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Research Notes

The Phytol-rich Essential Oil from Fresh *Medicago hispida* Exerts Significant Inhibitory Activity against *Escherichia coli*

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Abstract: *Medicago hispida* is one of the most widely distributed and commonly cultivated plants of the genus *Medicago* in China. It is mainly consumed as a vegetable. In this paper, the extraction, chemical composition and antimicrobial potential of the essential oil from fresh *M. hispida* (EOFMH) were conducted and evaluated for the first time. The extraction yield of EOFMH was found to be 0.27% and 27 compounds were identified in EOFMH by gas chromatography-mass spectrometer (GC-MS) with the highest level of phytol, accounting for 48.8%. EOFMH exhibited certain inhibitory effects against *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, *Bacillus pumilus* and *Bacillus subtilis*, particularly its antibacterial activity against *E. coli* was equivalent to that of chloramphenicol. Results obtained from present work provided strong evidence that EOFMH could be considered as a phytol-rich natural product and a new source of nature-based antibacterial agent to be applied in industry.

Keywords: *Medicago hispida*, Essential Oil, Phytol, Antibacterial Activity

Introduction

The safety issues of foods, pharmaceuticals and cosmetics regarding pathogenic microorganisms have become a particular concern for industries due to the fact that microbial contamination can lead to the deterioration of products, raising heath risk for consumers and the propagation of infectious diseases (Belletti *et al*., 2007). Preservatives are essential additives with microbial inhibition in industrial production, which can be divided into chemical synthetics and natural counterparts according to their sources. For example, sodium benzoate and potassium sorbate are widely used chemical preservatives in food industry owing to their high antimicrobial activities, long-acting time, convenient processing and low cost (Beales, 2004). However, long-term intake of chemically obtained preservatives could cause certain harmfulness to human health, leading to serious diseases eventually (Li *et al*., 2012). Nowadays, natural preservatives such as Essential Oils (EOs), organic acids, bacteriocins and phenolic compounds have aroused increasing attentions (Aziz and Karboune, 2016). EOs possess a broad spectrum of physiological and pharmaceutical functions such as antimicrobial, anticancer and antioxidant properties, which have been fully confirmed by literatures (Prakash *et al*., 2015). Especially, in the preservation of meat, dairy products, egg-based products and cosmetics, etc., EOs can inhibit pathogenic microorganisms effectively (El Abed *et al*., 2014; Nostro *et al*., 2004). Therefore, as a nature-based efficient and safe preservative, EOs have gained broadly applicable prospects.

The genus *Medicago*, a perennial leguminous herb comprises 87 species of flowering plants worldwide (Gholami *et al*., 2014), mainly distributed in the Mediterranean region, Europe, southwest Asia, central Asia and Africa. There are approximately 15 species belonging to plants of the genus *Medicago* in China, of which *Medicago sativa* and *Medicago hispida* are two of the most widely distributed and commonly cultivated species with total cultivation area of about 188 hm^2 (Michaud *et al*., 1988; Gao *et al*., 2006). At present, the active compounds of *M. sativa*, including saponins, flavonoids, phytoestrogens, coumarins, alkaloids and terpenes have been well investigated and these beneficial

ingredients could contribute to the pharmacological effects of neuroprotective, cholesterol-lowering, antioxidant, antibacterial and lipid-lowering capacities (Bora and Sharma, 2010). However, as for *M. hispida*, the species that is mainly consumed as a vegetable in China, there has been little information on its chemical components and activities.

Herein, the Essential Oil from Fresh *M. hispida* (EOFMH) was extracted and analyzed for the first time. Meanwhile, the antimicrobial potential of EOFMH was also evaluated for the discovery of novel nature-based preservative.

Materials and Methods

Materials and Chemicals

The fresh *M. hispida* used in this experiment were collected from the farmland of Southeast Development Zone, Changshu city, Jiangsu province, China, on December 10, 2018 and authenticated by Prof. Yongchun Su, Changshu Institute of Technology, Changshu, Jiangsu, China. A voucher specimen (No. SBFE-0018) was stored at the herbarium, School of Biology and Food Engineering, Changshu Institute of Technology. All chemicals in this work were analytical grade and microbial strains were purchased from Beijing Zhongke Quality Inspection Biotechnology Co., Ltd. (Beijing, China).

Essential Oil Extraction

One hundred grams of fresh *M. hispida* were treated with wet grinding and immersed in distilled water for 2 h at liquid-to-solid ratio of 40: 1 (mL/g), which were then transferred into a Clevenger apparatus for 6 h of extraction starting from water boiling. The obtained EOFMH with light yellow color was dried over anhydrous sodium sulfate and stored at 4℃ until analysis (Cui *et al*., 2018).

Gas Chromatography-Mass Spectrometry Analysis

Chemical components of EOFMH were analyzed by using a QP 2010 Gas Chromatography Mass Spectrometry (GC-MS) (Shimadzu, Kyoto, Japan) equipped with a Rxi-5Sil MS column (30 m ×0.25 mm ×0.25 μm). The analytical conditions were as follows: Helium was used as carrier gas at flow rate of 1.0 mL/min; column temperature was set at 40℃ (maintained for 4min), reached to 60℃ at heating rate of 5℃/min (maintained for 2 min), increased from 60℃ to 110℃ at 15℃/min (maintained for 4 min), then climbed to 180℃ at 5℃/min (maintained for 5 min), finally raised from 180℃ to 280℃ at 25℃/min (maintained for 5 min); transmission line temperature was 250℃; ion trap temperature was 230℃; ionization energy was 70 eV and scan range was from 35 to 500 amu. One microliter of EOFMH solution (dissolved in ether solution at concentration of 12 mg/mL) was injected into the column at split ratio of 1: 30. Compounds in EOFMH were identified by comparing both the retention index relative to standards of C_8-C_{40} *n*-alkanes and the mass spectra library (NIST 05) (Elkady and Ayoub, 2018).

Antimicrobial Activity

Staphylococcus aureus ATCC 25923, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 10231, *Bacillus pumilus* ATCC 7065 and *Bacillus subtilis* ATCC 6633 were used as test microbial strains to evaluate the antimicrobial potential of EOFMH. In brief, EOFMH solution (dissolved in dimethyl sulfoxide at concentration of 200 mg/mL) was diluted to serial dilutions (100~0.78 mg/mL) by 2-fold dilution method. Fifty microliters of solution were added into a 96-well plate and 150 μL of microbial liquid made of fresh medium containing 10^7~10^8 CFU/mL microbial strains were added into each well, which were placed in an incubator at 37℃ for 24 h, then the absorbance at 600 nm was determined. Chloramphenicol solutions (100~0.78 mg/mL) were used as positive control and 20% dimethyl sulfoxide was served as negative control. The Minimum Inhibitory Concentration (MIC) was the lowest concentration that completely inhibited the growth of tested microbial strains in the wells (Mann and Markham, 1998).

Results and Discussion

Chemical Components

The extraction yield of EOFMH by hydrodistillation was found to be 0.27% (w/w). As shown in Table 1, twenty seven compounds were identified in EOFMH with the highest level of phytol, accounting for 48.8%, followed by some alkane compounds with total content of 29.8%, suggesting that EOFMH could be used as a phytol-rich natural product.

Antimicrobial Activity

The antimicrobial activities of EOFMH were measured by the inhibition of *E. coli*, *S. aureus*, *C. albicans*, *B. pumilus* and *B. subtilis*, respectively. The MIC values of EOFMH against theses microbial strains were listed in Table 2. It can be seen that EOFMH possesses certain inhibitory effects on the above-mentioned microbial strains, particularly its antibacterial activity against *E. coli* was equivalent to that of chloramphenicol, a well-known antimicrobial agent available on the market. The pronounced

antibacterial activity of EOFMH against *E. coli* could be attributed by phytol, which has been found in EOFMH with total content of 48.8%. Previous study has confirmed that phytol elicits obvious bacterial activities and the underlying antibacterial mechanism may involve in the oxidative stress-mediated DNA damage to bacteria (de Santos *et al*., 2013). In addition to the antibacterial activities, phytol also exerts several other beneficial functions, including antioxidation, antitumor and immune enhancement (Jeong, 2018; de Santos *et al*., 2013). Thus the search for natural resources of phytol remains ongoing concern.

Nowadays, due to the pronounced antimicrobial capacities, different kinds of EOs have been widely used in food preservation (Ju *et al*., 2018), which has been fully verified in the storage of meat, dairy products, eggs and other animal-derived foods (El Abed *et al*., 2014; Nostro *et al*., 2004). In present investigation, it was found that the obtained EOFMH exhibits significant inhibitory effects against *E. coli*, which is one of the most common indicative bacteria that must be measured in the hygiene control of foods, pharmaceuticals, biotechnological products and cosmetics (Lues *et al*., 2006; Ragheb *et al*., 2012).

Table 1: Chemical profiles of EOFMH

No.	Compounds	Molecular formula	Rt/min	RI	RI*	Type	%
1	3-Methoxy-1,2-propanediol	$C_4H_{10}O_3$	6.270	900	924	Alcohol	0.06
2	*β*-Caryophyllene	$C_{15}H_{24}$	24.74	1494	1528	Alkene	1.06
3	Hexadecane	$C_{16}H_{34}$	25.77	1612	1564	Alkane	0.05
4	2, 4-Ditert-butylphenol	$C_{14}H_{22}O$	27.21	1555	1600	Phenol	0.08
5	Heptadecane	$C_{17}H_{36}$	29.46	1711	1701	Alkane	0.08
6	2,6,10,15-Tetramethylheptadecane	$C_{21}H_{44}$	31.93	1852	1807	Alkane	0.29
7	Hexadecanal	$C_{16}H_{32}O$	32.35	1800	1810	Aldehyde	0.15
8	Nonadecane	$C_{19}H_{44}$	35.18	1910	1901	Alkane	0.25
9	Eicosane	$C_{20}H_{42}$	37.75	2009	2001	Alkane	0.31
10	Ethyl heptadecanoate	$C_{19}H_{38}O_2$	39.05	2077	2096	Ester	0.23
11	Elaidyl alcohol	$C_{18}H_{36}O$	39.35	2061	2115	Alcohol	0.73
12	Heneicosane	$C_{21}H_{44}$	39.50	2109	2142	Alkane	0.68
13	Docosane	$C_{22}H_{46}$	40.05	2208	2200	Alkane	2.74
14	Phytol	$C_{20}H_{40}O$	40.18	2200	2218	Alcohol	48.8
15	2-[[2-[(2-Ethylcyclopropyl)methyl]cyclopropyl]methyl] cyclopropaneoctanoic acid methyl ester	$C_{22}H_{38}O_2$	40.55	2266	2266	Ester	1.07
16	Ethyl linolenate	$C_{20}H_{34}O_2$	40.61	2201	2258	Ester	1.79
17	*Cis*-9-tricosene	$C_{23}H_{46}$	40.88	2315	2311	Alkene	3.28
18	Tetracosane	$C_{24}H_{50}$	41.25	2407	2364	Alkane	0.13
19	Pentacosane	$C_{25}H_{52}$	42.21	2506	2500	Alkane	3.48
20	9-Hexacosene	$C_{26}H_{52}$	42.78	2614	2577	Alkene	2.28
21	Diisooctyl phthalate	$C_{24}H_{38}O_4$	43.47	2704	2662	Ester	1.08
22	Hexacosane	$C_{26}H_{54}$	43.79	2606	2675	Alkane	5.35
23	1-Hexacosanol	$C_{26}H_{54}O$	44.55	2848	2779	Alcohol	3.22
24	Methyl pentacosanoate	$C_{26}H_{52}O_2$	45.12	2773	2832	Ester	2.47
25	Nonacosane	$C_{29}H_{60}$	45.92	2904	2900	Alkane	4.16
26	Triacontane	$C_{30}H_{62}$	47.34	3003	3000	Alkane	7.53
27	Dotriacontane	$C_{32}H_{66}$	50.37	3202	3161	Alkane	4.78
	Total						96.1
	Diterpene alcohol compounds						48.8
	Sesquiterpenoids						0.29
	Dicyclic sesquiterpenes						1.06
	Alkane compounds						29.8
	Others						16.2

RI is the retention index determined by the database NIST 05; RI* is the retention index calculated according to the retention time of C_8 – C_{40} *n*-alkanes.

Table 2: MIC values of EOFMH

	E. coli	*S. aureus*	*C. albicans*	*B. pumilus*	*B. subtilis*
EOFMH (mg/mL)	< 0.78	1.56	6.24	50	50
Chloramphenicol (mg/mL)	< 0.78	< 0.78	12.5	< 0.78	< 0.78

EOFMH refers to the essential oil from fresh *M. hispida*

Compared to use phytol directly, the advantages to apply EOFMH for bacteria inhibition could be summarized as the following two points: (1) In addition to phytol, other ingredients present in EOFMH such as sesquiterpenes may potentiate the antibacterial effects (Bartikova *et al*., 2014). (2) Alkanes in EOFMH belong to liposoluble compounds, which could promote the dispersity of phytol in medium, enhancing the bioavailability of phytol in organisms, thereby improving the bioactivities (Chaud *et al*., 2010; Wang *et al*., 2014). These superiorities could make EOFMH more applicable than that of phytol in food-related preservation.

Conclusion

With the aim to obtain essential oil-based natural antimicrobial agent, in this work, the extraction, chemical constituents and antimicrobial activities of the essential oil from fresh *Medicago hispida* (EOFMH) were explored for the first time. The extraction yield of EOFMH was found to be 0.27% and twenty seven components were identified in EOFMH with dominant compound of phytol, accounting for 48.8%. The obtained EOFMH exerted inhibitory effects against five tested microbial strains, especially prominent antibacterial activity against *E. coli*. Moreover, further study should be made to fully assess its potential use in industry.

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Author's Contributions

Lixue Zheng, Bin Qi and Yang Zhang: Designed and carried out the experiments.

Leijie Ben and Qian Fu: Performed the experiments of extraction and antimicrobial evaluations.

Limei Wang: Conducted antimicrobial experiments.

Zhumei Cui: Processed data.

Lixue Zheng: Wrote manuscript.

Yang Zhang: Revised the manuscript.

Ethics

All authors declared no ethical issues that may arise after the publication of this manuscript.

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