Chemical composition, antioxidant and anticancer activity of the essential oil from myric rubra leaves

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Abstract. The Myric rubra leaves essential oil (MEO) was extracted by steam distillation and its major component was analyzed by gas chromatography mass spectrum (GC-MS). The result showed that 20 kinds of essential oils were identified. with humulene (26.03%), caryophyllene (19.74%), ledene oxide-(II) (16.37%) and γ-himachalene (7.7%) being the major constituents. The antioxidant activity of MEO were analyzed by 2,2-diphenyl-1-picrylhydrazyl assay (DPPH) and oxygen radical absorbance capacity (ORAC) assay respectively, Analogues of vitamin E (Trolox) was positive control. The results showed that the IC50 of DPPH radical scavenging was 0.0576 mg/mL and the ORAC value of fluorescence spectrometry is 1.32mmol TE/g. The anticancer effect of obtained MEO was tested in A549 lung cancer cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. MEO IC50 value of 163 μg/mL was obtained with significantly inhibited cell proliferation in concentration-dependant manner. MEO could be useful for pharmaceutical, food and cosmetic industry, because of its strong antioxidant and anticancer activity.

1. Introduction
Chinese bayberry belongs to Myricaceae, with crown spherical, single leaf alternate, and widely grown in Zhejiang Province. The kind of Myrica rubra cv. DongKui Orient Pearl originated in Zhejiang Huangyan, is famous for its largest fruit size in the world, known as "Bayberry king". The fruit can help produce saliva, slake thirst and digestion, having high medicinal and food value. Their roots, stems, leaves have also been used externally for dysentery, diarrhea, rectal bleeding, rheumatism pain, stomach pain, and cardiovascular disease in Chinese traditional medicine [1,2]. There are many active components in Chinese bayberry leaves, such as procyandinis, tannins, quercetin, bayberry, coumarin derivatives, triterpenes, etc., which have the functions of anti-depression, regulating lipid and glucose metabolism, anti-oxidation, anti-inflammatory, analgesic, tumor inhibition and anticancer. Chinese bayberry leaf essential oil is a kind of water insoluble volatile liquid extracted by steam distillation, supercritical extraction, simultaneous distillation and extraction, etc. The composition is complex and diverse, mainly terpenes. Significant anti-oxidation [3], anti-viral [4], anti-microbial [5,6], anti-tumor [7], anti-allergic activity of MEO has been reported in many studies. The anti-proliferation effect of MEO was tested in human colon cancer cell lines SW480, SW620, CaCO2, HCT8 and HT29. Concentration dependent proliferation of cells in all cell lines could be inhibited by MEO, and Caco2 is the most sensitive [8]. The antioxidant activity of essential oils of Myrica rubra Var. astropurea Tsen was reported on [9].
The antioxidant activity of the non-volatile compounds of Myrica rubra Var has been further reported [10]. One of the aims of the current study was to investigate EMO anti-oxidant activity by DPPH and ORAC methods and anticancer activity by MTT method for their future application in either pharmaceuticals or cosmetics.

2. Materials and methods

2.1. Plant materials
The naturally dried leaves of Myrica rubra cv. DongKui Orient Pearl was collected from Xianju, Zhejiang Province, China in Jun-2014.

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) and Trolox was from Sigma–Aldrich (USA), Stock solutions of DPPH were prepared in methanol.

2.2. Extraction of the essential oils
MEO was isolated by steam distillation. Air-dried Myrica rubra (500g, at room temperature, 6d) was crushed into powder with a mixer (HF-6300, Biaohang Co., Ltd., Hangzhou, China). The powder was mixed with water (1: 6), and refluxed for 6-8 hours at 160 °C to extract the essential oils. The obtained oil was dried with anhydrous sodium sulphate and stored in a 4 °C sealed vial in the refrigerator before analysis.

2.3. Gas chromatography-mass spectrometry (GC-MS)
GC–MS analysis was performed on a combined GC-MS instrument (Agilent 6890N GC with 5973 inert MSD) fitting with a HP-5 fused silica capillary column (30 m length, 0.25 mm diameter, 0.25 μm film thickness). GC-MS conditions: injection volume was 0.5 μL, solvent delay time was 2 min, split ratio was 1:40, injector temperature was 250 °C, carrier gas was helium at 1.0 ml/min constant flow mode, the initial column temperature was from 50 to 280 °C at 8 °C/min. Mass spectra: electron impact (EI+) mode, 70 eV and ion source temperature was 200 °C. Mass spectra was recorded over 50–500 amu range.

The relative percentage of MEO components was expressed as percentage by peak area normalization method. According to the gc-ms spectra from NIST’98 literature data of the United States, the composition of MEO was identified according to the retention time.

2.4. Antioxidant activity
DPPH radical scavenging activity [11,12] has been detected by the following method:

Stock solutions of DPPH (25mg/ml) were prepared in methanol. Various concentrations of oil sample in methanol (0.001, 0.005, 0.01, 0.025, 0.05, 0.075 and 0.10mg/mL) were added to 2 mL (25mg/ml) DPPH and the volume was made up to 2.2 mL with methanol. The mixture was shaken thoroughly and left at room temperature for 30 min. The mixture's absorbance was then measured on a spectrophotometer at 517 nm. Trolox was used as a positive control, and all determinations were performed in triplicate (n = 3).

The percentage inhibition was calculated by the following equation:

\[ \text{DPPH radical scavenging (\%) = } \left( \frac{\text{Ac} - \text{As}}{\text{Ac}} \right) \times 100\% \]

where, Ac is the absorbance of the control (only DPPH in methanol) and As is the absorbance of the sample.

2.5. ORAC assay
The ORAC assay for essential oils was carried out using an automated multimode reader with 96-well plates. Trolox standard was formulated into 6.25, 12.5, 25, 50μmol/L with phosphate buffer (pH7.4). Fluorescein (0.12 μmol/L) was used as the substrate and 2,2’-azobis (2-methylpropion amidine) dihydrochloride (AAPH) (40 mmol/L) was used as radical generator. The trolox standard or test compound solution (20μl, used as the control phosphate buffer) was added to each of the 96-well black microporous plates and 120μl of fluorescein was added to the phosphate buffer solution. The plate was incubated at 37 °C for 20 minutes, and the machine was programmed to inject 60 μl of AAPH into each well. Fluorescence
conditions were excitation at 485nm and emission at 538nm. A standard curve was drawn and the ORAC value of the tested compounds was obtained as Trolox equivalent (TE).

2.6. Inhibition of A549 lung cancer cells
A549 lung cancer cells (3000 cells/well) were inoculated into a 96-well plate and preincubated for 3 hours for cell adherence. Test samples of 10 μl EMO for various concentrations (100, 150, 200, 250, 300, 350 and 400 μg/mL) and the control cells without EMO were added into the cultures and incubated at 37°C for 72 h under humidified air containing 5% CO2 respectively.

After removing the medium from the wells, 10 μL of tetrazolium salt solution (MTT) (5mg/ml) was added. After being incubated at 37°C for 4h, the medium was removed and added into 150 μl of DMSO and the formazan crystals were dissolved in the shaker for 10 minutes. Absorbance was measured in a multimode reader at 570 nm. The cell viability ratio (%) was calculated from the following equation: viability % = (absorbance of test sample/absorbance of control) × 100%.

3. Results
MEO was obtained as a light-yellow oil, looked clear and transparent. The yield was 0.129% of the dried plant material.

Table 1. Identified chemical composition and the relative content of Myric rubra leaves essential oil.

| NO | RT (min) | Compound                        | Molecular formula | Composition (%) |
|----|----------|---------------------------------|-------------------|-----------------|
| 1  | 15.681   | Caryophyllene                   | C15H24            | 19.74           |
| 2  | 15.951   | Alloaromadendrene               | C15H24            | 0.56            |
| 3  | 16.216   | Humulene                        | C15H24            | 26.03           |
| 4  | 16.299   | Calarene                        | C15H24            | 0.18            |
| 5  | 16.501   | γ-Murolene                      | C15H24            | 1.65            |
| 6  | 16.703   | (-)-α-Selinene                  | C15H24            | 3.68            |
| 7  | 16.792   | β-copaene                       | C15H24            | 0.13            |
| 8  | 17.092   | (-)-g-Cadinene                  | C15H24            | 4.72            |
| 9  | 17.259   | Simvastatin                     | C25H38O5          | 1.07            |
| 10 | 17.44    | α-Murolene                      | C15H24            | 0.34            |
| 11 | 18.41    | Caryophyllene oxide             | C15H24O           | 1.64            |
| 12 | 18.566   | Ledene oxide-(II)               | C15H24O           | 16.37           |
| 13 | 18.794   | 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)- Naphthalene | C15H24 | 0.1   |
| 14 | 18.94    | 4,4-Dimethyl-3-(3-methylbut-3-enylidene)-2-methylenebicyclo[4.1.0]heptane | C15H22 | 6.85 |
| 15 | 19.033   | γ-Himachalene                   | C15H24            | 7.7             |
| 16 | 19.204   | β-Guaiene                       | C15H24            | 2.31            |
| 17 | 19.272   | Retinol, acetate                | C22H32O2          | 0.25            |
| 18 | 19.458   | Cadalin                         | C15H18            | 0.73            |
| 19 | 21.56    | 2-Pentadecanone, 6,10,14-trimethyl- | C18H36O | 1.44 |
| 20 | 24.808   | Dehydroisophytol                | C20H40O           | 0.77            |
3.1. Chemical composition of MEO

Chemical constituents of Myrica rubra cv. DongKui Orient Pearl essential oil analyzed by GC-MS is shown in Table 1, and Figure 1 shows the total ion flow of the essential oil. 20 chemical components were identified by GC/MS in MEO, covering 96.25% of the total peak area. The major constituents of the essential oil were Humulene (26.03%), Caryophyllene (19.74%), Ledene oxide-(II) (16.37%), γ-Himalchale (7.7%), 4,4-Dimethyl-3-(3-methylbut-3-enyldene)-2-methylbenz(bicyclo[4.1.0]heptane (6.85%), (-)-g-Cadinene (4.72%), (-)-α-Selinene (3.68%),β-Guaiene (2.31%), γ-Muurolene (1.65%), Caryophyllene oxide (1.64%).

![Figure 1](image1.png)

**Figure 1.** Identified chemical composition and the relative content of Myric rubra leaves essential oil.

3.2. Antioxidant activity of MEO

Free radical scavenging activities of MEO and trolox measured by DPPH assay are reported in Figure 2. It was observed that the radical scavenging activities enhanced with increasing concentration. The linear function obtained after plotting Radical scavenging versus the concentration were y = 633.76x + 35.926, R² = 0.9543 and y = 734.08x + 7.6931, R² = 0.9738 within 0.005-0.1mg / mL. IC50 of Trolox and essential oils (IC50) were 0.0222 mg / mL and 0.0576 mg / mL from the fitted curve, the radical scavenging activities of trolox was stronger than MEO’s.

![Figure 2](image2.png)

**Figure 2.** The fitting curve of DPPH radical scavenging activity of EMO and Trolox.

The antioxidant capacity of MEO and trolox determined by ORAC assay is reported in Figure 3. The linear function obtained after plotting net AUC versus the concentration was y = 4.8589x + 11.296, R²= 0.9973 within 6.25-50 μmol/L. The ORAC value of MEO, 1.32mmol TE/g from the fitted curve, showed good oxygen radical scavenging ability. Seen from component analysis of essential oils, the reason why MEO had the radical scavenging
activity was MEO had high content of antioxidant ingredients, such as Himachalene, Caryophyllene, Ledene oxide-(II), γ-Himachalene having strongly radical scavenging activities.

\[ y = 4.8589x + 11.296 \]

\[ R^2 = 0.9972 \]

**Figure 3.** Standard curve for the trolox concentration and the net area under the FL.

3.3. **Anticancer activity of MEO**

The yellow MTT (3-(4, 5-dimethyldiazole-2-group) -2, 5-diphenyltetrazolium bromide) can be reduced to purple formazan by the living cell mitochondria. Anti-proliferation effects of MEO at various concentrations (100-400 mug/mL) were tested in A549 lung cancer cells. The survival rates after 48h exposure shown in Figure 4 showed MEO inhibits cell line proliferation in a concentration-dependent manner. Essential oils were found to be very active at a concentration of 250 mug/mL, where the survival rate is 15%. The linear function was obtained after plotting survival rates versus the concentration: \[ y=-0.3215x+102.43, \]

\[ R^2=0.9757 \] within 0-300μg/mL. The values of IC50 is found to be 163μg/mL.

**Figure 4.** Effect of MEO on proliferation of human lung cancer cell A549. Values are mean ±SD of three experiments.

4. **Discussion**

The results obtained were 20 components in MEO, with α-Humulene (26.03%), β-Caryophyllene (19.74%), Ledene oxide-(II) (16.37%) and γ-Himachalene (7.7%) determined as the most abundant molecules among the principal compounds. While our team reported that the main components of MEO from Chaoshan carbon variety are Caryophyllene (44.01%), Humulene (34.40%), Ledene oxide-(II) (4.81%), α-cis-Himachalene (2.83%) [13]. Composition differences are due to different varieties of Myric rubra leavies and seasonal variability.
In the last few years, an increasing interest on the antioxidant and anticancer activity of the different essential oils has been reported and in many cases these bioactivities are due to the presence of active constituents such as α-Humulene, Caryophyllene oxide and β-caryophyllene [14,15]. β-Caryophyllene is reported to possess antioxidant [16], antimicrobial [17, 18], anti-inflammatory [19-21] and anticancer activities [22]. β-Caryophyllene induces apoptosis in tumor cells with the catalytic activity of DNA ladder and caspase-3. Intraperitoneally administered caryophyllene oxide exhibits anti-injury sensitivity and might play a role on a variety of mechanisms that involve central and peripheral pathways [23]. The essential oils with high content of caryophyllene and caryophyllene oxide have a strong anti-tumor activity. Therefore, Antitumor activity of MEO possibly associated with the major components of Humulene, caryophyllene and caryophyllene oxide, this result needs further study yet.

Free radical scavenging activities of MEO and trolox measured by DPPH assay have been researched above. It was observed that the radical scavenging activities enhanced with increasing concentration. IC50 of Trolox and essential oils (IC50) were 0.0222 mg / mL and 0.0576 mg / mL from the fitted curve, the radical scavenging activities of trolox was stronger than MEO’s.

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