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Properties and Exactions of Essential Oil from the Fruit of Clausena Lansium(Lour.) Skeels in Zhaoqing Area

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Abstract: *The water vapor distillation method was used for extracting the volatile oil in Wampee fruits, the volatile oil was identified by thin layer chromatography (TLC), and the component extraction for volatile oil was determined by GCMS computer combined with identification instrument the composition and the peak area normalization method to get the relative content of each component. Results showed that the 25 peaks isolated were identified 23 components which are phellandrene (36.97%), terpinen-4-ol (17.58%), sabinene hydrate (13.76%). These three substances are relatively high. In the study of the antioxidant activity of the volatile oil, the effect of the scavenging rate of hydroxyl radical was studied. It was found that the concentration of volatile oil in a certain concentration range was positively correlated with the clearance rate of hydroxyl radical. The filter paper method for study the antimicrobial activity of the essential oil presented that it all had a certain antibacterial effect to Staphylococcus aureus, Bacillus subtilis and Escherichia coli. And inhibition of different concentrations of essential oils was different for different types of bacteria. This bacteriostasis becomes smaller as with the decrease of concentration. And the effect of essential oil on mouse pancreatic cancer cells was also studied. The experiments showed that the essential oil had an effect of tumor inhibition.*

Keyword: *Fruit of clausena lansium(Lour.) skeels, essential oil;chemical components, properties*

1. INTRODUCTION

Wampee, one of the popular tropical fruit is Rutaceae evergreen small trees or large shrubs and has a high nutritional value. This fruit has a high medicinal value including the leaf, fruit and seeds as medicine, It could help stomach health and digestion, relieve thirst , gas, cough, pain and other effects(LI Ruizhen et al， 2007), Wampee fruit is rich in amino acids, sugars, organic acids, pectin, vitamin C, essential oils, flavonoids and other effective components(Tang Wenning et al.,2002).Zhaoqing also has many of the specific Wampee in China, and Wampee seeds are rich of oil belonging to the traditional chinese medicine volatile oil with a variety of biological activities as antibacterial, anti-inflammatory, anti-cancer, analgesic, diuretic and so on (Jiang Zitao et al.,**2012**). Therefore, the research on the volatile components and the development of its medicinal value would have an important significance. Volatile oil from Clausena lansium used making perfume process has not been reported up to now. So fitting of essential oil being made of perfume from wampee fruit is necessary for the antioxidant and inhibitory effects cancer effect analysis, It would provide a theoretical and experimental basis for the study of yellow fruit essential oil and also have a profound significance on the development prospect and application development.

2. MATERIAL AND METHODS

2.1. Extraction of Volatile Oil

The volatile oil extractor was used in yellow volatile oil extraction. As follows, 100g fresh fruit of chinese wampee were added distilled water for homogenation, and ground with a micro-plant grinding machine (FZ102; Tianjin Taisite Instruments, Tianjin, China) and then were extracted for 6h in volatile oil extractor. The fluid extracted including oil-water was divided by using high speed refrigerated centrifuge (Eppendorf). The fluids adding a little of anhydrous sodium sulfate were filtered by using a 0.45um membrane filter. At last a transparent, colorless volatile oil was got.

2.2. Chromatographic Analysis

The yellow volatile oil identification would be used by TLC method (Zhang Zhi et al, 2009). The volatile oil comparison was analyzed in mass spectrometry (Shimadzu QP2010GCMS). The parameters as follows, chromatographic column for RTX-5MS, quartz capillary column (30m*0.25mm*0.25um), Ion source for EI source, at a temperature of 230 degrees, the electronic energy 70eV, the interface temperature of 230 degrees, solvent delaying for 4min, scanning range 30~600amu. After heating program 60 □C, carrier gas for helium, column flowing speeds for 1.5ml/min, sample quantity for 1ul, split-flow ratio of 10:1,staying for 5 min and then to 4 degree / min increasing up to 250 □□C, retention for 7min.

2.3. Preparation of the Perfume

8% of the essential oils which included 3.6% of volatile oil from Clausena, 2.4% of essential oil of lavender (bought, made in China) and 2.0% sandalwood essential oil (bought, made in China) and ethanol of 92% would be mixed according to a certain proportion, After 2 hours of ultrasonic emulsified, the insoluble impurities were removed by 6000g centrifugation at 4C for 10 minutes to get the clarified liquid perfume.

2.4. Scavenging Hydroxyl Radical

The Yellow volatile oil properties were determined (Jiang Zitao et al,2012,Chen Sijia et al,2015,Zhang Fuping et al, 2013), Preparation of volatile oil solution were diluted with 60% as solvent into 1, 2, 4, 8, 16mg/ml of volatile oil solution and 2.502g of ferrous sulfate (Sigma) 7 H2O were dissolved in 1000 ml of deionized water and prepared for 9mmol / L of ferrous sulfate solution and placed in the dark. Additionally, 1.243g salicylic acid crystal (Sigma) will be dissolved into 1000ml with ethanol solution, and 9mmol / L salicylic acid- ethanol solution was prepared. Then the solution would be again dissolved in peroxide hydrogen of 30% about 889ml into 1000ml, 8.8mmol/L of hydrogen peroxide solution for use.

2.5. Determination of Hydroxyl Free Radical Scavenging Rate of Volatile Oil

1ml of 9 mmol/L ferrous sulfate solution, 1ml of 9 mmol/L salicylic acid ethanol solution, 1ml of different concentrations of samples were mixed, then the 1ml of 8.8mmol/L hydrogen peroxide was added finally for reaction30min at 37 ^{o}C as a control of distilled water. The OD value of each concentration was measured at a 510nm of wavelength in spectrophotometer (type 722, China). The measurements were repeated three times, and the average values were got as the measured results. The formula on calculation OH clearance rate as follows.

A0 - absorbance of blank control solution.

Ax - absorbance after adding sample solution.

Ax0 - no color reagent H2O2 only the optical absorption of sample solution.

volatile oil solution preparation (dichotomy)

Oil wampee pure oil obtained after filtering the water (anhydrous sodium sulfate) was diluted with acetone into7 different concentrations as 10%, 5% and 2.5%, 1.25%, 0.625%, 0.3125% and 0.156% respectively.

2.6. Antibacterial Activity of the Essential Oil

The antimicrobial activities of the essential oil were tested against three different bacteria. Two Gram-positive strains were Staphylococcus aureus and Bacillus subtilis, one Gram-negative strain was Escherichia coli .Staphylococcus aureus, Bacillus subtilis and Escherichia coli are three of common bacteria. These three kinds of bacteria are common and easy to cultivate, so we can use these three kinds of bacteria to explore the antibacterial activity of the perfume. The strains were provided by the Centre of Biopharmaceutical Engineering in Zhaoqing University. The bacteria incubation was placed for growth in the shelter to save the light. In order to make the experimental result standard and accurate, the method of the turbidity of wheat to measure the concentration of the diluted liquid were used. The bacteria solution was 1ml, and the bacterial concentration was about 1.5x10^8cfu/ml, which was diluted with normal saline to the turbidity and 0.5 wheat's standard tubes. And the essential oil from Zhaoqing wampee needs to be filtered. Then the antibacterial activity was investigated by agar disc diffusion method which a hole was punched into a 6mm filter paper in diameter. The micro broth

dilution was as follows 2×106CFU/mL. A control was also performed in containing inoculated broth supplemented with only DMSO. The maximum final concentration of DMSO was 2% in each medium which the test strains were incubated at 37°C for 24h. Then the size of inhibition ring was examined for evidence of antimicrobial (Zhang Chunle et al, 2006, Su Xiufang et al, 2011.).

2.7. Detection of the Cell Proliferation Inhibition the Perfume by MTT Method

The human colon carcinoma LoVo cells in DMEM culture medium containing 10% fetal bovine serum, 100u / ml penicillin and 100u / ml streptomycin) culture, were placed in 37 ℃, 5% CO2 and 80% - 90% of humidity in the incubator, the medium was replaced once every 2-3 days for daily observation of cell grow, and then they were .adjusted the cell for 5 x 106 / ml ~ 1 * 107 in density. The cells in Logarithmic growth phase were discarded the old medium, and washed with 2-3ml PBS washing twice, then was degraded with 0.25% trypsin for 2min, adding complete medium termination of digestion and spheroid cells into single cells (under inverted microscope observation), counting, cell concentration was adjusted to 40000-60000 / ml. The suspended cells were seeded in three of 96 well plates, the cells of each plate were divided into 8 lines for testing. Each line for five wells, including control line (line 1), the experimental wellswere5lines,10 wells each line, and the control line for five wells zero adjustments, 100ul of each well, only adding a complete incubating medium. All the plates were incubated at 37 C, 5% CO2 and under the condition of 80%-90% humidity in CO2 incubator. The morphology and growth of cells were observed every day. After 24 hours until the cells adhered to the wall, the experimental wells were added 100ul of perfume (ling 2, line 3, line 4 and line 5. and the control wells were added 100ul of 95% ethanol including the holes in line 6, line 7 and line 8 of the plate. The supernatant was sucked out in the super clean working table, and 20ul of MTT solutions of 0.5mg/ml were added to each well of the plate, placed in 37 degrees Celsius, 5% CO2 and humidity of 80%-90% to continue cultivating. The growth and morphology of the cancer cells experimented were observed and counted at 0, 1, 4, 18, 24h of different time under the inverted microscope. The supernatants in the plates were sucked out gently and added into 150uLDMSO solutions in each hole for shaking 5-10 minutes gently, after standing for 5 minutes, absorbance values were examined at 490 nm- 630 nm of wavelength on the enzyme mark analyzer and repeated the examination twice. (XingYan Bian, 1998).

The inhibition rates were calculated according to the following formula.

The inhibition rate = (OD value of the cell control hole -OD value of the experimental hole / OD value of the control hole x 100%

2.8. Statistical Methods

Measurement data were conducted by using the methods F deviation and LSD (Xiao-Hua Zhou, et al,2011)

3. RESULTS

3.1. Preparation of Volatile Oil

The extracting rates on zhaoqing yellow fruit are 0.4% by using steam distillation. The volatile oil was identified by thin plate chromatography method (Fig 1).

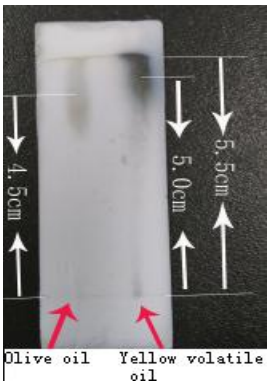

Fig1. *Thin plate chromatography identification of essential oils*

3.2. Components Analysis

The various components were identified according to the parameters designed and the peaks spectrum obtained by scanning the mass. The relative percentage contents of each component were got by NIST27 and NIST147 mass spectrometry spectra library and the artificial analysis with control, It was got that 25 components from the characteristics of Zhaoqing yellow fruits were separated and 23 components including terpenes and alcohols were identified. And the content of the highest is beta phellandrene, accounting for 36.97%, secondly another both of terpinen-4-ol and sabinene hydrate, accounting for the 17.58% and 13.76% respectively (Table 1).

Table1. *Composition analysis of yellow peel naphtha*

Number	retention time (min)	chemical name	chemical formula	molecular weight	relative percentage content /%
1	6.109	α- cedar ene	C10H16	136	0.27
2	6.354	α- pinene	C10H16	136	4.76
3	7.811	Juniper ene	C10H16	136	13.76
4	7.907	β-Beta pinene	C10H16	136	0.46
5	8.451	β-Beta myrcene	C10H16	136	2.48
6	8.948	α-Alpha water dropwort ene	C10H16	136	4.26
7	9.423	(+)-4- carene (+) -	C10H16	136	3.01
8	9.758	cymene	C10H14	134	2.56
9	9.988	β-- water dropwort ene	C10H16	136	36.97
10	11.090	terpinene	C10H16	136	5.19
11	12.224	2-- carene	C10H16	136	1.22
12	12.726	linalool	C10H18O	154	0.14
13	13.545	1 - methyl - 4 - (1 - methyl ethyl) - anti - 2 - cyclohexene - 1 - alcohol	C10H18O	154	0.75
14	14.253	1 - methyl - 4 - (1 - methyl ethyl) - shun - 2 - cyclohexene - 1 - alcohol	C10H18O	154	0.47
15	15.732	4- terpene alcohols	C10H18O	154	17.58
16	16.096	Implicit ketone	C9H14O	138	0.48
17	16.197	α- terpineol	C10H18O	154	0.76
18	16.826	Cis menthol	C10H18O	154	0.19
19	17.847	unidentification			0.14
20	20.890	unidentification			0.27
21	24.031	β- caryophyllene	C15H24	204	1.22
22	26.799	β- red myrrh ene	C15H24	204	1.50
23	27.264	β-half water dropwort	C15H24	204	0.15
24	27.508	α- sweet limonene	C15H24	204	1.21
25	31.819	santalol	C15H24O	220	0.22

3.3. Influence of Hydroxyl Free Radical Clearance on the Oil

The perfume from chinese wampee fruit and commercially available perfume were diluted into different concentrations gradient to determine the ability of hydroxyl radical scavenging for the two kinds of perfume. It was found that the hydroxyl removal rate of yellow pear perfume was better than the selection of commercially available perfume, The determination of volatile oil of hydroxyl free radical clearance rate showed that hydroxyl free radical clearance rate curve was increasing in the range of 1 to 16 mg/ml with the increase in the concentration, and the maximum clearance rate was 90.8% in concentration of 16 mg/ml(see table 2 and figure 1).

Table2. *Free radical clearance measurement of volatile oil of different concentrations*

sample con（mg/ml）	1	2	4	8	16
clearence %	13.9	28.1	51.5	69.8	90.6

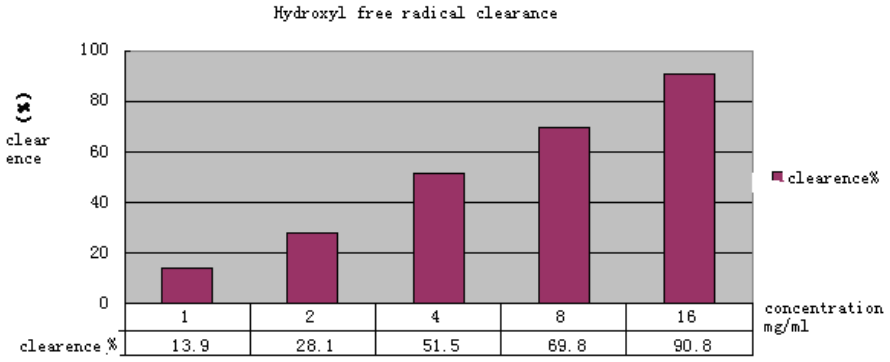

Figure1. *The volatile oil of different concentrations of hydroxyl free radical clearance*

3.4. Bacteriostasis of Zhaoqing Yellow Fruit Oil

The perfume solutions of different concentrations all showed inhibitory effects for the different types of bacteria. The bacteriostatic ring became small with essential oil concentrations decreasing. Both of them occurred a positive correlation. The inhibitory effect on Staphylococcus aureus and Bacillus subtilis were better, and the minimum inhibitory concentration (MIC) was 0.3125% for concentration of volatile oil. The antibacterial effect on Escherichia coli was relatively smaller, minimum inhibitory concentration (MIC) was 0.625% for concentration of volatile oil(Seeing Figure 2 and table 3).

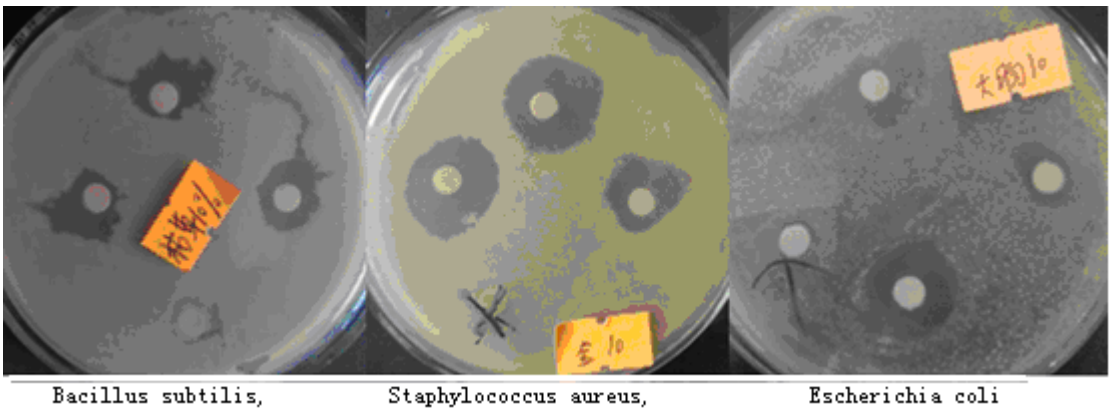

Fig2. *Zhaoqing yellow fruit antibacterial effect of essential oil*

Table3. *The diameter of inhibition zone (mm) of different concentrations of volatile oil to each test bacterium*

Concentration of volatile oil percent	10	5	2.5	1.25	0.625	0.312	0.156	acetone
Bacillus subtilis	15.36±0.24	12.70±0.17	11.17±0.28	11.00±0.47	10.50±0.2	9.58±0.29	----	---
Escherichia coli	11.80±0.23	11.00±0.58	10.67±0.28	10.42±0.32	9.75±0.375	----	----	---
Staphylococcus aureus	16.57±0.44	15.17±0.42	14.06±0.56	11.75±0.28	11.33±0.43	11.17±0.14	----	---

--- noninhibited

3.5. Comparison of Inhibit Cancer Cells on the Perfume

Recovery of mouse pancreatic cancer cells with each containing an equal amount of perfume and 95% ethanol culture flask culture observed and recorded each time the two culture flask cancer cell number

and cancer cell death. It was found that the perfume on cancer cell growth inhibition was better than the control when comparing with 95% ethanol anti cancer effect (P<0.05, Table 4).

Table4. *Comparison of inhibit cancer cells on the perfume and 95% ethanol (X±S) P*

Time of incubation		0h	1h	4h	18h	24h
95%**ethanol**	cell（n）	95±12	84±09	73±07	42±08	21±03
	mortality（%）	---	11.58	13.10	42.46	50
perfume	cell（n）	60±04	50±05	40±06	20±02	0
	mortality（%）	---	16.67	20	50	100

4. DISCUSSION

The studies showed that origin of climate and environment is different, it could affect the internal composition difference (Liu Youjie. 2014). So it is necessary to study the characteristics of the essential oil from yellow skin peel in Guangdong zhaoqing region in order to explore the pharmacological effects for making full use of the yellow skin fruit and knowing it's function.

On the volatile oil yields , the extracting rates of zhaoqing wampee fruit were close to that of chinese wampee from guangzhou fruit for 0.4 % (Li Ruizhen et al, 2007), however, slightly higher than that of volatile oil production from Hainan fruit of Chinese wampee for 0.39 % (Tang Wenning et al,2002).There is no much difference. The main difference lies in the composition and contents. Zhaoqing specific wampee fruit essential oil composition is mainly phellandrene (36.97%), terpinen-4-ol (17.58%), sabinene hydrate (13.76%), and Guangzhou fruit of Chinese wampee volatile oil content higher is terpinen-4-ol (21.06%), gamma Terpinene (12.9%), 3, 7, 7 - trimethyl - bicyclo [4.1.0] has - 2 ene (29.3%); Haikou wampee fruit essential oil content is higher for terpinen-4-ol (28.549_%%), sabinene (14.53 9%), gamma Terpinene (4 4_.868_%%). Comparing that of Chinese wampee, although most of the components same, its essential oil contents is different. It is the first time to analyze Zhaoqing wampee of volatile oil for its pharmaceutical function and application. Preliminary studies on volatile oil of Zhaoqing specific wampee fruit antioxidant showed that volatile oil on hydroxyl free radical had a good scavenging effect in a certain concentration range; with increasing of concentration of volatile oil its scavenging of hydroxyl radical rate was raising. There was a good correlation in 1 - 16mg / ml range for positive correlation. in the essential oil concentration and clear effects Hydroxyl radical scavenging rate is an important indicator of the antioxidant properties of substance. It is found that zhaoqing specific wampee fruit volatile oil had a certain resistance to oxidation through the point of view of the experimental results (Jiang Zitao et al., 2012, Chen Sijia et al., 2015, Zhang Fuping et al., 2013). And the result of hydroxyl radical scavenging rate increased as increasing the concentration of volatile oil in a certain range of concentration was also conformed to that of the references for 0.72mg/ml in lemon essential oil(Liu Yinhua,2009). And it was proved that the salicylic acid method used to study the caraway seed oil on hydroxyl free radical scavenging rate influence could achieve better hydroxyl free radical scavenging effect (Chen Sijia, 2015). The volatile oil (essential oil) of specific yellow peel in Zhaoqing on hydroxyl free radical scavenging effect is relatively weak in the two kinds of essential oils. Currently, the yellow fruit essential oils in antioxidant research haven't been reported. This experimental result showed that the scavenging action to hydroxyl free radical as indicators of antioxidant properties would be an important antioxidant indicator. And it would provide a theoretical basis for the use of wampee fruit.

It is known that hydroxyl radical is the active oxygen of the human body, which have antioxidant capacity. The inhalation of essential oils existed in the different plant roots, stems, leaves, flowers, fruit had some different effects (Chen Sijia et al, 2015), and pure natural essential oil as a volatile oil contained large amounts of terpenes, aldehydes, lipids, ketones, phenols or phenolic ether terpene hydrocarbon mono olefin and diene, in which single graphene had antiseptic, analgesic and also could increase the body's immune function. It was reported that a half proportion of graphene could be anti-inflammatory, antipruritic, anti fungal and balance hormone secretion(Liang Qiaohui et al, 2015) Therefore, yellow volatile oil which used as a spice for the preparation of perfume is valuable in theoretical and practical significance. The successful wampee fruit essential oil perfume has a better smell of wampee fruit, and the long persistence in the filter paper holding incense is better than the commercially available Mayerton perfume.

The knowledge of pharmacological activity on wampee volatile oil was not clear, and antimicrobial activity of the volatile oil on zhaoqing wampee fruit have not been reported up to now. The experiment showed that the inhibited bacteria effects of the yellow volatile oil increased with the contents increasing, it showed that the oil would become a potential antibacterial drug, but its effective antimicrobial components in isolation, identification and bacteriostasis mechanism need to be a further exploration.

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