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Molecular Characteristics of Biomedical and Bacteriostasis Extractives of *Illicium verum* Fruit

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Illicium verum **is an medicinal plant containing many valuable active ingredients. However, the rich extractives from its fruit are invariably wasted for inefficient separation processes. To further utilize these resources, the four extractives were obtained, and BPME extractives were subsequently analyzed by GC-MS. The first- and second-stage extractives contained 34 and 61 components, respectively. The third- and fourth-stage extractives only contained anethole. And the four methods were suitable for extraction of anethole. Furthermore, the BPME extractives contained many biological active molecules, such as anethole, stigmast-4-en-3-one and γ-sitosterol. It therefore suggested that BPME extractives could be used as biomedicines, rare spices, liquid bioenergy, etc.**

Key words: *Illicium verum* fruit, Wood derived biomedicine, Biomedical extractives, Bacteriostasis extractives.

Illicium verum Hook. f., which is also sometimes referred to as octagonal large fennel, aniseed, fennel on a ship, ship fennel, anise beads, fragrant anise, star anise, fennel oil, anise, and Chinese anise, originates from northeast Vietnam and southwest China[1]. This plant typically grows in moist, warm environments and is an evergreen tree that can grow up to 20 m in height with seeds that ripen in spring and autumn. *Illicium verum* has subsequently been widely planted throughout China, where it has spread out over more than 90 counties of the Guangxi, Guangdong, Yunnan, Fujian, Anhui, Jiangxi, and Hunan provinces. The planted area of *Illicium verum* in China is now currently greater than 3.3×10^5 ha, and provides a 1.25×10^4 ton yield of *Illicium verum* fruits, as well as more than 700 ton of anise oil. China is already the world's largest producer of *Illicium verum*, with cultivation in the Guangxi province accounting for approximately 90% of the total output.

Illicium verum is well known throughout the world for its medicinal and economic benefits, and the wood of this plant has been used for its fragrance and vermifuge effects, as well as being used for the construction of sculptures, furniture, and timbers for interior decoration. The fruits, seeds and leaves of this plant are rich in aromatic oils that have been used as important raw materials in the manufacture of soap, toothpaste, cosmetics, wine, beer and food[2]. The fruits of *Illicium verum* have a strong flavor and are fragrant and sweet, and have been used historically to create a famous condiment for the therapeutic efficacy of warming Yang and eliminating cold and arrest pains, although the ingestion of too much of this condiment can lead to head aches and other complications. The roots, stems, bark and leaves of *Illicium verum* have all been used as medicines to the treatment of rheumatoid arthritis, bruises,

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vomiting and dyspepsia[3]. Furthermore, the fruits have been used to treat vomiting, bloating, gas pain, and snake bites, whereas the extract anethole has been used to treat leucopenia[1]. Red water extractives have been used as pesticides. *Illicium verum* fruits currently trade internationally at 15.8 CNY/Kg and the cultivation of *Illicium verum* in Southeast Asia will continue to expand as a consequence of economic and medicinal value associated with this plant.

Illicium verum fruits have been used in medicine in China for a long time, and their use was first documented in 1505 in a book entitled "Herbal Essentials Collection", where it was reported to heal fistula and cholera. Given the variety of different therapeutic applications of *Illicium verum*, this material is still used today in many traditional Chinese medicines. The fruits of *Illicium verum* are antibacterial, diuretic, carminative, stimulatory, odontalgic and stomachic[4]. Furthermore, *Illicium verum* has been prescribed as an digestive aid to nursing mothers to promote breast-milk production, as well as being used as a breath freshener, as sleeping aide, and for its anti-bacterial and anti-fungal effects against asthma, bronchitis and dry coughs[1,3]. The essential oil of *Illicium verum* contains 75-90% anethole, which was a estrogenic effect, and has been useful applied to provide relief from rheumatism and lower back pain[1,5]. Research towards identifying the pharmaceutical ingredients of *Illicium verum* has been underway since 1948, and Kouno et al. reported the isolation and identification of neolignans and a phenylpropanoid glucoside from *Illicium* defengpi in 1993[6]. Thomas et al. studied novel seco-prezizaane sesquiterpenes from North American IIIicium species in 1999[7]. And the main biologically active ingredients to have been determined to date were volatile and fatty oils, proteins, and resins. In 2005, *Illicium verum* biomass was used in the production of Tamiflu, and two independent reports appeared in the literature demonstrating that the key intermediate in the synthesis of Tamiflu (i.e., shikimic acid) could be extracted from *Illicium verum*[8,9]. Unfortunately, however, the rich extractives of the *Illicium verum* biomass have traditionally been wasted because of inefficient extraction and separation processes. In the current study, we have obtained four extractives using the four-stages extraction technique, and subsequently analyzed and identified the active molecules from the four extractives using GC-MS with the aim of further utilizing the high quality resources contained in *Illicium verum* fruit.

MATERIALS AND METHODS

The *Illicium verum* fruits were provided by the Guangxi Academy of Forestry, and were collected in August 2012 from the Nanning Forest Farm, Guangxi province, P. R. China. The fresh fruits were air-dried indoors and subsequently sieved through 40 mesh powder AS200 Sieving Instrument (Retsch Co., Ltd, Germany). Benzene, methanol, ether, petroleum ether and ethanol were purchased in the chromatographic grade and used without further purification. The quantitative filter paper, cotton bag and cotton were all extracted with a mixture of benzene/ethanol for 12 h. The benzene/ethanol solution was made-up as a 1:4 mixture (v/v), the methanol/ethanol solution was made-up as a 1:3 mixture (v/v), the ether/ethanol solution was made-up as a 1:9 (v/v) mixture, and the petroleum ether/ethanol solution was made-up as a 1:1 mixture (v/v).

Four-stages of extraction

Thirty two pieces of the sieved powder were weighed out (about 10 g with 1.0 mg accuracy) and parceled in a cotton bag tied with a cotton thread and signed. The four-stages of extraction were then carried out using a large-calibre Soxhlet extractor with 800 mL of solvent which were benzene/ethanol solution, petroleum ether/ethanol, ether/ethanol solution and methanol/ethanol, successively. The extraction times for the benzene/ethanol, petroleum ether/ethanol, methanol/ethanol, and ether/ethanol solutions were 9, 5, 3 and 5 h, respectively, with an extraction temperature in the range of 85 to 90°C being used in all cases. Following the extractions, the four extraction solutions obtained were reduced in volume to 10 mL under vacuum (0.05–0.07 MPa) at 45°C to give the residues from the benzene/ethanol (BY03), petroleum ether/ethanol (SY03), methanol/ethanol (JY03), and ether/ethanol (YY03) extractives. The BY03, SY03, JY03, and YY03 extractives be called as BPME extractives by a joint name.

GC-MS analysis

The above extractives were analyzed

using an online linked gas chromatograph-mass spectrometer (GC-MS). The GC/MS analyses were carried out on an Aglient 6890N+5975C GC-MSTM system (Aglient Co., Ltd, USA) linked to a mass selective detector. An elastic quartz capillary column DB-5MS (30 m × 250 μm × 0.25 μm) coated with a neutral phase (hewlett-packard-5 cross-linked 5% phenyl methyl silicone) was used. The carrier gas was helium and the injection port temperature was 250°C. The temperature program for the GC started at 50°C and increased at the rate of 8°C/min to 250°C, and then at a rate of 5°C/min until it reached 300°C, followed by a split injection at a ratio of 15:1. The MS program scanned over the molecular weight (m/z) range of 35 to 335 atomic mass units (AMU), with an ionizing voltage of 70 eV and an ionization current of 150 μA of electron ionization (EI). The flow velocity of helium was 1.2 mL/min. The ion source and quadrupole temperatures were set at 230°C and 150°C, respectively.

RESULTS AND DISCUSSION

Components of the BPME extractives from *Illicium verum* fruit

According to the optimum extraction method, four extractives (benzene/ethanol, petroleum ether/ethanol, methanol/ethanol, and ether/ethanol) were respectively obtained. The total ion chromatograms of these four extractives by GC/MS are shown in Fig.1. The relative content of each component was counted by area normalization. Subsequent analysis of the MS data using the NIST standard MS map by computer, as well as open-published books and papers[9-24], allowed for the individual components to be identified.

According to GC/MS result, 34 components were identified from the 41 peaks produced by the benzene/ethanol extractives from the *Illicium verum* fruit. The results showed that the main components were anethole (74.93%), benzene, cyclohexyl-(3.40%), 2- hydroxyl-2- (4-methoxy-phenyl)-*N*-methyl-acetamide (2.68%), benzaldehyde, 4-methoxy-(2.65%), benzene acetic acid, α-hydroxy-4-methoxy-, methyl ester (2.64%), 1,6-octadien-3-ol, 3,7-dimethyl-(1.43%), *trans*-4-meth -oxycinnamaldehyde (1.33%), 2-propanone, 1-(4-meth -oxyphenyl) -(1.31%), D-limonene (1.23%), estragole (%1.01), 1-(3-methyl-2-butenoxy)-4-(1-propenyl) benzene (0.85%), (+)-4-carene (0.77%), S-(*p*-methoxy -benzoyl) thiohydroxylamine (0.65%), bicyclo[3.1.1]hept -2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)- (0.59%), terpinen-4-ol(0.44%), stigmast-4-en-3-one (0.38%), benzene, 1-(1,1-dimethylethyl)-4-ethenyl-(0.34%), caryo -phyllene (0.34%), eucalyptol (0.34%), catechol (0.29%), ethyltetramethylcyclopentadiene (0.28%), *n*-hexadecanoic acid (0.26%), ethanone, 1-(2-hydroxy-5- methylphenyl) - (0.25%), naphthalene, 2,6-bis(1,1-di -methylethyl)- (0.23%), naphthalene, 2,7-bis(1,1-dimeth ylethyl)- (0.22%), benzamide, *N*-(3-nitrophenyl) -2- methoxy- (0.17%), ²-bisabolene (0.15%), *cis*-β- farnesene (0.15%), cyclohexanol, 2-[2-pyridyl]- (0.15%), 1-phenanthrenecarboxylic acid, 1,2,3,4,4a,9,10,10a-octahydro-1,4a- dimethyl-7-(1-methylethyl)-, [1S-(1α, 4aβ,10aβ)]- (0.14%), 1-propanone, 1-(3-methoxy -phenyl)-(0.14%), 2-propenal, 3-(4-hydroxy-3- methoxy phenyl)- (0.10%), 2,3'-bipyridine, 1'-acetyl-1',3,4,4',5,5', 6,6'- octahydro-(0.08%), dehydroabietic acid (0.07%).

According to GC/MS results, 61 components were identified from the 64 peaks produced by the petroleum ether/ethanol extractives from the *Illicium verum* fruit. The result showed that the main components were anethole (46.32%), undecane (5.22%), decane (4.27%), dodecane (3.25%), tridecane (2.11%), benzaldehyde, 4-methoxy- (1.78%), nonane (1.78%), 2(1H)-pyridinone, 1,4,6-trimethyl-(1.52%), mesitylene (1.47%), naphthalene (1.23%), *p*-xylene (1.16%), benzene, 1,2,3- trimethyl- (1.15%), *trans*-decalin, 2-methyl-(1.14%), benzene, 1-methyl-3-(1-methylethyl)- (1.01%), decane, 4-methyl- (0.99%), nonane, 3-methyl- (0.89%), decane, 2-methyl-(0.88%), cyclohexane, butyl- (0.85%), benzene, 1-ethyl-2-methyl- (0.81%), naphthalene, decahydro-, *trans*- (0.79%), naphthalene, decahydro -2-methyl-(0.78%), sulfurous acid, cyclohexylmethyl heptadecyl ester (0.77%), 3-methoxy-cinnamaldehyde (0.76%), 2-propanone, 1-(4-methoxyphenyl)- (0.74%), undecane, 2-methyl-(0.73%), nonane, 2-methyl- (0.72%), benzene, 2-ethyl-1,4-dimethyl- (0.71%), benzene acetic acid, ±-hydroxy-4-methoxy-, methyl ester (0.70%), 2-cyclohexen-1-ol, 3,5,5-trimethyl- (0.66%), cyclohexane, pentyl- (0.64%), undecane, 2,6-dimethyl- (0.61%), ±-terpineol (0.60%), heptane, 3-

ethyl-2-methyl- (0.59%), octane, 3,6-dimethyl- (0.59%), cyclohexane, propyl- (0.57%), benzene, 1-methyl-3-propyl- (0.56%), decane, 3-methyl- (0.56%), octane (0.56%), cyclohexane, 1-ethyl-1-methyl- (0.54%), tetradecane (0.54%), undecane, 4-methyl- (0.54%), benzene, 1,3-dimethoxy- 5-[(1E)-2-phenylethenyl]- (0.52%), stigmasta-4,6,22- trien-3±-ol (0.52%), undecane, 3-methyl- (0.52%), 2(1H) -naphthalenone, octahydro-4a-methyl-, *cis*- (0.50%), cyclohexanol, 2-[2-pyridyl]- (0.50%), benzene, 1,2,4,5 -tetramethyl- (0.44%), phenol, 2,4-bis(1,1-dimethyl -ethyl)-(0.44%), 2H-pyran, 2-(1,1-dimethyl ethoxy) etrahydro- (0.40%), naphthalene, 2-methyl- (0.39%), cyclohexane, 1,1,3-trimethyl- (0.37%), cyclohexane, ethyl- 0.36%), dodecane, 2-methyl- (0.36%), 1-ethyl-4- ethylyclohexane (0.33%), caryophyllene (0.32%), bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-ethyl-3- entenyl) (0.31%), dibutyl phthalate (0.31%), cyclohexane, 1,2,4-trimethyl-, (1α,2β,4β)- (0.29%), norbornane (0.29%), 15-hydroxydehydroabietic acid, methyl ester (0.26%), 1-ethyl-3-methylcyclohexane (c,t) (0.25%), 7,9-di-*tert*-butyl-1-oxaspiro(4,5)deca-6,9 diene-, -dione (0.25%).

According to GC/MS result, 1 component was identified from the many peaks provided by the methanol/ethanol and ether/ethanol extractives from the *Illicium verum* fruit. The results showed that the one component was anethole.

Molecular distribution of BPME extractives from *Illicium verum* fruit

The results of the GC-MS analysis showed that the molecular distribution of the BPME extractives from *Illicium verum* was the richest in terms of the number of components present after the first-stage of the extraction with the benzene/ ethanol system, with compounds such as anethole (74.93%), and cyclohexylbenzene (3.40%) being identified in the extractives. The relative hydrocarbon, alcohol (phenol alcohols), aldehyde/ ketone, ether, and acid/ester contents, as well as other unknown compounds, accounted for 11.38, 2.65, 1.83, 75.94, 0.47 and 7.73% of the benzene/ ethanol extractives, respectively. The most abundant components from the second-stage extraction with the petroleum ether/ethanol system were anethole (46.32%) and undecane (5.22%). The relative hydrocarbon, alcohol (phenol alcohols), aldehyde/ketone, ether, and acid/ester contents, as well as the other unknown compounds, accounted for 43.63, 2.72, 1.77, 46.32, 1.73 and 3.83% of petroleum ether/ethanol extractives, respectively. The one component of the third- and fourth-stage extractions using the methanol/ ethanol and ether/ethanol extraction systems, respectively, was anethole (100%). Taken together, the results suggested that the four extraction systems were suitable for the extraction of anethole, and that the second-stage extraction was better suited to the extraction of hydrocarbons.

The retention times of different components in different extractions of *Illicium verum* showed a particular trend. For the first-stage extraction, the molecules with retention times of ≤10, ≤20, ≤30, ≤40 min and ≥ 40 min were 0.00, 89.89, 9.55, 0.17 and 0.38%, respectively. For the second-stage extraction, the molecules with retention times of ≤10, ≤20, ≤30, ≤40 min and ≥40 min were 16.51, 77.73, 5.00, 0.26 and 0.52%, respectively. For the third- and fourth-stage extractions, the molecules with retention times of 16.82 to 16.95 min were 100%. The results showed that most of their components from four extractives of the *Illicium verum* fruit had retention times between 10-20 min.

Resource utilization of BPME extractives from *Illicium verum* fruit

Illicium verum is a medicinal wood-type plant, and its extractives and derivatives could be used as novel lead compounds to create new drugs. Research in this area has shown that there were many rare biomedical components in the BPME extractives of the *Illicium verum* fruit. Given its officinal value, pentadecane heneicosanoic and nonadecene were identified as the main components of the volatile oil from Qinghai Red King, and these components can be used for the treatment of pneumonia, coughs, hemoptysis, vaginal discharge, and the swelling of the limbs, as well as being used as a topical treatment for bruises, and scalding burns[10]. Nonadecene is used as the main component in many flavors, whereas hexadecyl ester, 9,12-octadecadienoic acid ester, 9-hexadecenoate twenty-enoate has been identified as the main medical component of dried worms, and has diuretic, swelling and detoxification properties[11]. Anethole has been widely used as a flavoring substance, and is present in the essential oil derived from guarana which has been reported to cause psychoactive effects. Furthermore, this material has shown potent antimicrobial properties,

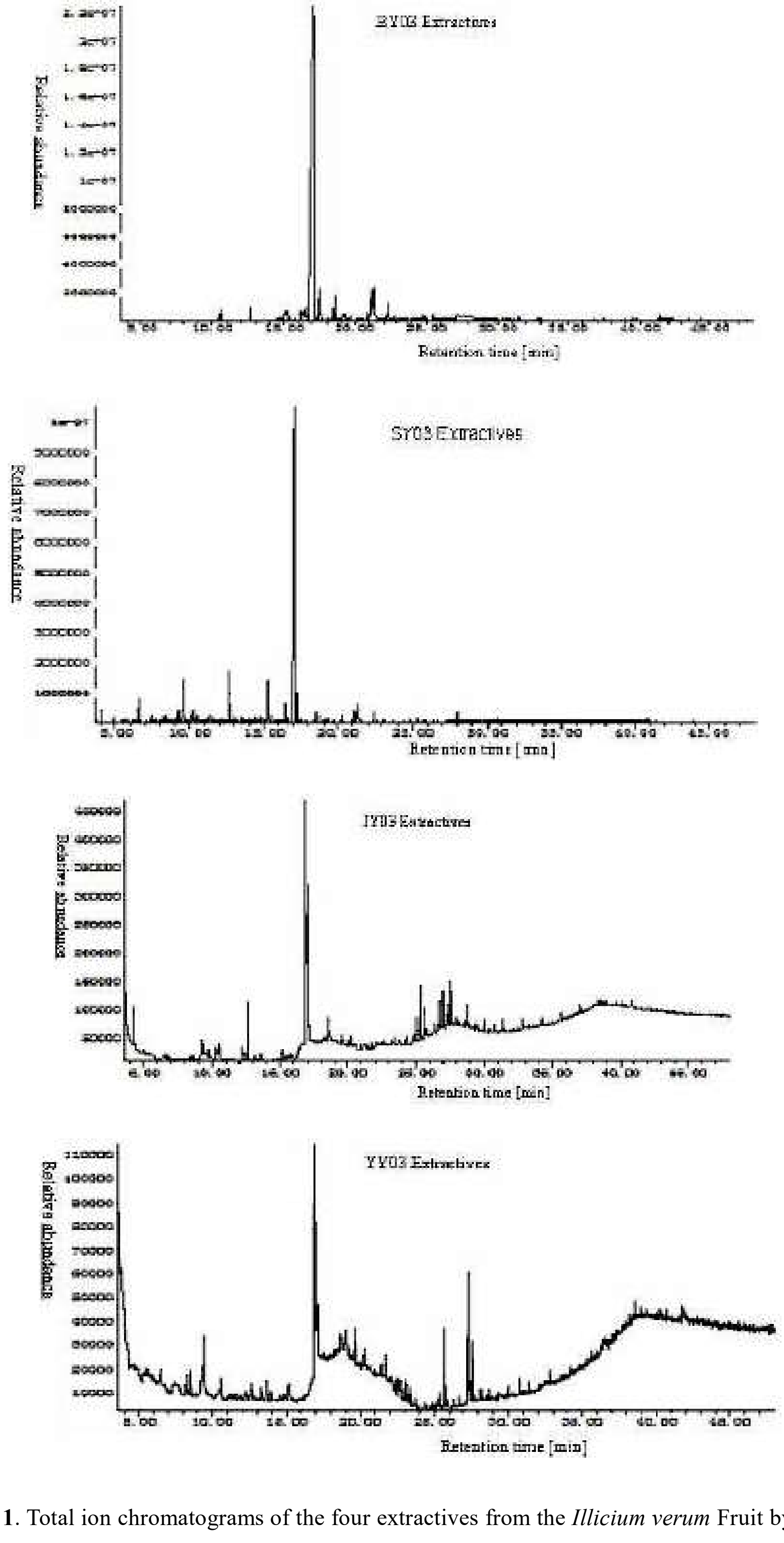

Fig. 1. Total ion chromatograms of the four extractives from the *Illicium verum* Fruit by GC/MS

against bacteria, yeast, and fungi[1]. In vitro, anethole has shown anthelmintic activity towards the eggs and larvae of the sheep gastrointestinal nematode *Haemonchus contortus*, and nematicidal activity against the plant nematode Meloidogyne javanica in vitro and in pots of cucumber seedlings[1]. Caryophyllene is the one of the aroma components of the *Illicium verum* fruit, and has been used as a flavoring of cloves, pepper, nutmeg, citrus and herbs[12]. Eucalyptol is a colorless natural organic compound that has a fresh camphor-like smell and a spicy, cooling taste. Eucalyptol can be used to reduce inflammation and pain, and destroy leukemia cells in vitro, and has also been used as a mouthwash and cough suppressant, as well as a treatment for headaches, sensitivity of the pressure points of the trigeminal nerve, nasal obstruction, impairment of general condition, and nasal secretions[13-17]. D-Limonene is a natural monoterpenoid which has been widely used in the perfume industry. This compound also exhibits efficacy as an anti-oxidation, anti-inflammatory, cholagogic and litholytic agent. D-Limonene can also be used for the prevention and treatment of cancers of the colon, breast, gut, lung, skin, liver, and pancreas induced by chemical carcinogens[18]. 3,7-Dimethyl-1,6-octadien-3-ol with its fragrance of muguet odorants is found in most common spices. This material can also induce low mood, depression and even life-threatening respiratory diseases. Terpinen-4-ol has been reported to inhibit bacterial, fungal and viral infections, as well as enhancing immunity and preventing baby diaper rash. (+)-4-carene is the main active ingredient of the essential oils from rosemary, and also exhibits a repellent effect towards *Aedes albopictus*[19]. cis-β-frnesene is the main active ingredient of Chinese dalbergia and has shown efficacy to quicken the blood and treat qi, analgesia and hemostasis. β-bisabolene is an aromatic volatile oil that can have a mild stimulating effect on the digestive tract that can lead to bowel tone, rhythm and enhanced motility promoting gastrointestinal digestive function. 2-[2-Pyridyl]-cyclohexanol is an aromatic medicine, which has also been used as a flavoring agents and carminative for the treatment of pain. Dehydroabietic acid possesses anti-fungal and hemostasis properties. Tetradecane, 2,6-dimethyl-undecane and 3-methyl-undecane are the main active ingredients of *Anemone Altaica Fisch* which is known to elicit sedative and analgesic effects. Tridecane is the main active ingredient of *Anemone Altaica Fisch* and exhibits activity against tumors and anti-pathogenic microorganisms, as well as improving immune function. α-terpineol can be used as a disinfectant, antioxidant, and medicine. Squalene, which can be used to resist fatigue and strengthen the body's resistance, as well as protecting the liver and improving human immunity, has been used in a number of nutraceutical and pharmaceutical products[20]. [1S-(1.alpha., 4a. alpha., 10a.beta.)]-1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-1- phenanthrene-carboxylic acid is the active ingredient in a number of skin care products and can be used to heal facial peeling[21]. β-cedrene and β-cedrene are used as the raw materials for advanced odorants[22]. α-Cadinol possesses anti-fungal and hepatoprotective properties and has also been proposed as a possible remedy for drug-resistant tuberculosis[23,24]. Stigmast-4-en-3-one, stigmasta-4,6,22-trien-3β-ol and γ-sitosterol are the physiological active of several natural medicines, and have been used as growth hormones in plants and animals, as well as being used as anti-inflammatory, antipyretic, and anti-ulcer treatments. Furthermore, ³-sitosterol has been used for the treatment of cervical cancer and skin cancer, and is one of the major active ingredients of hair perfume, shampoo, cream and other cosmetics products that are used for the moisturization of dry skin and keratinization, and to inhibit the formation of corns, and improve skin texture[15]. These medicines have effectively enhanced the economic value of *Illicium verum* fruit extractives for the future. As well as the medicinal properties of the *Illicium verum* fruit extractives, there are also were many toxic substances in the extractives, such as catechol, which is a known mutagen and carcinogen, ethyl-cyclohexane, which can elicit irritant and narcotic effects, and estragole, which is known to cause liver cancer. These toxic materials should clearly be avoided when *Illicium verum* fruit extractives are used. Based on its relative content of biomedical components, the BPME extractives of the *Illicium verum* fruit would be suitable for the extraction of anethole.

There were large number of common components in the extractives of *Illicium verum* fruit. The by-product components are often discharged and pose a serious pollution risk to the surrounding environment and lead to the generation of a lot of waste fruit. There were many components of liquid bioenergy including octane, cyclohexane, ethylcyclohexane, 1,1,3-trimethylcyclohexane, 1,2,4-trimethyl-, (1α,2β,4β)-, *p*-xylene, 1-ethyl-3-methylcyclohexane (c,t), nonane, norbornane, cyclohexane, propylnonane, 3-methylheptane, 3-ethyl-2-methyl-, 2H-pyran, 2-(1,1-dimethylethoxy) tetrahydro-, 2-cyclohexen-1-ol, 3,5,5-trimethyl-, benzene, 1-ethyl-2-methyl-, nonane, and 2-methyl-, mesitylene, and the by-products could also be used as a source of liquid bioenergy.

CONCLUSIONS

The BY03, SY03, JY03 and YY03 extractives of the *Illicium verum* fruit gave 34, 61, 1 and 1 components, respectively, that could be identified by GC-MS. The most abundant components of first-stage extractives were anethole (74.93%) and cyclohexylbenzene (3.40%), whereas the most abundant components in the second-stage extractives were anethole (46.32%) and undecane (5.22%). Only one component was identified in the third- and fourth-stage extractives, and this was anethole (100%). All four of the extractives of the *Illicium verum* fruit had their majority of their retention times between 10 and 20 min. Furthermore, the four extractives were suitable for the extraction of anethole, whereas only the second-stage extraction was suitable for the extraction of hydrocarbons.

The functional analytical result suggested that the BPME extractives of the *Illicium verum* fruit was rich in bioactive components that could be used in biomedicines, rare spices, and high-grade cosmetics and skin care products. Furthermore, the by-products could also be used as a source of liquid bioenergy. There were however, some toxic compounds in the BPME extractives of the *Illicium verum* fruit.

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