The effect of different drying methods on non-volatile and volatile components and immunomodulatory activity of *Osmamthus fragrans* flowers

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Abstract

Background: *Osmamthus fragrans* (*O. fragrans*) has high ornamental, edible and medicinal value in China. The components of its flowers have been gradually revealed, but the active ingredients with immunoregulatory activity have been unknown. Also, it needs to be studied that which groups and drying methods can make *O. fragrans* fowers produce more immunomodulatory activity. This study aimed to investigate the effects of drying methods on non-volatile and volatile components of *O. fragrans* flowers from three groups, and to further explore if their groups and drying methods had an ovious effect on their immunoregulatory activity.

Methods: *O. fragrans* flowers from three groups such as "*Aurantiacus*", "*Latifolius*" and "*Thunbergii*" were dried with shade drying, sun drying, quick-lime drying, oven drying and microwave drying method, respectively. The non-volatile components such as salidroside, verbascoside, oleanolic acid and ursolic acid and volatile components such as linalool, linalool oxide, geraniol, α-ionone and β-ionone of *O. fragrans* flowers above were determined by high-performance liquid chromatography (HPLC) and gas chromatography (GC), respectively. The immunomodulatory activities of chemical components above were investigated by the neutral red uptake assay.

Results: There are more non-volatile components and less volatile components in *O. fragrans* flowers from *Aurantiacus* group and *Latifolius* group than *Thunbergii* group. Microwave drying and oven drying at high temperature were beneficial for the preservation of the bioactive non-volatile components for killing enzyme and protecting glycoside. Other drying methods such as shade drying, sun drying, quick-lime drying and oven drying at low temperature were beneficial to their preservation of the fragrant volatile components. Salidroside, verbascoside, linalool, and linalool oxide had the better immunoregulatory activity than other ingredients. In addition, non-volatile components played a more important role in the contribution to the immunoregulatory activity than the volatile components as the former was almost 1000 times as much as the latter.

Conclusions: *O. fragrans* flowers from *Aurantiacus* group with microwave drying (high fire) method had the best immunoregulatory activity. The research could provide some evidence in choosing drying method of *O. fragrans* flowers as food or medicine.

Background

Osmamthus fragrans (Thunb.) Lour. (*O. fragrans*) is a typical representative of the *Oleaceae* family [1]. It is mainly distributed in China, partly in other regions of Asia, Europe, America and Oceania [2].It is not only a famous aromatic and ornamental plant, but also an excellent edible and medicinal plant [3]. Its ornamental value has been developed in China and its cultivation history can be traced back to the Han Dynasty [4]. Now *O. fragrans* has been widely planted in gardens, courtyards and scenic spots as a unique tourist landscape in China. Its flowers could be made into the scented *O. fragrans* tea, wine, cake and so on [5, 6]. In addition, its flowers can be used to treat stomach, liver and kidney diseases [7]. The

consensus has been formed in Chinese Landscape Circles that there are four *O. fragrans* groups, including *Semperfloren*, *Aurantiaeus*, *Latifolius* and *Thunbergii* groups [8]. There are almost 18 varieties in *Semperfloren* groups, 36 varieties in *Aurantiaeus* groups, 57 varieties in *Latifolius* groups, and 43 varieties in *Thunbergii*, respectively [9].

O. fragrans flowers mainly contain volatile and non-volatile components. The volatile components from *O. fragrans* flowers predominantly included terpenes, aldehydes, esters, ketones and alcohols [10]. Their fragrance components which emitted directly in the flowering process contained more monoterpenes with low boiling point than their *O. fragrans* flower extracts [11, 12]. The harvest stages, flower tissues and organs, cultivated varieties, extraction methods, and preservation methods all had a great impact on the volatile components of *O. fragrans* flowers [13–20]. The non-volatile components from *O. fragrans* flowers mainly include flavonoids, terpenoids, lignans and phenylpropanoids [21]. However, only these quantitative non-volatile components from *O. fragrans* flowers were reported as follows: 0.27–0.95% salidroside in dry flowers, 1.82–7.29% verbascoside in dry flowers, 0.03% quercetin in fresh flowers and 0.1% forsythigenin in *O. fragrans* flower extracts [22–26].

After *O. fragrans* flowers were picked for six hours, the fragrance became significantly weaker [27]. Therefore, it was particularly important for *O. fragrans* flowers to choose the best method from various drying methods, such as hot-air drying, microwave drying, infrared drying, freeze drying, vacuum oven drying and so on [28, 29]. Different drying methods had significant influences on the chemical composition and bioactivity from mulberry leaves, tea and finger citron [30–32]. Also, the contents and compositions of the essential oil from *thymus daenensis* Celak. and *Laurus nobilis* L. leaves changed due to different drying methods [33, 34]. It was reported that sensory quality from mulberry leaves, jujube fruits and bitter gourd slices were also affected by different drying methods [35–37].

However, these chemical components of *O. fragrans* flowers did not exactly reflect the bioactivities of *O. fragrans* flowers. Therefore, the immunomodulation functions of these components were estimated to develop the health functions of *O. fragrans* flowers [38]. Macrophages perform several functions such as host defense, inflammatory regulation, and tissue remodeling and so on [39]. They play a vital role in both innate and adaptive immune response to pathogens through phagocytosis, antigen presentation, and cytokine secretion [40]. The stimulation of macrophages is one of the important strategies to increase the human defense system. So far people have found many bioactive components which promote macrophage energy, such as polysaccharides, glycosides, alkaloids and flavonoids [41].

Though the drying process of *O. fragrans* flowers is usually mentioned in some references, little detailed datum currently exist. In addition, it was urgent to make clear which components of *O. fragrans* flowers have immunomodulatory activities. To the best of our knowledge, it also remains unknown whether the traditional methods such as sun drying, shade drying and quick lime drying will keep from losing immunoregulatory active components. Therefore, it was aimed to screen the drying methods of various *O. fragrans* flowers based on the aromaticity and immunoregulatory activity of their chemical components.

Materials And Methods

Chemicals and reagents

Acetonitrile and methanol of HPLC grade were purchased from Merck (Darmstadt, Germany). The standard products such as salidroside, verbascoside, oleanolic acid and ursolic acid were obtained from National Institutes for Food and Drug Control (Beijing, China). L (-)-2-Octanol (as the internal standard), linalool, linalool oxide, geraniol, α-ionone and β-ionone were bought from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Petroleum ether with the boiling range 80–120 °C was purchased from J&K Chemical Ltd. (Beijing, China). Water of HPLC grade was purified by reverse osmosis systems (Millipore, Ireland). All the reagents of HPLC grade should be filtered through a 0.45 μm membrane before experiment. Dimethyl sulfoxide(DMSO), 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), trypan, Concanavalin A (Con A), penicillin G, and strepto-mycin sulfate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Gibico 1640 medium and fetal bovine serum (FBS) were purchased from Gibco Invitrogen Corp. (San Diego, CA, USA). Other reagents and chemicals were of analytical grade.

Plant material

Fresh *O. fragrans* flowers were harvested from three different *O. fragrans* groups in our campus whose flowers were almost orange, silver and golden, respectively. They were identified separately as *O. fragrans var. Aurantiacus*, *O. fragrans var. Latifolius* and *O. fragrans var. Thunbergii* by Dr. Feng Li from Shandong University of Traditional Chinese Medicine. To narrate bellows conveniently, they were tentatively called as "*Aurantiacus*", "*Latifolius*" and "*Thunbergii*".

Drying methods

Shade drying (SHD), sun drying (SUD), quick-lime drying (QLD), oven drying (OD) and microwave drying (MD) were conducted on fresh flowers from three *O. fragrans* groups, respectively. The processing of SHD and SUD were separately carried out in doors or in the sun. The processing of QLD was carried out by placing the fresh flowers in the upper glass dryer whose lower layer was filled with quick lime. The processing of OD was operated separately at 40, 50, 60 and 70 °C in the drying oven (DHG-9070B, Shanghai Pei for Experimental Instrument Co., Shanghai, China). The processing of MD was carried out separately at medium-low fire (MLF), medium fire (MF), medium-high fire (MHF) and high fire (HF), respectively, in the microwave oven (M1-L213B, Midea Group Co., Foshan, China). All the dried *O. fragrans* flowers above should remain their constant weights before completions of their drying processing and be stored in the glass dryer filled with the desiccant silica gel.

Sample extraction and preparation

The dried flowers from three *O. fragrans* groups were extracted and prepared to determine 4 kinds of non-volatile components as HPLC samples as follows. 0.1 g of the dried *O. fragrans* flowers were measured

precisely and placed into 50 mL conical flask with its stopper. Then 10 mL precise methanol was dumped into the above flask and extracted for 30 min under 100 w power and 40 kHz frequency at room temperature using an ultrasonic cleaner (KQ-100 DE, Kunshan Ultrasound Instrument Co., Suzhou, China). The lost weight was compensated with methanol after being cooled to room temperature and the extracts was filtrated through the double-layer filter paper. The continuous filtrates above was collected and stored at 4 °C for the subsequent content determination of 4 kinds of non-volatile components.

The dried flowers from three *O. fragrans* groups were extracted and prepared to determine 5 kinds of volatile components as GC samples as follows. 10 g of the dried *O. fragrans* flowers were measured precisely and put into 500 mL round-bottom flask. Then 300 mL of deionized water were added into the above flask. With the Soxhlet apparatuses utilized, they were extracted for 4 h. 2 mL of L (-)-2-Octanol petroleum ether solutions with the boiling range 80–120 °C were added into the distillated extracts. The above liquids were mixed homogeneously and their supernatants were separated and stored at 4 °C for the next determination of the volatile components. All experiments were carried out in triplicate.

High Performance Liquid Chromatography (HPLC)

4 kinds of non-volatile components of HPLC samples were analyzed by E2695 LC instrument (Waters Instruments Co., Massachusetts, USA) with an on-line degasser, a quaternary pump, a 2489 ultraviolet-visible detector (UVD) and an anto-sampler. 10 μL of the HPLC samples were subjected to HPLC analysis with a Symmetry C-18 analytical column (250 mm × 4.6 mm, 5 μm, Waters Corporation, USA), using a linear gradient with water- phosphoric acid (100:0.05, v/v) as solvent A and acetonitrile as solvent B. The gradient program was carried out as follows: 10% B in 0–8 min, 10–30% B in 8–22 min, 30–85% B in 23–36 min, 85–10% B in 37–66 min. The velocity of flow was 1.0 mL min^{-1} and the column temperature was kept at 30 °C. Detection wavelength was performed at 220 nm in 0–25 min and 210 nm in 26–66 min.

Gas chromatography (GC)

5 kinds of volatile components of GC samples were analyzed by GC-4000A GC instrument (East &West Analytical Instruments Co., Beijing, China) with a nitrogen canister, a flame ionization detector (FID), a hydrogen generator, an air pump and a 1μL injection needle. 1μL of the GC samples were injected to GC instrument with an AE.SE-54 nonpolar analytical column (30 m × 0.32 mm, Lanzhou Atech Technologies co., Lanzhou, China) and the high purity nitrogenas as carrier gas, using a temperature gradient program. The gradient program was carried out as follows: 50℃ for 2 min, 50–160 °C at the rate of 2 °C min^{-1}, 160–250 °C at the rate of 22.5 °C min^{-1}, 250 °C for 12 min. The temperature of gasification chamber and flame ionization detector was all set at 250 °C. The pressure of nitrogen, air and hydrogen was set at 0.4, 0.2 and 0.09 MPa, respectively.

Assessment of cell viability

The cytotoxicity of relevant chemical components of *O. fragrans* flowers with various concentrations was evaluated by MTT test with some modification [42]. 1×10^5 of RAW 264.7 cells were seeded in triplicates in 96-well plates and cultured 3 h to allow cell attachment, then cultured with salidroside, verbascoside, linalool oxide, linalool, geraniol, α-ionone and β-ionone at 3.125, 6.25, 12.5, 25, 50, 100 μg mL^{-1} for 24 h. Subsequently, 20 μL of MTT solution (5 mg mL^{-1}) was added to each well, and the cells were incubated for 4 h at 37 °C. The supernatants were discarded and 150 μL of DMSO were added to each well and homogenized. Absorbance values were estimated at 570 nm by Epoch2 microplate spectrophotometer (Bio Tek Instruments Co., USA). Cell viability was calculated by the following equation.

$$\text{Cell viability (\%)} = \frac{A_2}{A_1} \times 100 \quad (1)$$

Where A_1 is the absorbance value of blank control group, A_2 is the absorbance value of treatment group.

Phagocytosis assay

The phagocytic activities of RAW 264.7 cells treated with various concentrations of relevant chemical components of *O. fragrans* flowers were examined by neutral red uptake assay [43]. RAW 264.7 cells were seeded at 2.7×10^6 cells per well in 96-well microplates. After incubation for 3 h, the supernatant was discarded. RAW 264.7 cells were pretreated with salidroside, verbascoside, linalool oxide, linalool and geraniol at different concentration (3.125, 6.25, 12.5, 25, 50, 100 μg/mL) in the presence or absence of LPS (1.0 μg mL^{-1}) for 24 h. Then, 100 μL of neutral red solutions (0.1%, w/v) were added into each well and incubated for another 30 min at room temperature. The supernatants were discarded and the cells were washed with phosphate buffered saline (PBS, 0.01 M, pH 7.4) thrice, the cells were in lysis for 30 min at room temperature by adding 150 μL of cell lysis buffer ($V_{ethanol}$: $V_{acetic\ acid}$=1:1) and homogenized. The absorbance values were measured at a wavelength of 540 nm using Epoch2 microplate spectrophotometer.

$$\text{Neutral red phagocytosis rate (\%)} = \frac{A_2}{A_1} \times 100 \quad (2)$$

Where A_1 is the absorbance value of blank control group, A_2 is the absorbance value of treatment group.

Statistical analysis

All data were reported as means ± standard deviation of three samples. Statistical analysis was performed with SPSS 19.0. Significance differences were tested by using the ANOVA procedure, using a significant level of p□0.05.

Results

The dry flower yields of *O. fragrans* flowers with different drying methods

Table 1
Dry flower yields ($X \pm s$, $n = 3$) of 100 g fresh *O. fragrans* flowers with different drying methods

Drying methods	*Auranticacus* (g)	*Latifolius* (g)	*Thunbergii* (g)
OD(40°C)	22.15 ± 1.25	23.60 ± 1.45	20.45 ± 1.13
OD(50°C)	19.95 ± 1.15	23.20 ± 1.23	19.65 ± 1.18
OD(60°C)	19.58 ± 1.16	23.10 ± 1.31	19.40 ± 1.23
OD(70°C)	19.35 ± 1.35	22.70 ± 1.17	19.10 ± 1.16
MD(MLF)	22.90 ± 1.25	22.20 ± 1.09	18.30 ± 1.27
MD(MF)	22.40 ± 1.16	22.30 ± 1.16	17.90 ± 1.15
MD(MHF)	21.00 ± 1.13	22.20 ± 1.30	16.90 ± 1.18
MD(HF)	20.20 ± 1.31	22.50 ± 1.21	16.45 ± 1.07
SHD	20.40 ± 1.08	22.50 ± 1.14	17.90 ± 1.21
SUD	21.00 ± 1.19	23.00 ± 1.22	17.80 ± 1.20
QLD	20.50 ± 1.12	22.80 ± 1.13	17.50 ± 1.11

Drying is a physicl process to remove free water from materials by using heat energy. However, the process may be accompanied by changes in chemical composition. Different drying methods have different drying efficiency. In order to remove the influence of water on chemical composition content, we calculated and compared the effects of different drying methods on dry flower yields of O. fragrans flowers (Table 1). The results showed that different drying methods had no significant effect on the yield of dried flowers (P□0.5).

Effect of different drying methods on non-volatile components of *O. fragrans* flowers

Both Salidroside and verbascoside structurally belong to phenylethanoid glycosides, and both oleanolic acid and ursolic acid are typical pentacyclic triterpenoids. The characteristic spectra of HPLC were shown in Fig. 1, displaying the composition of the key phenylethanoid glycosides and related pentacyclic triterpenoids.

It was showed in Fig. 2 that it was impossible for quantify of oleanolic acid and ursolic acid. The contents of 4 kinds of non-volatile components in *Aurantiacus* flowers with different drying methods were showed in Fig. 2a. MD displayed the greater advantage in the contents of salidroside, with the maximum of 0.867 ± 0.07 g (MF). Moreover, OD (70 °C) and QLD had a good performance and were second only to MD. The salidroside contents approximately increased with the increase of firepower of MD or drying temperature of OD. The local high temperature inside of MD had the rapider and greater function of killing

the large amount of enzymes and preserving glycosides than that of OD [44, 45]. Other drying methods kept the activity of enzyme and caused the hydrolysis of salidroside for their absence of high temperature. The similar results occurred to verbascoside, with the maximum of 2.306 ± 0.06 g (HF). It was showed in Fig. 2b that the maximum content of oleanolic acid appeared in SUD and the lowest point appeared in OD for *Latifolius* flowers. The gap of salidroside and verbascoside contents diminished among different drying method in *Latifolius* flowers had the small amount of enzymes. The drying temperature had little effect on the salidroside and verbascoside contents. It was showed in Fig. 2c that there was absence of oleanolic acid and ursolic acid in fresh *Thunbergii* flowers. The four extremes of salidroside (0.192 ± 0.05 g and 0.244 ± 0.04 g) and verbascoside (1.766 ± 0.05 g and 2.24 ± 0.06 g) appeared in OD (50 °C) and MD (HF), respectively. It was showed that the salidroside and verbascoside contents were sensitive to temperature. In a word, Enzyme content and activity played an important role in non-volatile components in fresh *O. fragrans* flowers of three *O. fragrans* groups. On the whole, MD (HF) was the most suitable way to keep highest contents of non-volatile components. The maximum total contents of salidroside and verbascoside were 3.029 ± 0.11 in 100 g *Aurantiacus* flowers, 2.092 ± 0.13 g in 100 g fresh *Latifolius* flowers and 2.484 ± 0.12 g in 100 g fresh *Thunbergii* flowers.

Effect of drying methods on volatile components of *O. fragrans* flowers

Linalool oxide, linalool and geraniol belong to chain terpene alcohols, and α-ionone and β-ionone belong to cyclic terpene alcohols. These components in three *O. fragrans* groups could be quantified. Gas chromatograms were shown in Fig. 3, displaying the composition of the key chain terpene alcohols and cyclic terpene alcohols [46].

It was showed in Fig. 4 that the contents of dibutyl phthalate, dihydroactinolide and δ-decanolide were could not be quantified in three *O. fragrans* groups. It may be related to the different *O. fragrans* flowering stages [13]. 5 kinds of volatile component contents in 100 g fresh *Aurantiacus* flowers with different drying methods were showed in Fig. 4a. The contents of α-ionone were obviously affected by drying mothed and the maximum content (51.14 ± 2.81 mg) appeared in SUD. The maximum contents of both linalool oxide and linalool were present in OD (40 °C). There were three maximum contents of geraniol in OD (40 °C), MD (MF) and SUD, respectively. There was little β-ionone in 100 g fresh *Aurantiacus* flowers. On the whole, drying methods at the low temperature such as SUD, MD and QLD were instrumental in the preservation of volatile components in 100 g fresh *Aurantiacus* flowers. The correlative results were showed in Fig. 4b that the maximum content (72.63 ± 3.64 mg) appeared in SUD in 100 g fresh *Latifolius* flowers. The maximum contents of linalool oxide and linalool appeared in QLD and SHD, respectively. The contents of geraniol and β-ionone in 100 g fresh *Latifolius* flowers were similar to those in 100 g fresh *Aurantiacus* flowers. It was showed in that in Fig. 4c there was quite a lot of geraniol in 100 g fresh *Thunbergii* flowers and its maximum (89.93 ± 3.14 mg) content was present in SHD. The maximum contents of both linalool oxide and linalool were also present in OD (40 °C). There was little α-ionone and β-ionone in *Thunbergii* 100 g fresh flowers. There were significant differences in different *O. fragrans* groups. Temperature obviously has a different effect on different volatile components in different *O. fragrans* groups. The maximum total contents of linalool oxide and linalool were 44.57 ± 3.08 mg in

100 g fresh *Aurantiacus* flowers, 43.14 ± 3.89 mg in *Latifolius* flowers and 89.64 ± 4.75 mg in 100 g fresh *Thunbergii* flowers.

Effect of chemical components of *O. fragrans* flowers on cell viability of RAW264.7 cells

MTT test was used to response the number of viable cells and cell viability by detecting the activity of mitochondrial succinate dehydrogenase of live cell. MTT colorimetric method has a very wide range of applications in cell proliferation and differentiation, and is related to immunological experiments in cell metabolism. As shown in Fig. 5, the survival rates of RAW 264.7 cells were basically above 90% after the disposition of salidroside, verbascoside, linalool oxide and linalool in the concentration range of 3.125-50 μg mL^{-1}. The survival rates of RAW 264.7 cells decreased to less than 50% after the disposition of geraniol, α-ionone and β-ionone in the concentration range of 50–100 μg mL^{-1}. It was showed that geraniol, α-ionone and β-ionone had a great cytotoxic effect. Therefore, geraniol, α-ionone and β-ionone were not chosen to carry on the next phagocytosis assay because of their severe cell cytotoxicity.

Effect of relevant chemical components of *O. fragrans* flowers on the phagocytic activity of RAW 264.7 cells

The phagocytosis of macrophages is the first step in an immune response, and increasing phagocytic activity is characteristic of activated macrophages [47]. Thus, the phagocytic activities of RAW 264.7 cells treated with various concentrations of relevant chemical components were investigated by neutral red uptake assay. When the tissues and organs undergo pathological changes, macrophages can eliminate antigen through intracellular cytotoxicity. Phagocytosis is a key indicator of evaluating macrophages' activities, activated macrophages help initiate specific defense mechanisms by recruiting other immune cells and play a critical role in the immune system [47]. Salidroside and verbascoside (3.125–100 μg mL^{-1}) could increase the phagocytosis of RAW 264.7 cells over the control cells in Fig. 6a. Linalool oxide could also increase the phagocytosis at 3.125–6.25 μg mL^{-1}, but not did at 12.5–100 μg mL^{-1}. Linalool had no effect of increasing the phagocytosis. Therefore, salidroside and verbascoside had more abilities to enhance phagocytic activity of RAW 264.7 cells than linalool oxide and linalool.

Phagocytosis of pathogens, an important function of macrophages, not only participates in activating the adaptive immune system, but also in the regulation of immune regulation [47]. The phagocytic rate was significantly suppressed ($P < 0.05$) when the cells were exposed to LPS after pretreated with salidroside, verbascoside, linalool oxide and linalool at 3.125-100 μg mL^{-1} (Fig. 6b). The phagocytic rates ($P < 0.05$) in LPS-stimulated RAW 264.7 cells through treating with salidroside, verbascoside, linalool oxide and linalool at 3.125-100 μg mL^{-1} was lower than that (155.1 ± 5.6%) of LPS-stimulated RAW 264.7 cells. But linalool oxide and linalool had obvious advantages than salidroside, verbascoside in reducing phagocytic activity of LPS-stimulated RAW 264.7 cells.

Effects of cell viability and phagocytic activity on drying method of *O. fragrans* flowers

According to the former results, geraniol, α-ionone and β-ionone had the bad cell proliferation and viability. Salidroside and verbascoside possessed the notable effects on enhancing the phagocytic activity of natural RAW 264.7 cells. Salidroside, verbascoside, Linalool oxide and linalool all had the remarkable advantages in reducing the phagocytic activity of LPS-stimulated RAW 264.7 cells. Therefore, salidroside and verbascoside had two-way immunoregulation of phagocytic cells. However, the contents of linalool oxide and linalool, only in the order of mg, were lower than salidroside and verbascoside, in the order of g in 100 g fresh *O. fragrans* flowers. Therefore, salidroside and verbascoside played a main part in regulating the activities of phagocytic cells in the immune system. The total content of salidroside and verbascoside in *"Aurantiacus", "Latifolius" and "Thunbergii"* was the highest with MD method in HF. Therefore, the rapid drying method represented by microwave drying was more conducive to maintaining the health care ability of *O. fragrans* flowers, which should be advocated. It was better to keep the unique fragrance of *O. fragrans* flowers and be used in food industry by the traditional drying, such as shade drying or lime drying.

Discussion

People liked to eat *O. fragrans* flowers and its preparations to a greater extent because of its good osmanthus fragrance. Their health care function was easily overlooked. Furthermore, their volatile components were paid more attention to than their non-volatile components. It would be very interesting that their osmanthus fragrance and health care function are both considered. Therefore, the study investigated the effects of drying methods on their non-volatile and volatile components and immunoregulatory activity of *O. fragrans* flowers.

Immunomodulation is a process of adapting the immune system to obtain the optimal response, in means of either stimulation or suppression of the immune reaction [48]. Immunotherapy has already been applied in cancer therapy. In this case, the immunity needs to be enhanced [49]. Natural products with immunomodulatory activity are widely used in treatment of many diseases including autoimmune diseases, inflammatory disorders in addition to cancer. Plants rich in flavonoids, isothiocyanates, carotenoids, organosulfur, flavonolignans, polysaccharides, and polyphenols may possess immunomodulatory and antitumor activities [50]. *O. fragrans* flowers as natural medicine and food plants should be investigated fully and taken seriously.

In this study, *O. fragrans* flowers from were dried with different drying methods. Their weight changes were contrasted before and after drying and their yields were calculated to ensure that moisture did not affect the content determination of the non-volatile and volatile components. These measures had the same effect to the determination of water contents in bitter gourd slices [37]. It was confirmed by comparison that these volatile components were similar to the reported literature with GC-MS method [51]. But their relative contents were grately different. It could be caused by the different extracting method. In this study, vapor distillation method was used to extract the essential oil. But it dispersed in the water only little. This extracting method was nearer the actual consumption mode than other methods. Therfore, it could reflect the actual application. The contents of salidroside and verbascoside

were similar to the contents with UPLC-MS method [52]. Similarly, the other non-volatile components were little. The verbascoside content was still the highest. Therefore, HPLC method was enough for the determination of the non-volatile components.

The final results showed that *O. fragrans* flowers from *Aurantiacus* group with the microwave drying method had the more salidroside, verbascoside, linalool oxide, and linalool.

Macrophages are immune myeloid cells with an extreme ability to modulate their phenotype in response to insults and/or pathogens. The immunomodulatory capacity of macrophages is also patent [53]. RAW 264.7 macrophages were usually used to evaluate the immunomodulatory activity of chemical substances. The immunotoxicity of salidroside, verbascoside, linalool oxide, linalool, Geraniol, α-ionone and β-ionone on RAW 264.7 macrophages were determined firstly to ensure that the investigated components did not produce the cytotoxic effects. Geraniol, α-ionone and β-ionone were toxic to macrophages. Salidroside, verbascoside, linalool oxide, and linalool were further screened according to their phagocytic activity of normal or LPS-stimulated RAW 264.7 cells. Both salidroside and verbascoside had the better phagocytic activity of normal or LPS-stimulated RAW 264.7 cells. According to above results, *O. fragrans* flowers from Aurantiacus group with the microwave drying method had the best immunomodulatory activity.

In this study, we addressed the effects of drying methods on non-volatile and volatile components and immunoregulatory activity of *O. fragrans* flowers from three groups by using HPLC, GC and MTT test. Though only several representative components were chosen and only in vitro immunomodulatory activity was investigated, it had the important reference value for evaluation of immunomodulatory activity of traditional Chinese Medicine.

Conclusions

O. fragrans flowers from three *O. fragrans* groups such as "*Aurantiacus*", "*Latifolius*" and "*Thunbergii*" were dried with five different methods. The non-volatile and volatile components such as salidroside, verbascoside, linalool oxide, and linalool were determined. The different characteristics appeared in the effects of drying methods on the non-volatile and volatile components of various *O. fragrans* flowers. The effects of the former quantitative components on the cell viability and phagocytic activity of normal or LPS-stimulated RAW 264.7 cells were investigated. 4 kinds of components such as salidroside, verbascoside, linalool oxide, and linalool all had a good immunoregulatory effect. Considering their different contents, the best drying method depended on the total contents of salidroside, verbascoside. Microwave drying and oven drying at high temperature were beneficial for the preservation of the bioactive non-volatile components for killing enzyme and protecting glycoside. Other drying methods such as shade drying, sun drying, quick-lime drying and oven drying at low temperature were beneficial to their preservation of the fragrant volatile components. The research will provide some evidence in choosing drying method of *O. fragrans* flowers as food or medicine.

Abbreviations

O. *fragrans*:*Osmamthus fragrans*;HPLC:high-performance liquid chromatography;GC:gas chromatography;DMSO:Dimethyl sulfoxide;3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide:MTT;ConA:Concanavalin A;FBS:fetal serum bovin;SHD:Shade drying;SUD:sun drying; QLD:quick-lime drying;OD:oven drying;MD:microwave drying;MLF:medium-low fire; MF:medium fire;MHF:medium-high fire;HF:high fire;UVD:ultraviolet-visible detector;FID:flame ionization detector.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CY designed the whole experiment, established the method of determination and was a major contributor in writing the manuscript. LJ , JY, and PS performed the determination of non-volatile and volatile components and the immunomodulatory activity examination. GA and DY established the method of immunomodulatory activity examination. All authors read and approved the final manuscript.

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Figures

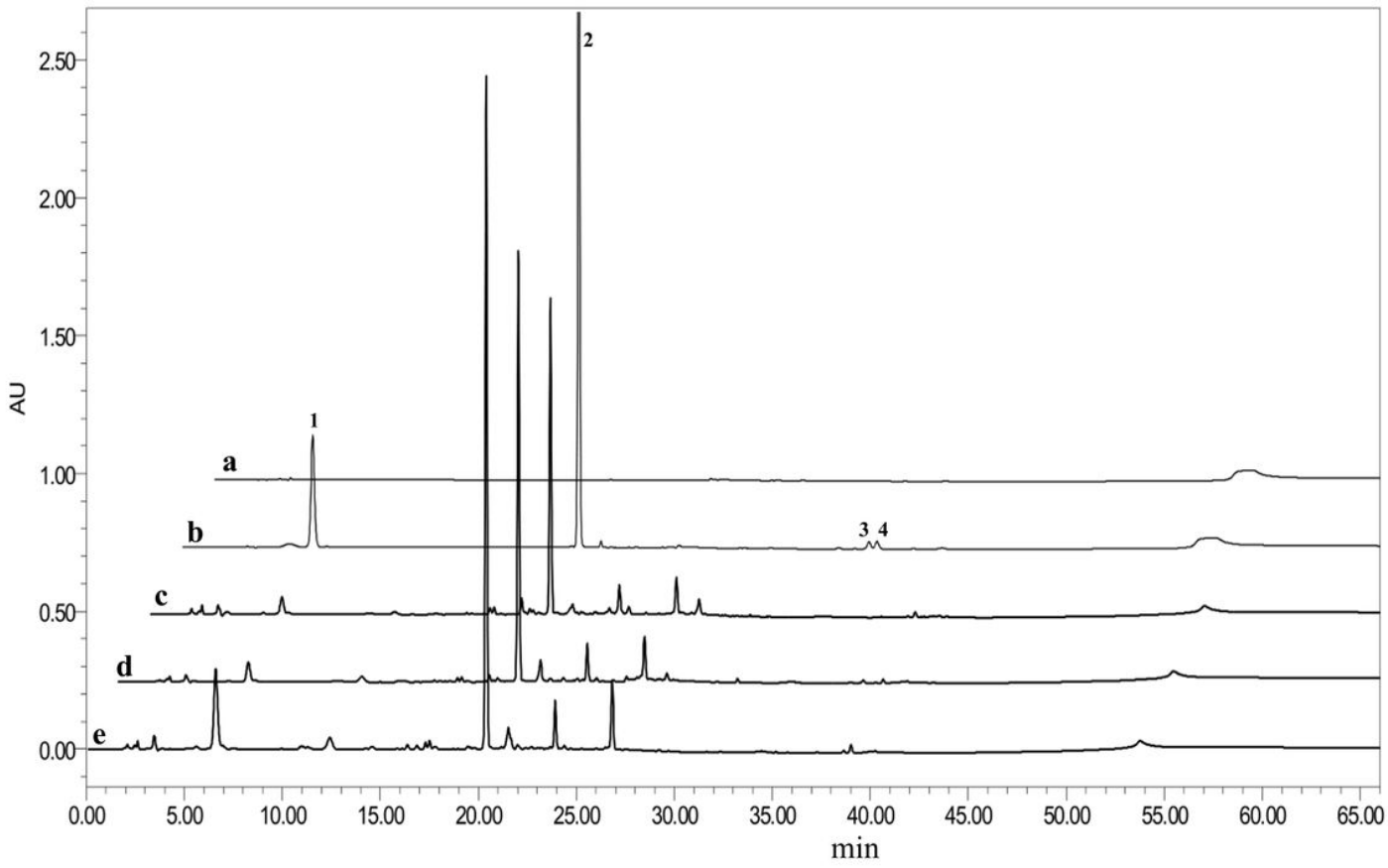

Figure 1

HPLC chromatogram of three O. fragrans groups (a. blank solution; b. mixed reference substance solution; c. Aurantiacus flowers; d. Latifolius flowers; e. Thunbergii flowers 1. salidroside; 2. verbascoside; 3. oleanolic acid; 4. ursolic acid)

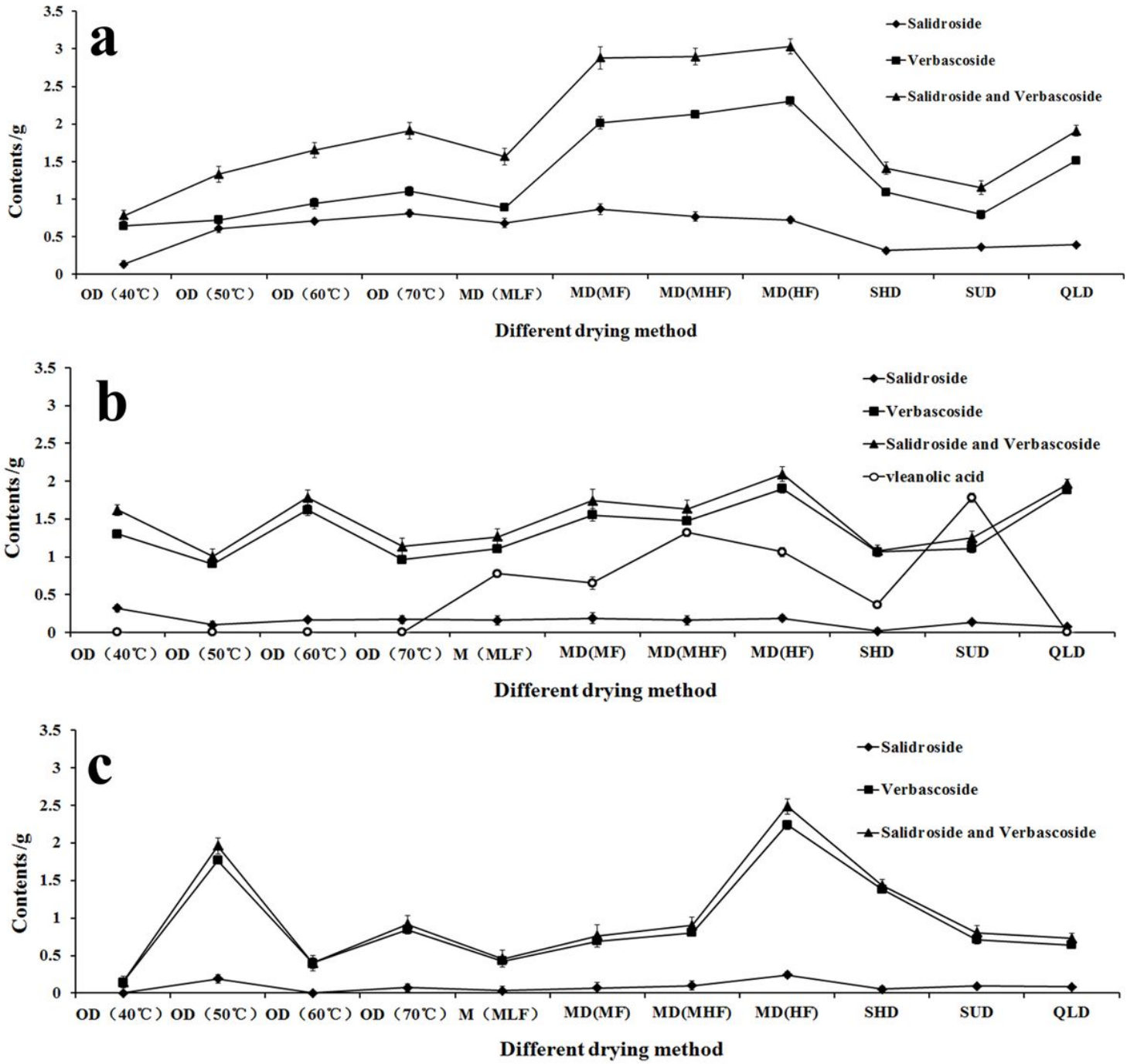

Figure 2

Effects of drying methods on the contents of salidroside, verbascoside, oleanolic acid and ursolic acid in three O. fragrans groups (a. Aurantiacus flowers; b. Latifolius flowers; c. Thunbergii flowers)

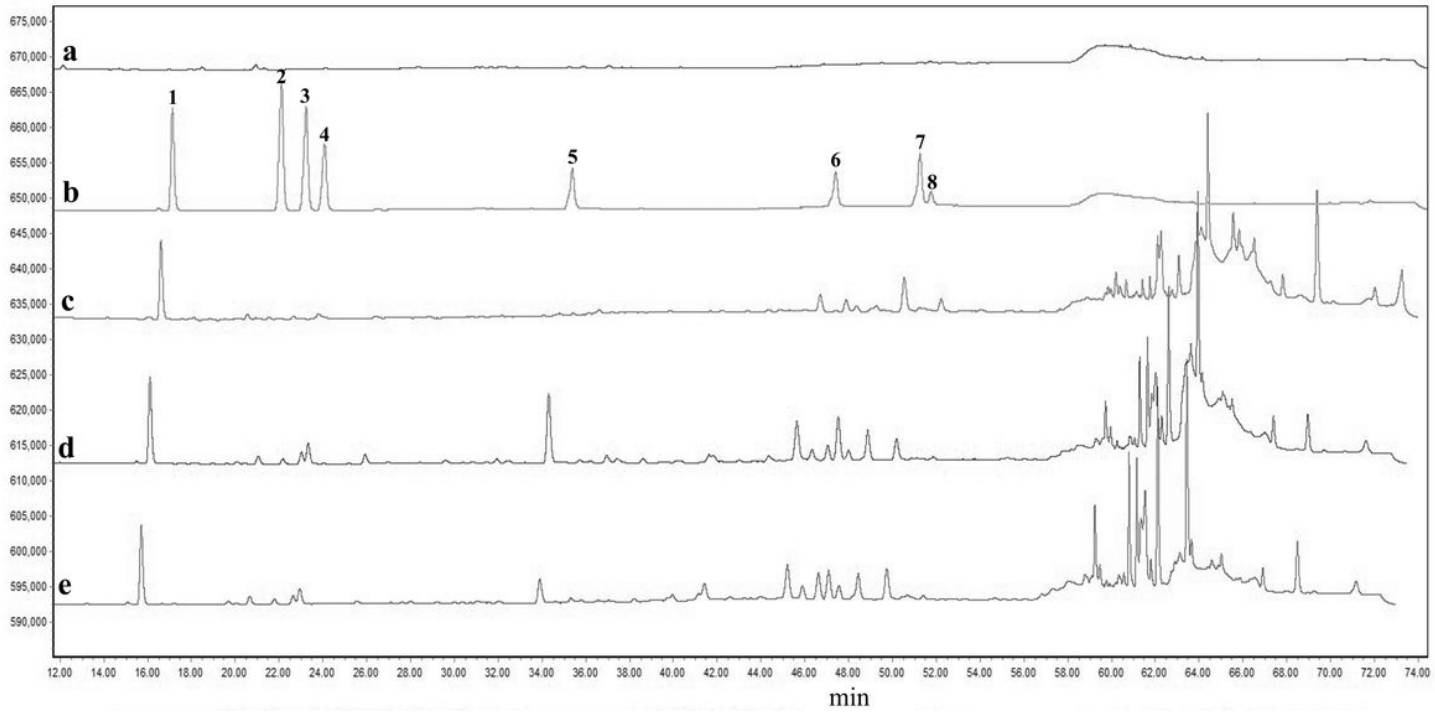

Figure 3

GC of three O. fragrans groups (a. blank solution ; b. mixed reference substance solution; c. Aurantiacus flowers; d. Latifolius flowers; e. Thunbergii flowers; 1. l (-)-2-octanol; 2-3. linalool oxide (isomer mixtures); 4. linalool; 5. geraniol; 6. α-ionone; 7.β-ionone; 8. δ-decanolide)

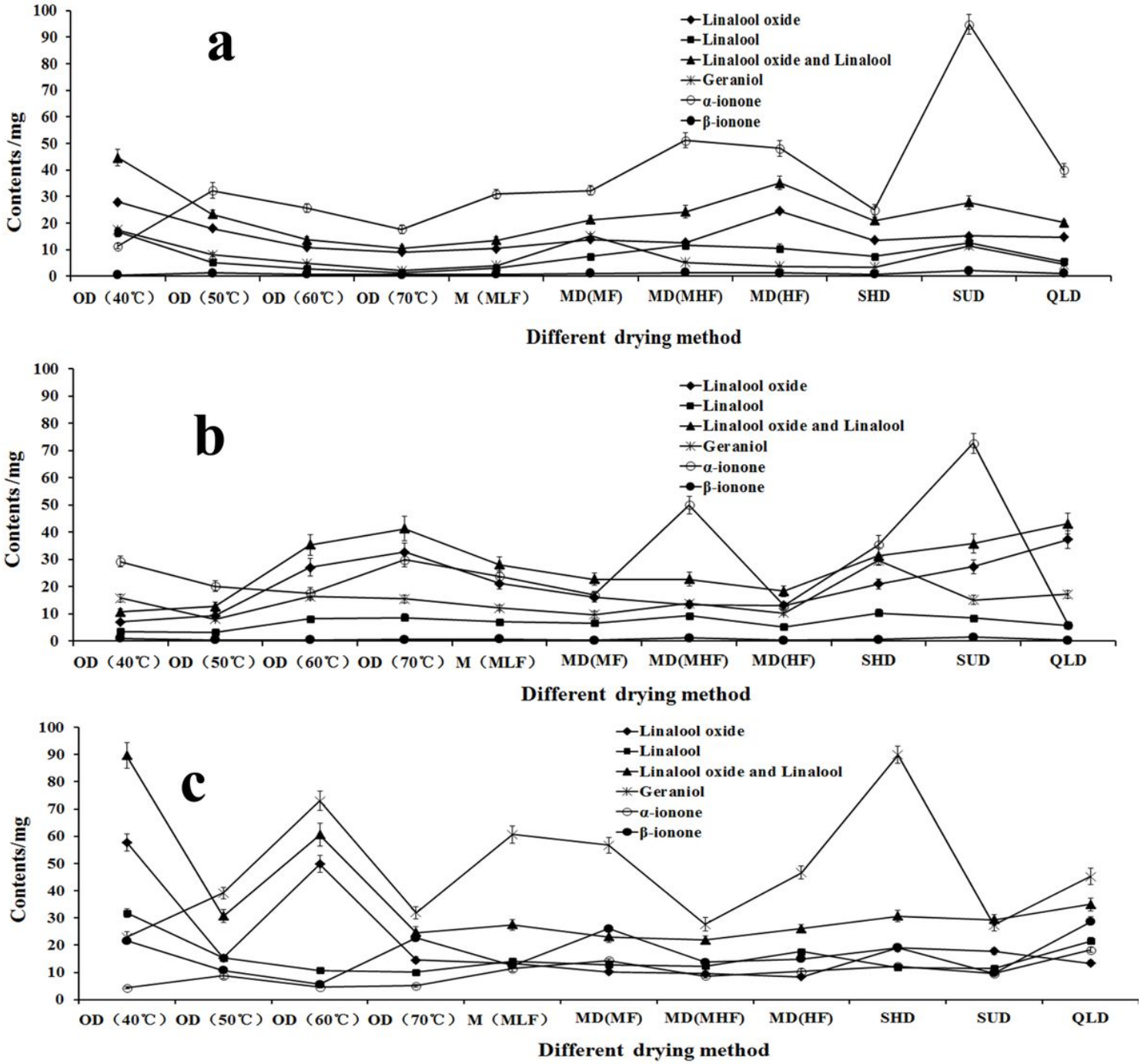

Figure 4

Effects of drying methods on the contents of Linalool oxide, linalool, geraniol, α-ionone and β-ionone in three O. fragrans groups (a. Aurantiacus flowers; b. Latifolius flowers; c. Thunbergii flowers)

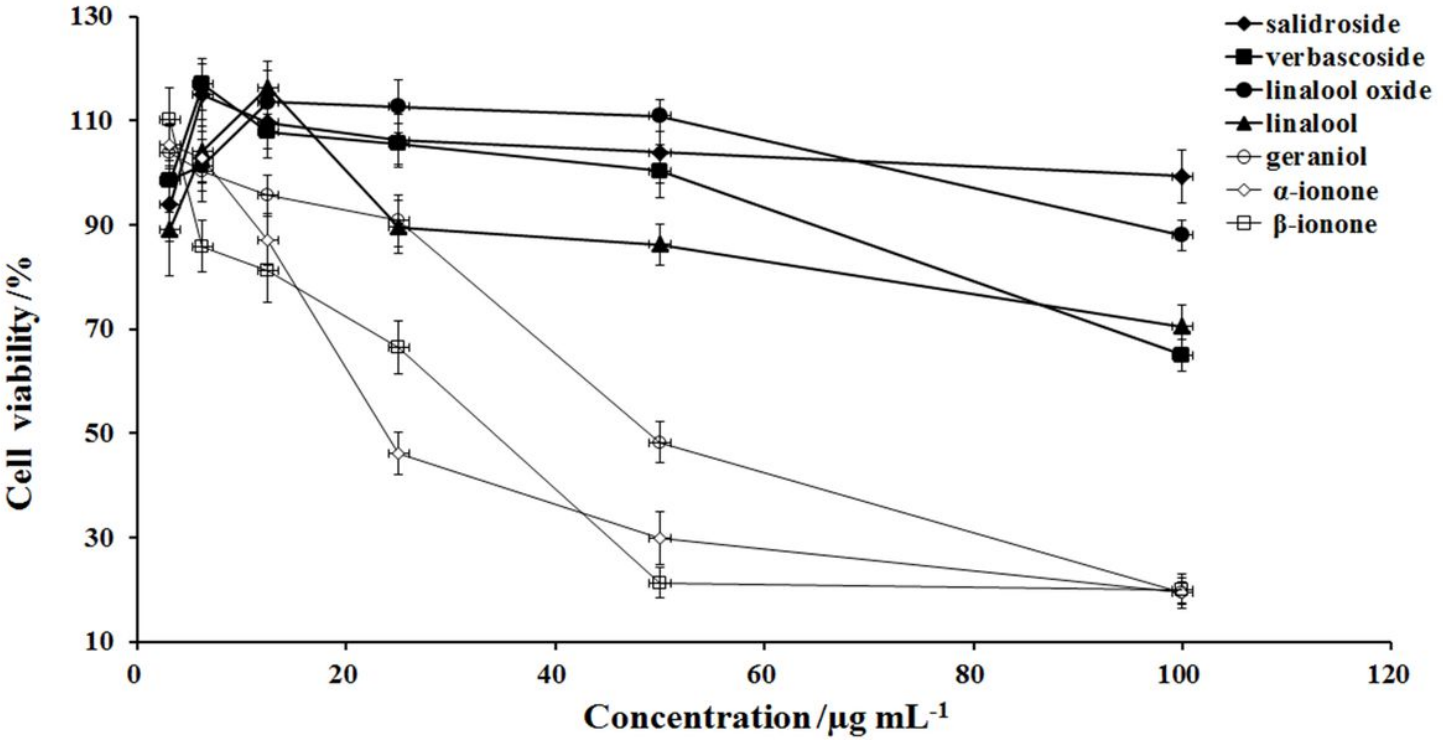

Figure 5

Effect of chemical components of O. fragrans flowers on cell viability of RAW 264.7 cells

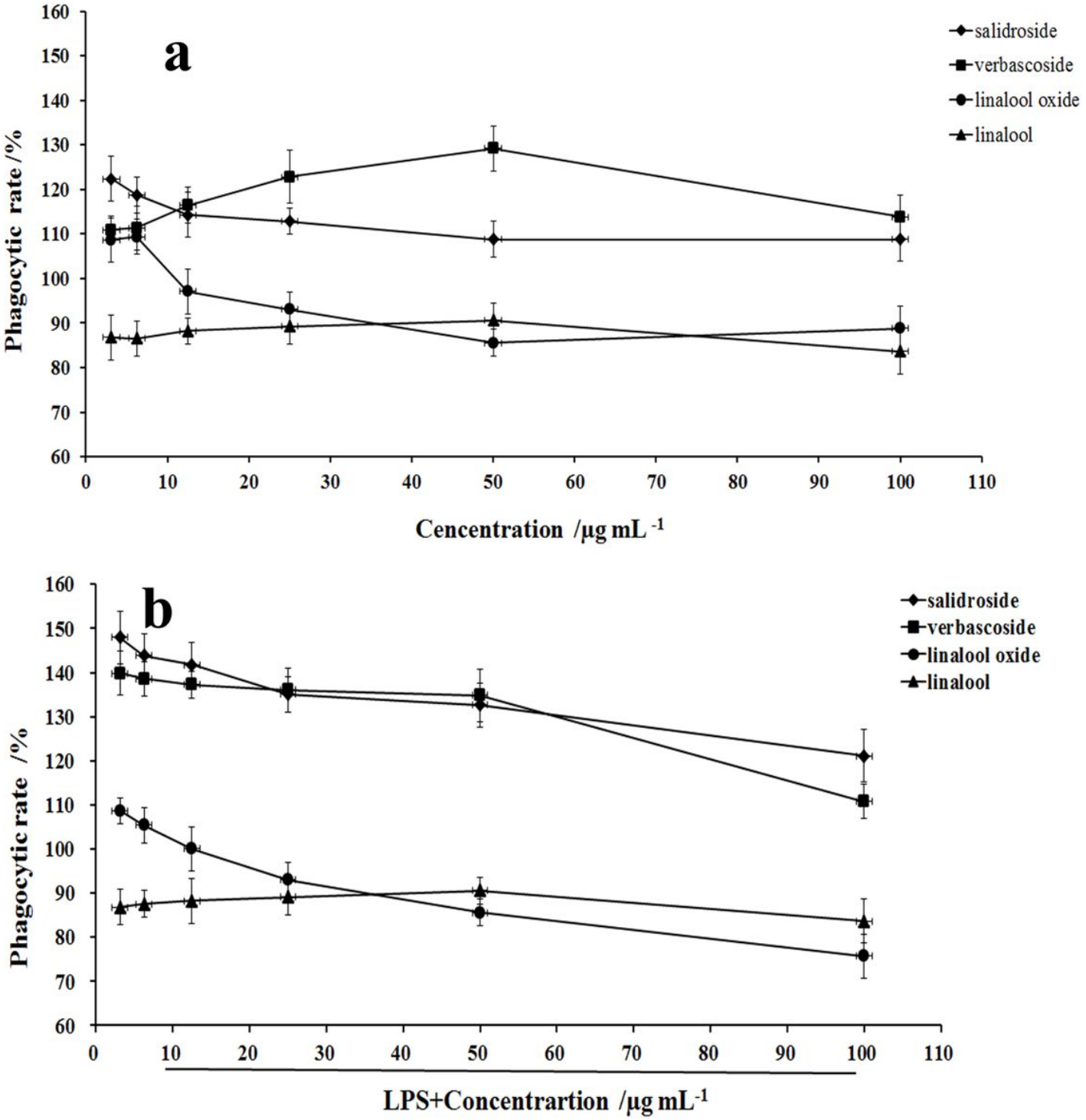

Figure 6

Effect of relevant chemical components of O. fragrans flowers on the phagocytic activity of RAW 264.7 cells