen is a non-steroidal anti-inflammatory drug (NSAID) normally used as a painkiller.

Another bioactive compound isolated from *C. vespertilionis* is the phytol compound. Phytol is a bioactive component derived from the chlorophyll of green plants. The compound was found in many plant extracts, such as *Majorana hortensis Moench*, *Thymus vulgaris* L., *Achillea ligustica*, *Marchantia convolute,* and *Porophyllum ruderale* (Conde-Hernández et al., 2017). Islam et al. (2015), in their review on the importance of phytol in the pharmaceutical industry, have reported that phytol portrayed numerous medicinal properties such as cytotoxic, anti-tumorous, anti-mutagenic, anti-teratogenic, antidiabetic, lipid-lowering, antispasmodic, anticonvulsant, anti-nociceptive, anxiolytic, antidepressant, hair growth facilitator, hair fall defense and antidandruff activities. Phytol is a non-polar compound; the SC-CO_2 tends to solubilize the compound from the plant materials. The

Table 2
Bioactive compounds identified in SC-CO_2 extraction of Christia vespertilionis *using GCMS for different sample pre-treatments (D, A, W & M)*

Compounds Names	Retention Time (min)	Percentage Area (%)	Formula	Molecular Weight (g/mol)	Chemical Structure	Biological Activity	References
Ibuprofen alcohol	A: 14.78 W: 14.80 D: 14.79 M: -	A: 2.37 W: 6.89 D: 7.97 M: -	$C_{13}H_{20}O$	192.30		Ibuprofen impurity P was used in the pharmaceutical study.	(Giacomini et al., 2007)
2-(4-isobutylphenyl) propanal	A: 17.01 W: 17.05 D: 17.04 M: -	A: 4.54 W: 8.06 D: 10.19 M: -	$C_{13}H_{18}O$	190.28		Synthesis of ibuprofen	(Roy, 2011)
α-Monolaurin	A: 22.64 W: - D: - M: -	A: 27.01 W: - D: - M: -	$C_{15}H_{30}O_4$	274.40		Anti-bacterial Antiviral Antimicrobial	(Lieberman et al., 2006)
Cis-phytol and Phytol	A: 29.95 W: 30.61 D: 30.46 M: -	A: 16.68 W: 10.61 D: 17.05 M: -	$C_{20}H_{40}O$	296.54		Antimicrobial Anti-cancer Anti-inflammatory Antioxidant	(Pejin et al., 2014) (Kalaivani et al., 2012)
L-ascorbyl 2,6-dipalmitate	A: - W: 22.81 D: 22.76 M: 22.58	A: - W: 8.20 D: 6.38 M: 4.13	$C_{38}H_{68}O_8$	652.94		Anti-bacterial	(Okwu & Ighodaro, 2010)
Limonene	A: - W: - D: 5.44 M: -	A: - W: - D: 17.57 M: -	$C_{10}H_{16}$	136.24		Antioxidant	(Bagheri et al., 2014)

highest peak area of phytol of 17.05% was obtained in the D sample compared to the A and W samples. It indicates that the doping technique with ethanol did not enhance the extraction of the phytol into the SC-CO_2. The phytol appeared at the retention time ranging from 21.22 to 22.00 min (not shown in Table 2), corresponding to isomer cis-phytol. In contrast, the phytol was identified at 29.95, 30.61, and 30.46 for the A, W, and D samples.

On the other hand, the maximum peak area of 27.01% that corresponds to the α-monolaurin appeared merely in the sample doped with absolute ethanol (A sample). The presence of the α-monolaurin was possibly due to the addition of ethanol, which increases the solvating power of the SC-CO_2 fluid to dissolve polar components in the *C. vespertilionis* plant. Although the oil yield of the A sample is slightly lower than the D sample, the oil quality was enhanced when the ethanol was added via doping technique, enabling the extraction of a polar compound from the plant. The discovery of α-monolaurin from *C. vespertilionis* was the first time discovered and reported in this study. This compound possesses many potential benefits that promote good health sustainability. A review study by Lieberman et al. (2006) has summarized that monolaurin compounds exhibit anti-bacterial, anti-fungal, and anti-virus effects, which are useful in treating infections caused by harmful organisms. The study on the anti-fungal activity of monolaurin was further continued by Seleem et al. (2016), where monolaurin was found could treat *Candida albicans*, i.e., a type of pathogen that existed in the gastrointestinal tract and mouth of the human body. According to a clinical study, the major source of monolaurin comes from the mother's breast milk which possesses an effective immune potion to protect newborn babies from bacterial infections due to the antimicrobial activity owned by the monolaurin compound.

On the other hand, l-ascorbic acid 2,6-dihexadecanoate, also known as l-ascorbyl 2,6-dipalmitate (ADP), was merely identified in D, W, and M samples with a peak area range from 4 to 8%. The absence of the ADP in the A sample could be due to its high molecular weight that hindered its solubility into the SC-CO_2, which cannot be improved even though it was doped with absolute ethanol. Sethupathy et al. (2017) demonstrated that ADP exhibits anti-biofilm, anti-pathogenic and anti-carotenogenic agents. The compound was also found in the leaf extract of *Alstonia boonei* and had anti-bacterial activity (Okwu & Ighodaro, 2010). Another study by Selvamangai and Bhaskar (2012) on the extraction of *Shorea robusta* leaves L. under methanolic extraction isolated the ascorbic compound and reported the compound possesses antioxidant, anti-scorbutic, anti-inflammatory, anti-nociceptive, anti-mutagenic and wound healing property.

Furthermore, *C. vespertilionis* extract contained L-limonene, classified as a cyclic terpene. Nevertheless, this compound was merely discovered from the dried and ground sample (D). The compound was detected at 5.44 min of retention time and had the highest relative abundance of 17.57% peak area compared to other compounds. Limonene is naturally a non-polar compound, thus, making it easy to solubilize in the SC-CO_2. The

discovery of limonene in the *C. vespertilionis* was in correspondence with the previous study conducted by Upadhyay et al. (2013), where the authors proved that limonene is present as one of the major components in *C. vespertilionis*. Terpenes were known to be the primary constituents of the essential oils of many types of medicinal plants.

The chemical structure of limonene comprises an alkene compound with double bonds that are insoluble in a polar solvent such as water or ethanol. It explains that limonene was not found in the extracts of the samples doped with absolute ethanol (A sample) and ethanol/water (W sample). A previous study demonstrated that the limonene compound could be extracted without additional co-solvent (Nautiyal, 2016). Another possible reason for the absence of limonene in the W and A type sample was the evaporation of the compounds during the sample collection. Therefore, it is suggested to place the test tube of the sample collector in an ice bath to reduce the loss of the volatile compounds at room temperature, as demonstrated by Conde-Hernández et al. (2017). As for the microwave pre-treatment (M sample), the limonene could not be identified. It could be due to the high amount of moisture in the sample that might prevent the SC-CO_2 from reaching the solute in solid particles during the extraction process.

CONCLUSION

The effect of different sample pre-treatments on the *C. vespertilionis* plant was reported in this work, including drying and grinding, the doping technique with absolute ethanol, 80% (v/v) ethanol/water as co-solvent, and the microwave pre-treatment. The findings showed that drying and grinding the sample without additional co-solvents obtained the highest extraction yield of 4.56 mg/g and solubility of 0.067 mg oil/g CO_2. Furthermore, applying microwave pre-treatment on fresh leaves prior to the SC-CO_2 did not enhance the oil yield and solubility. The best pre-treatment method for SC-CO_2 extraction of *C. vespertilionis* was by drying the fresh leaves to reduce their moisture content, then grinding them into smaller particle sizes. This technique has considerably increased the oil yield and solubility. The phytocompounds analysis of the *C. vespertilionis* extracts discovered the identification of four medicinal compounds with phytol present as the major component, followed by α-monolaurin, l-ascorbyl 2,6-dipalmitate, and limonene. The variation of compounds discovered from different types of sample pre-treatment methods suggests that the extraction of compounds of interest can be optimized by applying the appropriate techniques to increase their extractability from specific plant matrices.

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