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ORIGINAL ARTICLE

Molecular characteristics of *Illicium verum* extractives to activate acquired immune response

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KEYWORDS

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Abstract *Illicium verum,* whose extractives can activate the demic acquired immune response, is an expensive medicinal plant. However, the rich extractives in *I. verum* biomass were seriously wasted for the inefficient extraction and separation processes. In order to further utilize the biomedical resources for the good acquired immune response, the four extractives were obtained by SJYB extraction, and then the immunology moleculars of SJYB extractives were identified and analyzed by GC–MS. The result showed that the first-stage extractives contained 108 components including anethole (40.27%), 4-methoxy-benzaldehyde (4.25%), etc.; the second-stage extractives had 5 components including anethole (84.82%), 2-hydroxy-2-(4-methoxy-phenyl)-n-methyl-acetamide (7.11%), etc.; the third-stage extractives contained one component namely anethole (100%); and the fourth-stage extractives contained 5 components including cyclohexyl-benzene (64.64%), 1-(1-methylethenyl)-3-(1-methylethyl)-benzene (17.17%), etc. The SJYB extractives of *I. verum* biomass had a main retention time between 10 and 20 min what's more, the SJYB extractives contained many biomedical moleculars, such as anethole, eucalyptol, [1S-(1α,4aα,10aβ)]-1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-1-phenanthrenecarboxylic acid, stigmast-4-en-3-one, γ-sitosterol, and so on. So the functional analytical results suggested that the SJYB extractives of *I. verum* had a function in activating the acquired immune response and a huge potential in biomedicine.

1. Introduction

Illicium verum, which originated in southwest China and northeast Vietnam, is an evergreen plant. *I. verum* fruits have traditionally been used as flavors. Simultaneously, *I. verum* was also used in medicine for a long time. *I. verum* has been documented in the book "Herbal Essentials Collection" in 1505's for the treatment of many fistula and cholera. In 1769, HUANG Gongxiu found that *I. verum* could heal heavy cold and inveterate cold. It was recorded in the book "Herbal positive" that *I. verum* could remove teeth mouth disease,

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detoxify and descend Qi. WANG Fu discovered that *I. verum* played a role in the treatment of relieving the depressed liver, reinforcing the kidney, and healing beriberi. And *I. verum* is widely used in many traditional Chinese medicines today. *I. verum* fruits were diuretic, antibacterial, stimulant, carminative, odontalgic and stomachic (Chopra et al., 1956; Ashraf et al., 2011). *I. verum*, which was prescribed as an digestive aid which could make the nursing mothers promote breast-milk production, had the anti-bacterial and anti-fungal affection of asthma, bronchitis and dry cough, refreshed the breath, and ensured a good sleep (Cheng and Changli, 2007; Qin et al., 2005; Ashraf et al., 2012). Its essential oil, which contained 75–90% anethole and had the observed estrogenic effect, was useful in providing relief from rheumatism and lower back pain (Wang et al., 2011; Qin et al., 2005; Qureshi et al., 2015). The pharmaceutical ingredients of *I. verum* began research and analysis since 1948. Kouno et al. reported neolignans and a phenylpropanoid glucoside from *Illicium* defengpi in 1993 (Kouno et al., 1993). In 1999, Thomas et al. studied novel secoprezizaane sesquiterpenes from North American *IIIicium* species (Thomas and Schmidt, 1999). Chinese and Japanese scholars all studied some chemical compositions of *I. verum* biomass (Kenichi et al., 1983, 1984; Yoshiyasu et al., 1994). What's more, Chinese scientists have done extensive researches on the extraction technology, active ingredients identification and utilization of *I. verum* biomass (Lei and Jun, 2006; Zhi-ke and Shen, 2007; Wenyi et al., 2008; Jiahui et al., 2008; YuLing et al., 2006; Mingdong and Chenghuan, 2011; Nasreen et al., 2015). Especially, after *I. verum* was eaten, its extractives were dissolved in the blood which could activate the acquired immune response to resist the virus intrusion. Currently, the main biological active ingredients were resin, fatty oil, protein, volatile oil, etc. *I. verum* biomass was initiated for the production of Tamiflu in 2005. The Independent reported news that the medicinal ingredient of Tamiflu was shikimic acid which came from *I. verum* (Krämer et al., 2003; Johansson et al., 2005; Hashemi et al., 2015). It made *I. verum* a famous botanical anti-bird plant which has been planted more than 3.3 × 105 ha in south China. However, rich extractives in *I. verum* biomass were seriously wasted for the inefficient extraction and separation processes. the four extractives were obtained by SJYB extraction, and then the biomedical molecular of SJYB extractives were identified and analyzed by GC–MS so as to further utilize the biomedical resources.

2. Materials and methods

2.1. Materials

I. verum fruits were collected from Nanning Forest Farm, Guangxi province, P.R. China. The fresh fruits were dried in the indoor air, and about 40 mesh powder was sieved out using AS200 Sieving Instrument (Made in America). Benzene, methanol, ether, petroleum ether, and ethanol (chromatographic grade) were prepared for subsequent experiments. Quantitative filter paper, cotton bag, and cotton thread were all extracted in benzene-ethanol solution for 12 h. The benzene–ethanol solution was mixed according to $V_{\text{ethanol}}/V_{\text{benzene}}$ 4 double. The methanol–ethanol solution was mixed according to $V_{\text{ethanol}}/V_{\text{methanol}}$ 3 double. The ether–ethanol solution was mixed according to $V_{\text{ethanol}}/V_{\text{ether}}$ 9 double. And the petroleum ether–ethanol solution was mixed according to $V_{\text{ethanol}}/V_{\text{petroleum ether}}$ 1 double.

2.2. Methods

8 pieces of the above powders were weighed, each 10 g (1.0 mg accuracy), and then parceled by using the cotton bag and tied by using cotton thread, and signed. Extraction was gradually carried out by large-caliber Soxhletor and extracted in 800 ml petroleum ether–ethanol, methanol–ethanol, ether–ethanol, and benzene–ethanol solution, respectively. Parallel sample number was 2. Extraction time of petroleum ether–ethanol, methanol–ethanol, ether–ethanol, and benzene–ethanol solution was 5 h, 3 h, 5 h, and 9 h, respectively. Extraction temperature was 85–90 °C. After extraction, four extractive solutions were obtained and dried to 10 ml under the condition of 45 °C and a vacuum of 0.05–0.07 MPa. The petroleum ether–ethanol, methanol–ethanol, ether–ethanol, and benzene–ethanol extractives were obtained, respectively.

2.3. GC–MS analysis

The above extractives were analyzed by online linked gas chromatograph/mass spectrometer(GC–MS), respectively. The GC/MS analysis was carried out on a Aglient 6890 N + 5975C GC–MSTM (Aglient Co., Ltd, USA), which was 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 of GC began at 50 °C and increased at the rate of 8 °C/min until 250 °C, 5 °C/min until 300 °C was reached, followed by a split injection at a ratio of 15:1. The program of MS was scanned over the 35–335AMU (m/z) respectively, 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. Ion source temperature: 230 °C, quadrupole temperature 150 °C (Mateen et al., 2015).

3. Results and discussion

During SJYB extraction, four extractives (petroleum ether–ethanol extractives, methanol-ethanol extractives, ether–ethanol extractives, and benzene-ethanol extractives) were obtained respectively. The total ion chromatograms of four extractives by GC/MS are shown in Fig.1, respectively. Relative content of each component was counted by area normalization. Analyzing the MS data, the NIST standard MS map by computer, open-published books and papers [10–27], then components and their contents were identified.

3.1. Components of SJYB extractives from I. verum

According to GC/MS result, 108 components were identified on 124 peaks of SY04 extractives from *I. verum* fruit. The main components were anethole (40.27%), 4-methoxy-benzaldehyde (4.25%), 2-hydroxy-2-(4-methoxy-phenyl)-n-methyl-acetamide (3.53%), spiro [5.5]undecane (1.83%), 2,6-dimethyl-6-(4-methyl-

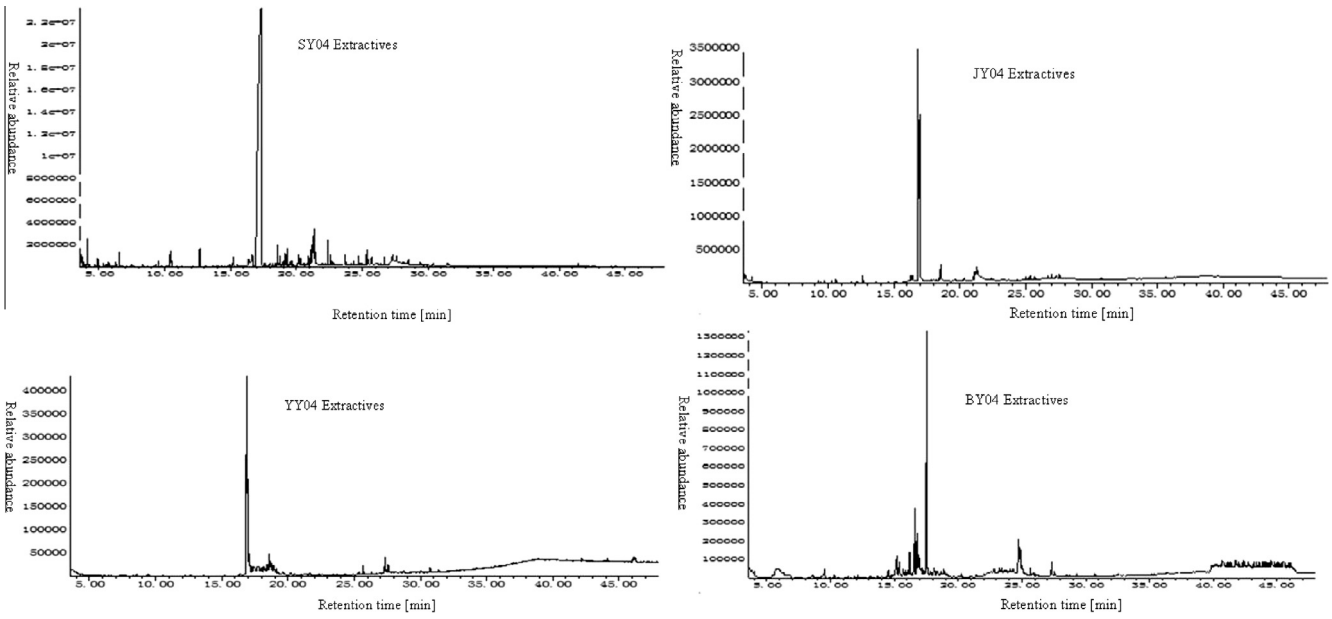

Figure 1 Total ion chromatogram of four extractives from *Illicium verum* Fruit by GC/MS.

3-pentenyl)-bicyclo[3.1.1]hept-2-ene (1.82%), docosane (1.71%), 2-methyl-naphthalene (1.69%), 4-methoxycinnamaldehyde (1.55%), 4-methoxy-benzoic acid methyl ester (1.44%), 1-(4-methoxyphenyl)-2-propanone (1.29%), 3-methoxy-acetophenone (1.24%), caryophyllene (1.24%), 2-[2-pyridyl]-cyclohexanol (1.19%), 1,6,7-trimethyl-naphthalene (1.18%), tetradecane (1.18%), β-bisabolene (1.08%), 1-methoxy-4-(1-methylpropyl)-benzene (1.08%), 4-methoxybenzoic acid, 2,3-dichlorophenyl ester (1.05%), 4-methoxyphenyl-propane-2-ol (1.03%), decahydro-4,4,8,9,10-pentamethylnaphthalene (0.95%), cis-β-farnesene (0.94%), and so on. Others were 2,3-dihydro-4,7-dimethyl-1h-indene, 1-(2-butenyl)-2,3-dimethyl-benzene, 1,4,6-trime-thyl-naphthalene, 4-dimethyl (ethenyl)silylbut-1-en-3-yne, [1s-(1α,4aα,10aβ)]-1,2,3,4, 4a, 9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-1-phenanthrenecarboxylic acid, hexadecane, heptadecane, 2-methyl-1, 1'-biphenyl, octadecane, 3,7-dimethyl-1,6-octadien-3-ol, 1,2,3,4-tetrahydro-1,4-dimethyl-naphthalene, 1,6-dimethyl-naphthalene, sulfurous acid butyl dodecyl ester, 1,2,3,4-tetrahydro-1,1,6-trimethyl-naphthalene, 2-methyl-tetradecane, 4-methyl-tridecane, 2,3,6-trimethyl-naphthalene, n-hexadecanoic acid, pentadecane, 1,4-dimethyl-naphthalene, 8-amino-5-fluoro-6-methoxy-2-methylquinoline, 2,6,10-trimethyl-pentadecane, 2,6-dimethyl-undecane, 2,7-dimethyl-naphthalene, octane, (r)-4-methyl-1-(1-methyl-ethyl)-3-cyclohexen-1-ol, (z)-(3,3-dimethylcyclohexy-lidene)-acetaldehyde, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-cyclohexane, d-limonene, α-farnesene, 1-(1-methylethenyl)-3-(1-methylethyl)-benzene, 1,2-dimethoxy-4-(1-propenyl)-benzene, dibutyl phthalate, 1,4-dimethoxy-2-methyl-benzene, nonadecane, pulegone, isobutyl undecyl phthalic acid ester, nonane, dehydroabietic acid, 2,6,10,14-tetramethyl-hexadecane, eicosane, p-xylene, hexadecanoic acid ethyl ester, linoleic acid ethyl ester, 9,10-dimethylanthracene, heneicosane, 1-methyl-anthracene, ethyl-cyclohexane, 2-methyl-phenanthrene, 2,3-dihydro-4-methyl-1h-indene, eucalyptol, 1-methyl-phenanthrene, 1,1,3-trimethyl-cyclohexane, cis-1,3-dimethyl-cyclohexane, decane, (e)-4,4-dimethyl-2-pentene, 3-methyl-4-(phenylthio)-5h-furan-2-one, stigmast-4-en-3-one, tricosane, 1,2,3-trimethyl-benzene, trans-1,2-dimethyl-cyclohexane, 2-carene, 9-[(4-methoxybenzoyl) oxy]-9-borabicyclo[3.3.1]nonane, (1α,2β,4β)-1,2,4-trimethyl-cycloh-exane, 4-methyl-octane, α-pinene, trans-1,3-dimethyl-cyclohexane, tetrahydro naphthyl methyl carbamate, (+)-3-carene, 1-ethyl-3-methyl-benzene, methyl-cycloheptane, 1-ethyl-2-methyl-cyclo-pentane, ethylbenzene, tetracosane, 2-(2-chlorophenyl)-1h-indole, 4-methoxy-n-[2-(4-methoxyphenyl) ethyl]-benzeneethanamine, 1-adamantan-1-yl-1,3-dihydro-benzoimidazol-2-one, 4-methylene-1-(1-methylethyl)-bicyclo[3.1.0]hexane, trans-1-ethyl-2-methyl-cyclohexane, propyl-cyclohexane, propyl-cyclopentane, 2,6-dimethyl-heptane, hexacosane, 1-methyl-2-propyl-cyclopentane, methabenzthiazuron, γ-sitosterol, and (z)-3,7-dimethyl-1,3,6-octatriene.

5 components were identified on 7 peaks of JY04 extractives from *I. verum* fruit. The components were anethole (84.82%), 2-hydroxy-2-(4-methoxy-phenyl)-n-methyl-aceta-mide (7.11%), 1-(4-methoxyphenyl)-2-propanone (4.18%), trans-4-methoxycinnamaldehyde (2.05%), and undecane (1.84%).

1 component was identified on many peaks of YY04 extractives from *I. verum* fruit. The component was anethole (100%).

5 components were identified on 5 peaks of BY04 extractives from *I. verum* fruit. The components were cyclohexyl-benzene (64.64%), 1-(1-methylethenyl)-3-(1-methyl ethyl)-benzene (17.17%), (3-methylcyclopentyl)-benzene (9.80%), 5,6,7,8,9,10-hexahydro benzocyclo octene (5.38%), 2,7-bis(1,1-dimethylethyl)-naphthalene (3.01%).

3.2. Molecular distribution of SJYB extractives from I. verum

The GC–MS analysis results show the molecular distribution of SJYB extractives from *I. verum*. The richest components of first-stage extractives (petroleum ether–ethanol extractives) were anethole (40.27%), 4-methoxy-benzaldehyde (4.25%), etc. Relative content of hydrocarbons, alcohols (phenol

alcohols), aldehydes/ketones, ethers, acid/esters, and other compounds occupied 45.17%, 3.08%, 0.19%, 40.27%, 5.26%, and 6.03% of petroleum ether–ethanol extractives, respectively. The richest components of second-stage extractives (methanol-ethanol extractives) were anethole (84.82%), 2-hydroxy-2-(4-methoxy-phenyl)-n-methyl-acetamide (7.11%), etc. Relative content of hydrocarbons, alcohols (phenol alcohols), aldehydes/ketones, ethers, acid/esters, and other compounds occupied 3.89%, 0.00%, 4.18%, 84.82%, 0.00%, and 7.11% of methanol–ethanol extractives, respectively. The only component of third-stage extractives (ether–ethanol extractives) was anethole (100%). The richest components of fourth-stage extractives (benzene-ethanol extractives) were cyclohexyl-benzene (64.64%), 1-(1-methylethenyl)-3-(1-methylethyl)-benzene (17.17%), etc. Relative content of hydrocarbons occupied 100%. The results suggested that the first-stage, second-stage and third-stage extractives were suitable to extract anethole, and fourth-stage extractives were suitable to extract hydrocarbons (Khaskheli et al., 2015).

The retention time of each stage extractive of *I. verum* showed a particular rule. Among the first-stage extractives, the molecules with the retention time of $\leqslant$10 min, $\leqslant$20 min, $\leqslant$30 min, $\leqslant$40 min and $\geqslant$40 min were 2.8%, 71.94%, 24.91%, 0.22% and 0.42%, respectively. Among the second-stage extractives, the molecules with the retention time of $\leqslant$10 min, $\leqslant$20 min, $\leqslant$30 min, $\leqslant$40 min and $\geqslant$40 min were 0.00%, 90.84%, 9.16%, 0.00% and 0.00%, respectively. Among the third-stage extractives, the molecules with the retention time of 16.82–16.95 min were 100%. Among the fourth-stage extractives, the molecules with the retention time of $\leqslant$10 min, $\leqslant$20 min, $\leqslant$30 min, $\leqslant$40 min and $\geqslant$40 min were 0.00%, 96.99%, 3.01%, 0.00% and 0.00% respectively. The results showed that the four extractives of *I. verum* biomass had a main retention time between 10 and 20 min (Nasreen et al., 2015).

3.3. Resource utilization of SJYB extractives from I. verum

There were many biomedical components in the SJYB extractives of *I. verum* biomass. Because of its officinal value, α-cedrene and β-cedrene were used for the raw materials of advanced odorants. Squalene, which was used in nutraceutical and pharmaceutical industries, could resist fatigue and strengthen the body's resistance, protect the liver, and improve human immunity (Kim and Karadeniz, 2012). α-Cadinol, which acted as anti-fungal and as hepatoprotective, was proposed as a possible remedy for drug-resistant tuberculosis (Bueno et al., 2011; Ho et al., 2011; Tung et al., 2011). Anethole, which was widely used as a flavoring substance, had potent antimicrobial properties, against bacteria, yeast, and fungi (De et al., 2002; Camurça-Vasconcelos et al., 2007). In vitro, anethole also had antihelminthic action, haemonchus contortus, and nematicidal activity against the plant nematode meloidogyne javanica in vitro and in pots of cucumber seedlings (Oka et al., 2000; Weiming, 2005). Eucalyptol, which had a fresh camphor-like smell and a spicy, cooling taste, could reduce inflammation and pain, kill leukemia cells in vitro, treat mouthwash and cough suppressant, lower headache on bending, frontal headache, sensitivity of pressure points of trigeminal nerve, nasal obstruction, impairment of general condition, and rhinological secretion (Wanxi et al., 2012a,b; Ashraf et al., 2013). [1S-(1α,4aα,10aβ)]-1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-1-phenanthrenecarboxylic acid was the active ingredient of skin care which could heal facial peeling[28]. Especially, stigmasta-4,6,22-trien-3β-ol and γ-sitosterol could reduce serum cholesterol and had effect on atherosclerotic lesion development (Gehui et al., 2002; Tabassum et al., 2014). According to the relative content of biomedicine components, the SJYB extractives of *I. verum* biomass were suitable to extract anethole.

4. Conclusions

The 108, 5, 1 and 5 components were identified on the peaks of SY04, JY04, YY04 and BY04 extractives from *I. verum* fruit, respectively. The richest components of first-stage extractives were anethole (40.27%), 4-methoxy-benzaldehyde (4.25%), etc. The richest components of second-stage extractives were anethole (84.82%), 2-hydroxy-2-(4-methoxy-phenyl)-n-methyl-acetamide (7.11%), etc. The only component of third-stage extractives was anethole (100%). The richest components of fourth-stage extractives were cyclohexyl-benzene (64.64%), 1-(1-methylethenyl)-3-(1-methylethyl)-benzene (17.17%), etc. And the four extractives of *I. verum* biomass had a main retention time between 10 and 20 min what's more, the first-stage, second-stage and third-stage extractives were suitable to extract anethole, and fourth-stage extractives were suitable to extract hydrocarbons.

The functional analytical result suggested that the SJYB extractives of *I. verum* biomass contained rich immunogenetic function components which had huge potential in biological medicine, especially including anethole, [1S-(1α,4aα,10aβ)]-1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-1-phenanthrenecarboxylic acid, stigmast-4-en-3-one and γ-sitosterol, and so on.

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