

Anti-cryptococcus Activity and Mechanism of Essential Oil from *Coreopsis tinctoria* Flowering on Cryptococcus

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Abstract

The essential oil (EO) extracted from the flower of *Coreopsis tinctoria* Nutt. is a kind of plant-derived natural therapeutics against *Cryptococcus neoformans*. In our study, the anti-cryptococcal effects of the EO were observed from various aspects, such as minimal inhibitory concentration, minimum fungicidal concentration, germ tube formation, and biomass. The results indicated that the EO had potent antifungal activities on *C. neoformans* and that it was more effective than the commonly used antifungal agents fluconazole and amphotericin B. To further elucidate the mechanism of the antifungal action, the effect of the EO on the plasma membrane and the mitochondria of *C. neoformans* were investigated. The lesion in the plasma membrane was detected through flow cytometry and was further verified through the inhibition of ergosterol synthesis. The essential oil caused morphological changes in the cells of *C. neoformans* and a reduction in the ergosterol quantity. Moreover, mitochondrial membrane potential, mitochondrial ATPase, succinate dehydrogenase (SDH), and malate dehydrogenase (MDH) were detected. The reactive oxygen species (ROS) accumulation was examined through fluorometric assay. Decreased SDH, MDH, and ATPase in *C. neoformans* cells were also observed in a dose-dependent manner. The above dysfunctions of the mitochondria caused ROS accumulation in *C. neoformans*. A reduction in cell viability was prevented through the addition of L-cysteine, and this reduction indicates that ROS is an important mediator of the antifungal action of the EO. In summary, EO from *C. tinctoria* flower showed anti-cryptococcus activity. Its mechanism of action is to disrupt the permeability barrier of the plasma membrane and the mitochondrial dysfunction-induced ROS accumulation in *C. neoformans*. With better antifungal activity than the commonly used antifungal agents and less possibility to induce drug resistance, the EO can be used as a potential anti-cryptococcal agent.

Keywords: *Coreopsis tinctoria*; Anti-cryptococcal; Mechanism; Essential oil

Introduction

Cryptococcus neoformans is a kind of environmental yeast that causes opportunistic infection [1]. The most common and serious type of cryptococcal disease is pulmonary cryptococcosis, which is uncontrolled and leads to cryptococcal meningitis or meningoencephalitis [2]. Cryptococcosis is one of the main pathogens which infections in patients with human hematological malignancies, immunodeficiency virus (HIV) and therapeutic immunosuppression [3,4]. The mortality of disseminated cryptococcosis is 70%-80% in untreated patients compared with 17% in patients treated with systemic antifungal agents [5]. Many clinically used antifungals (e.g., amphotericin-B, fluconazole, 5-fluorocytosine, miconazole, nystatin, etc.) suffer from various drawbacks in terms of toxicity, lack of fungicidal efficacy, high cost, drug-drug interactions, and emergence of resistant strains resulting from frequent usage [6,7]. As useful natural products from medicinal and aromatic plants, essential oils (EO) are known to possess antioxidant, antiviral, antibacterial and antifungal activities [8,9].

Coreopsis tinctoria Nutt., which belongs to the family Asteraceae, is a widely distributed medicinal plant in the Kunlun Mountains of the Xinjiang Uyghur Autonomous Region (altitude: approximately 3000 m)

in China [10]. *C. tinctoria* is widely used not only as an ornamental plant but also as functional food in China. It is rich in essential nutrients (e.g., vitamins, selenium, zinc, and amino acids [11,12], etc.) and many phytochemicals (e.g., flavonoids, polyphenols, polysaccharides, saponins, and EO) [13-19]. It has been demonstrated to have many nutraceutical properties, such as those for the management of diabetes, reduction of blood pressure and vasorelaxant effects, α-Glucosidase inhibitory effect and hypoglycemic activity, anti-coagulating activity, anti-aging activity, and antioxidant and anti-inflammatory activities [20-24]. In recent years, the *C. tinctoria* flowering top from the Kunlun Mountains in Xinjiang has become a popular health product used as herbal tea or beverage in China. However, to our knowledge and according to a literature survey, no reports have been made yet on the anti-cryptococcus activity and mechanism of EO from *Coreopsis tinctoria* flowering top. Accordingly, this study aimed to determine the mechanism of action of the EO on *C. neoformans*.

Materials and method

Plant material

The flower parts of *C. tinctoria* plants were harvested from the Xinjiang Uyghur Autonomous Region of China in September 2012 and were identified by Prof. Jianwei Hu (College of life science, Tarim

University). The No. 1-81 was deposited at the herbarium of the Institute of Traditional Chinese Medicine & Natural Products, Tarim University School of Life Sciences.

Gas chromatography-mass spectroscopy (GC-MS) analysis

Air-dried *C. tinctoria* flowers (200 g) were subject to hydrodistillation for 4 h using a Clevenger-type apparatus. The EO was dried over anhydrous Na_2SO_4 and preserved in a sealed vial at 4°C until further use. The EO yield was 0.15% (v/w).

The chemical composition of EO was analysed using GC-MS. The EO (20 μL) was dissolved in acetone (200 μL), and 1 μL of the solution was injected into the GC-MS (Agilent 7890A-5975C, USA). The capillary column was HP-5MS (length = 30 m, thickness = 0.25 μm , and i.d = 0.25 mm). Helium was used as the carrier gas at a flow rate of 1 ml·min⁻¹. The GC column oven temperature was increased from 50°C to 300°C at a rate of 10°C·min⁻¹, with a final hold time of 10 min. Injector and detector temperatures were maintained at 280°C. The EI mode was at 70 eV, mass spectra were recorded in the 30 amu-600 amu range, and the ion source temperature was 230°C. The EO components were quantified by the relative percent peak area of total ion current curve (TIC) from the MS signal and identified by comparing their mass fragmentation pattern with those stored in the spectrometer database using NIST05.LIB and NIST05s.LIB (National Institute of Standards and Technology).

Microorganisms

The *C. neoformans* strains were purchased from the China General Microbiological Culture Collection Center. The fungal strain cultures were maintained on a Sabouraud dextrose agar (SDA) slant at 4°C. The old cultures were transferred to a fresh slant every two months to avoid a decline in strain viability.

Antifungal activities of EO against *C. neoformans*

The *C. neoformans* strains were grown on SDA plates in the dark at 17 ± 2°C for four days. The concentrations of spores were adjusted to 1.0 × 10⁸ CFU/mL using a hemocytometer. The EO (80 μL) was dissolved in 1 mL 0.01% Tween 20 to obtain a working concentration.

The broth microdilution protocol based on the CLSI reference document M27-A3 with modification was used to determine minimal inhibitory concentration (MIC) of yeasts [25].

Briefly, tests were performed in sterile U-bottom 96-well plates. The drug was serially diluted twofold with RPMI 1640 (without sodium bicarbonate and L-glutamine, pH 7.0), and 100 μL of the drug solution at each concentration was used. The concentrations of EO, fluconazole (FCZ), and amphotericin B (AmB) ranged from 40 $\mu\text{L}/\text{mL}$ - 0.038 $\mu\text{L}/\text{mL}$ and 40 $\mu\text{L}/\text{mL}$ - 0.38 $\mu\text{g}/\text{mL}$, respectively. The inoculum *C. neoformans* suspension was diluted 10 times with RPMI 1640 broth to obtain a working suspension (approx. 1 × 10⁶ CFU/mL), and 100 μL of the diluted suspension was added to each well. Wells without inocula were used as negative controls, and wells with 0.01% (v/v) Tween 20 but without the EO were used as positive growth controls. The microplates were incubated at 17 ± 2°C for two days. MICs (the lowest concentrations of the test substances that prevented the visible growth of microorganisms) were then determined. To evaluate the minimum fungicidal concentrations (MFCs; the lowest concentrations yielding negative subcultures or only one colony) of the tested drugs, 100 μL solutions from each negative well and from the positive growth control

were transferred onto the SDA. The colonies were counted after four days' incubation at 17 ± 2°C. All experiments were performed in triplicate.

Germ tube formation assay

Germ tube formation was tested following the procedure described previously but with a slight modification [26]. The EO was dissolved in 0.1% Tween 20 to final EO concentrations of 0.391, 0.781, and 1.563 $\mu\text{L}/\text{mL}$, with a final Tween 20 concentration of 0.01%. The controls contained 0.01% Tween 20 without the EO. The final concentrations of AmB and FCZ (positive control) were 3.91 and 50 $\mu\text{g}/\text{mL}$, respectively. The drug solutions were inoculated with spore suspension of each *C. neoformans* containing 1.0 × 10⁶ CFU/mL and then homogenized by inverting the test tubes three or four times. A total of 100 μL of *C. neoformans* mixture was placed on separate glass slides and incubated in a moisture chamber at 17 ± 2°C for 8 h. The slides were then observed under the microscope for spore germination. About 200 spores were counted, and the percentage spore germination was calculated.

Determination of biomass

The EO was dissolved in 1 mL 0.01% (v/v) Tween 20 and added to 48 mL of the SDB culture medium to make final concentrations of 0.391, 0.781, and 1.563 $\mu\text{L}/\text{mL}$, and then inoculated with 1 mL *C. neoformans* containing 1.0 × 10⁶ CFU/mL. Control plates (with 0.1% Tween 20 but without the EO) were inoculated with the same procedure. The final concentrations of AmB and FCZ (positive control) were 1.563 and 50 $\mu\text{g}/\text{mL}$, respectively. The flasks were then incubated for 72 h shaking (180 rpm) at 17°C. After incubation, the cells were filtered with sterile Whatman paper and washed twice with sterile distilled water. The cells were dried at 60°C for 6 h and then at 40°C overnight, and weighed. Percent growth inhibition on the basis of dry weight was calculated as follows: (Control weight-Sample weight)/Control weight × 100 [27]. All experiments were conducted in triplicate.

Determination of ergosterol content in the plasma membrane of *C. neoformans*

Membrane damage was examined following the procedure described previously but with a slight modification [27]. Briefly, a single *C. neoformans* colony from an overnight SDA culture plate was inoculated into 60 mL of SDB containing different concentrations of the EO. The EO was dissolved in 0.1% Tween 20, and the final concentrations were 0.391, 0.781 and 1.563 $\mu\text{L}/\text{mL}$. FCZ (50 $\mu\text{g}/\text{mL}$) was used as the positive control. The negative control contained only 0.01% (v/v) Tween 20. The cultures were incubated for 24 h at 17°C in a shaker at 200 rpm. Stationary-phase cells were harvested by centrifugation at 5000 g for 5 min and washed once with sterile distilled water. The wet weights were then determined. About 10 mL of 25% alcoholic potassium hydroxide solution was added to each pellet and vortex mixed for 10 min. Cell suspensions were transferred into clean glass tubes and incubated in an 85°C water bath for 4 h. Sterols were extracted from each sample by adding a mixture of 1 mL sterile distilled water and 3 mL n-heptane, followed by vigorous vortex mixing for 10 min. The n-heptane layer was transferred into a clean tube and stored at -20°C for 24 h. Prior to analysis, 1 mL aliquot of sterol extract was diluted twofold with 100% ethanol, and the spectra between 230 nm and 300 nm were scanned with a spectrophotometer. The presence of ergosterol (282 nm) and the late sterol intermediate

24(28) dehydroergosterol (DHE, 230 and 282 nm) in the n-heptane layer led to a characteristic curve. The ergosterol content was calculated as a percentage of the wet weight of the cells, which was based on the absorbance and wet weight of the initial pellet. The calculation formula of the ergosterol content is as follows:

$$\% \text{ Ergosterol} + \% 24(28)\text{DHE} = [(A_{282/290} \times F) / \text{pellet weight},$$

$$\% 24(28)\text{DHE} = [A_{230/518} \times F] / \text{pellet weight},$$

$$\% \text{ Ergosterol} = [\% \text{ ergosterol} + \% 24(28)\text{DHE}] - \% 24(28)\text{DHE},$$

where F is the factor for dilution in ethanol, and 290 and 518 are the E values (in percentage per centimetre) determined for crystalline ergosterol and 24(28) DHE, respectively.

Determination of plasma membrane damage

Membrane damage was examined following the previously described procedure with a slight modification [28]. *C. neoformans* suspensions were grown in the SDB for 24 h at 17°C in a shaker at 200 rpm. The cell suspension (1×10^6 CFU/mL) of *C. neoformans* was then added into each glass tube. A certain amount of the EO was added into the tubes to make final concentrations of 0.391, 0.781, and 1.563 $\mu\text{L/mL}$. Samples with only 0.01% Tween 20 were used as the control, and those with 50 $\mu\text{g/mL}$ FCZ were used as the positive control. The mixtures were then incubated at $17 \pm 2^\circ\text{C}$ for 12 h in an incubator shaker. The cells were washed and re-suspended in 500 μL PBS and stained with 1 $\mu\text{g/mL}$ Propidium iodide (PI, Sigma Co.) in PBS for 30 min at $17 \pm 2^\circ\text{C}$. Unstained cell suspensions were always included as auto-fluorescence controls. For each sample, a scattergram analysis was performed to evaluate morphological changes, and the percentage of PI-positive cells was determined using FAScalibur (BD, USA) at FL2 channel (620 nm). All tests were performed in triplicate.

Measurement of mitochondrial membrane potential ($\text{mt}\Delta\psi$).

The effect of the EO on the $\text{mt}\Delta\psi$ of *C. neoformans* was measured by flow cytometry using rhodamine 123 (Rh123) according to a previously described procedure with some modifications [26]. Cells exposed to various doses of the EO (0.391, 0.781, and 1.563 $\mu\text{L/mL}$) at 17°C for 12 h were harvested by centrifugation at 5000 g for 5 min, washed twice, and then re-suspended at 6×10^6 CFU/mL in PBS. Rh123 was added to a final concentration of 0.1 mg/mL and incubated for 30 min in the dark at 17°C. For all cytofluorimetric experiments, forward and side scatters were gated on the major population of cells, and 6×10^6 CFU/mL cells per sample were measured. $\text{mt}\Delta\psi$ was expressed as the fluorescence intensity of Rh123, which was read using a FAScalibur (BD, USA) at FL1 channel (525 nm). The data were analyzed using Cell Quest Pro software. All tests were performed in triplicate.

Measurement of the reactive oxygen species (ROS) formation

The endogenous amounts of ROS in *C. neoformans* were measured by flow cytometry using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as a ROS indicator as described previously with minor modifications [27]. Exponentially growing *C. neoformans* cells were suspended in sterile PBS to 6×10^6 CFU/mL. Serial twofold dilutions of the EO (0.391, 0.781, and 1.563 $\mu\text{L/mL}$) were added to the cell suspensions for 12 h at 17°C. Samples with only 0.01% Tween 20 were used as control, and those with 50 $\mu\text{g/mL}$ FCZ were used as positive control. The cells were harvested by centrifugation at 5000 g for 5 min, washed twice, and re-suspended to 6×10^6 CFU/mL in PBS. DCFH-

DA was added from a stock solution of 1 mM to a final concentration of 10 mM using a ROS kit (C1300; Apply gen Technology Co.). After 30 min of incubation at 17°C, the cells were collected by centrifugation and re-suspended in PBS to 6×10^6 CFU/mL. ROS was expressed as the fluorescence intensity of DCFH-DA, which was read using a FAScalibur (BD, USA) at FL1 channel (520 nm). The data were analyzed using the Cell Quest Pro software. Additionally, the experiments were performed with the presence of the antioxidant Cys and the EO at concentrations of 60 mM and 0.781 $\mu\text{L/mL}$, respectively. Control group without Cys and EO. The results were corrected by subtracting the fluorescence value of dill oil $\pm$ antioxidant in the corresponding concentration without cells but with DCFH-DA. All tests were performed in triplicate.

Measurement of mitochondrial dehydrogenases

The effect of EO on the activities of mitochondrial dehydrogenases was determined using the XTT method as described previously with some modifications [29]. XTT (Sigma) was prepared as a saturated solution at 1 mg/mL in PBS. The solution was filter sterilized through a 0.22 mm pore size filter, aliquoted, and then stored at -80°C . Prior to each assay, an aliquot of stock XTT was thawed, and 10 mM menadione (Sigma), which was prepared in acetone, was added to a final concentration of 1 mM. *C. neoformans* was cultured and diluted to 6×10^6 CFU/mL. The cells were inoculated in 96-well flat-bottomed microplates. Different concentrations of the EO (0.391, 0.781, and 1.563 $\mu\text{L/mL}$) or the medium alone were added, and the cells were incubated at 17°C for 24 h. A 100 μL aliquot of the XTT/menadione solution was added to each well and to the control wells to measure the background XTT reduction levels. The plates were incubated at 17°C for 4 h in the dark. Fungal mitochondrial dehydrogenase reduced the XTT tetrazolium salt to XTT formazan, thus resulting in a colorimetric change correlated with cell viability. The colorimetric change was measured using a microtiter plate reader (KHB ST-360; Experimental System Co.) at 492 nm.

Preparation for the mitochondria

Briefly, *C. neoformans* cells (6×10^6 CFU/mL) were used to inoculate 60 mL of the SDB containing various concentrations (0.391, 0.781, and 1.563 $\mu\text{L/mL}$) of the EO. The cultures were incubated for 12 h shaking (200 rpm) at $17 \pm 2^\circ\text{C}$. The cells were harvested by centrifugation at 5000 g for 5 min, washed twice with sterile distilled water, suspended, and homogenized in a medium (50 mM Tris pH 7.5, 2 mM EDTA, and 1 mM phenyl methyl sulfonyl fluoride). Then, 2% glucose was added to the mixture and centrifuged at $2000 \times g$ for 5 min to remove cell debris and broken cells. The supernatants were collected and centrifuged at $10,000 \times g$ for 30 min, and the pellets were collected as mitochondria. The pellets was resuspended in 50 mM Tris (pH 7.5) - 2 mM EDTA-20 % (v/v) glycerol and stored at -80°C .

Measurement of the succinate dehydrogenase, malate dehydrogenase, and ATPase activities in the mitochondria of *C. neoformans*

The effects of the EO on the activities of mitochondrial succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) of *C. neoformans* were detected using SDH, MDH, and Micro-ATPase Assay Kit obtained from the Institute of Biological Engineering of Nanjing Jianchen (Nanjing, China) following the manufacturer's

protocol. Protein content was determined as described by Bradford, and bovine serum albumin was used as a standard [30].

Statistical analysis

The statistical analysis was performed using statistical software (SPSS, 13.0; Chicago, USA). All data are reported as means $\pm$ standard deviations. The significant differences between the mean values were determined by Duncan's Multiple Range test, following one-way analysis of variance. P value < 0.05 was considered statistically significant.

Results

Chemical composition of the EO

Twenty-two components of the essential oil, accounting for 99.4% of the total oil composition, were identified by GC-MS (Table 1).

Peak No	Compound	Composition (%)
1	1R-alpha-Pinene	11.70
2	Beta-Phellandrene	0.51
3	Beta-Myrcene	1.86
4	Alpha-Phellandrene	1.92
5	1-methyl-2-(1-methylethyl)benzene	1.09
6	Limonene	52.56
7	3-carene	1.44
8	(4E,6Z)-2,6-Dimethyl-2,4,6-octatriene	0.59
9	Bicyclo[3,1,1]hept-3-en-2-ol,4,6,6-trimethyl	0.83
10	1,3-Cycloheptadiene	0.61
11	Longifolene-(V4)	3.67
12	Caryophyllene	1.45
13	Tricyclene	1.22
14	Sabinene	0.61
15	Zingiberene	7.63
16	Campholenic aldehyde	1.79
17	Caryophyllene oxide	0.59
18	Apiole	0.39
19	Camphene	0.78
20	9-Fluorene-methanol	4.28
21	3-methylcyclohexene	0.76
22	2-Amino-6-methylbenzothiazole	1.20
	Total	99.40

Table 1: Chemical composition of the EO isolated by hydrodistillation from *C. tinctoria* flower.

The most abundant components of the EO were limonene (52.56%), pinene (11.70%), and zingiberene (7.63%). Several other components such as 9-fluorene-methanol (4.28%), longifolene-(V4) (3.67%), phellandrene (1.92%), myrcene (1.86%), 3-carene (1.44%) and caryophyllene (1.45%) were less in amounts. Oxygenated sesquiterpenes, sesquiterpene hydrocarbons, hydrocarbons, and others were found in trace amounts only.

Anti-*C. neoformans* effects of the EO

The MIC and MFC values indicated that the EO was active against *C. neoformans* (Table 2) and exhibited strong antifungal activity. The MIC and MFC levels of the EO against *C. neoformans* were 0.781 and 1.563 μ L/mL, respectively. The MIC levels of AmB and FCZ against the *C. neoformans* were 3.906 and 50 μ g/mL, respectively. In recent years, the increasing social and health implications of *C. neoformans* have suggested the constant endeavoring to develop safe and new natural antifungal agents to cure human fungal disorders. In this study, the EO showed a pronounced antifungal efficacy against *C. neoformans* and a MIC value lower than the positive control (FCZ and AmB).

	MIC	MFC
AmB μ g/mL	3.906	7.812
FCZ μ g/mL	50	100
EO μ L/mL	0.781	1.562
Limonene μ L/mL	0.781	1.562
Pinene μ L/mL	1.562	3.124

Table 2: MIC and MFC values of EO, FCZ, and AmB against *C. neoformans*.

The germ tube formation is a transition state from budding to hyphal cells, and it represents an essential stage to virulence. Therefore, the effect of the EO on the germ tube formation was tested (Figure 1A).

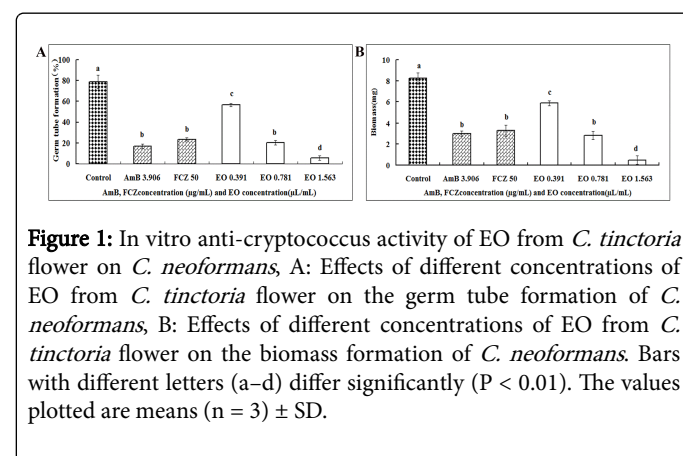


Figure 1: In vitro anti-cryptococcus activity of EO from *C. tinctoria* flower on *C. neoformans*, A: Effects of different concentrations of EO from *C. tinctoria* flower on the germ tube formation of *C. neoformans*, B: Effects of different concentrations of EO from *C. tinctoria* flower on the biomass formation of *C. neoformans*. Bars with different letters (a–d) differ significantly (P < 0.01). The values plotted are means (n = 3) $\pm$ SD.

Various concentrations of the EO and positive control showed inhibitory effects on the germ tube formation compared with the controls (P < 0.05). The inhibition calculated on the 8th hour was found to be equal to 56.33%, 20.31%, and 5.38 % for *C. neoformans*, at the EO final concentrations of 1.563, 0.781, and 0.391 μ L/mL. The positive controls FCZ and AmB exhibited inhibition on the germ tube formation at 50 and 3.906 μ g/mL, respectively, and the EO showed

comparable activities at 0.781 $\mu\text{g}/\text{mL}$ and higher activities at 0.391 $\mu\text{g}/\text{mL}$ (Figure 1A).

Inhibition of the biomass by the EO was also estimated by measuring the dry cell weight (Figure 1B). The EO significantly inhibited the growth of *C. neoformans* in a dose-dependent manner ($P < 0.05$). Similar to the results of MIC, MFC, and germ tube formation experiments, the EO at 0.391 $\mu\text{L}/\text{mL}$ showed comparable activity with the positive controls FCZ (50 $\mu\text{g}/\text{mL}$) and Amb (1.563 $\mu\text{g}/\text{mL}$).

Acting mechanism of the EO against *C. neoformans*

Flow cytometry was used to investigate the membrane integrity of *C. neoformans* cells after treatment with the EO, with PI as the fluorescent marker. Substantial morphological changes were observed on the scattergrams of *C. neoformans* cells after 12 h incubation with 0.391, 0.781, and 1.563 $\mu\text{L}/\text{mL}$ EO (Figure 2D-2F), and the percentage of PI-stained *C. neoformans* cells increased along with the concentration.

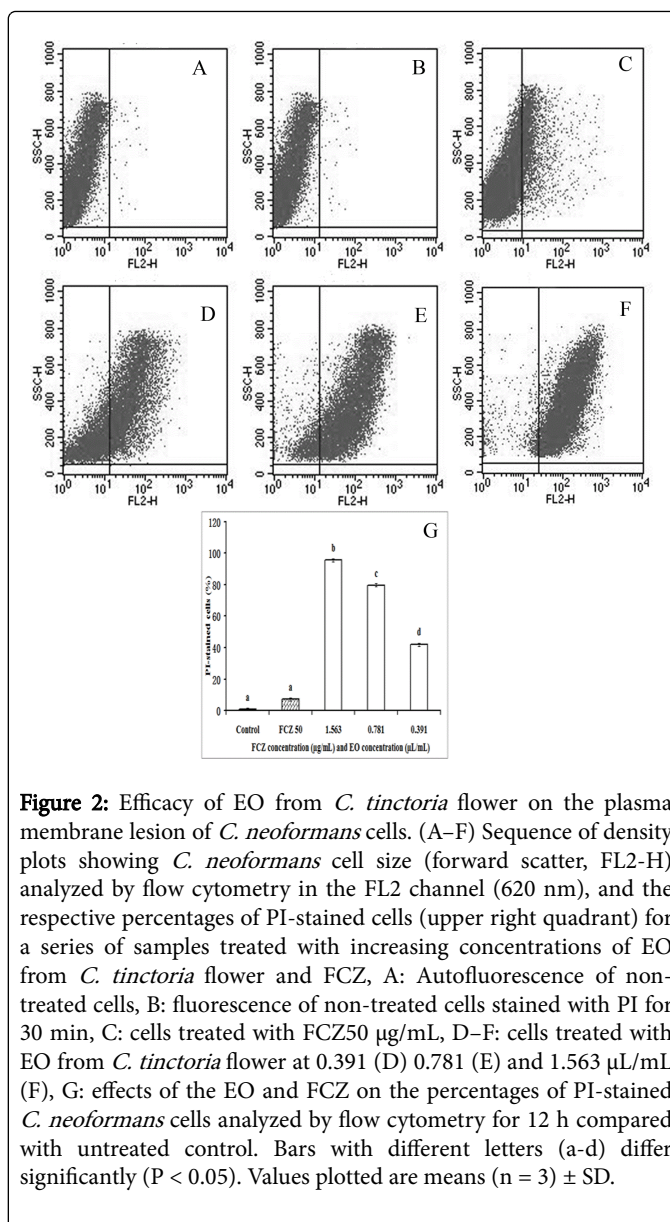


Figure 2: Efficacy of EO from *C. tinctoria* flower on the plasma membrane lesion of *C. neoformans* cells. (A–F) Sequence of density plots showing *C. neoformans* cell size (forward scatter, FL2-H) analyzed by flow cytometry in the FL2 channel (620 nm), and the respective percentages of PI-stained cells (upper right quadrant) for a series of samples treated with increasing concentrations of EO from *C. tinctoria* flower and FCZ, A: Autofluorescence of non-treated cells, B: fluorescence of non-treated cells stained with PI for 30 min, C: cells treated with FCZ50 $\mu\text{g}/\text{mL}$, D–F: cells treated with EO from *C. tinctoria* flower at 0.391 (D) 0.781 (E) and 1.563 $\mu\text{L}/\text{mL}$ (F), G: effects of the EO and FCZ on the percentages of PI-stained *C. neoformans* cells analyzed by flow cytometry for 12 h compared with untreated control. Bars with different letters (a–d) differ significantly ($P < 0.05$). Values plotted are means ($n = 3$) $\pm$ SD.

The percentages of stained cells with 1.563, 0.781, and 0.391 μL EO were 95%, 79%, and 42%, respectively. The results suggested that the cell membrane structure of the *C. neoformans* was disrupted by the EO in a dose-dependent manner. Under the same experimental conditions, 50 $\mu\text{g}/\text{mL}$ FCZ induced only 7.18% of cell death (Figure 2C). These results indicated that the mode of action of the EO involved a primary lesion of the cell membrane, thus leading to cell death. Furthermore, the effect of EO on the cell membrane might be different from that of FCZ.

The total ergosterol content of the plasma membrane of *C. neoformans* was determined after treatment with different concentrations of EO (0.391 $\mu\text{L}/\text{mL}$ –1.563 $\mu\text{L}/\text{mL}$) (Figure 3). The resultant ergosterol contents were 0.037 ± 0.01 , 0.023 ± 0.09 , 0.017 ± 0.06 , and 0.013 ± 0.02 , respectively, and the total ergosterol content of the positive control (FCZ at 50 $\mu\text{g}/\text{mL}$) was 0.024 ± 0.03 . This finding demonstrated that the ergosterol content of the plasma membrane of *C. neoformans* was significantly reduced when treated with different

concentrations of the EO in a dose-dependent manner. The decrease in ergosterol content after treatment with 0.781 $\mu\text{L/mL}$ EO (decreased by 46.74%) was comparable with that of the positive control of 50 $\mu\text{g/mL}$ FCZ (decreased by 45.78%), and it was further decreased when treated with 1.563 $\mu\text{L/mL}$ EO (decreased by 73.26%) (Figure 3).

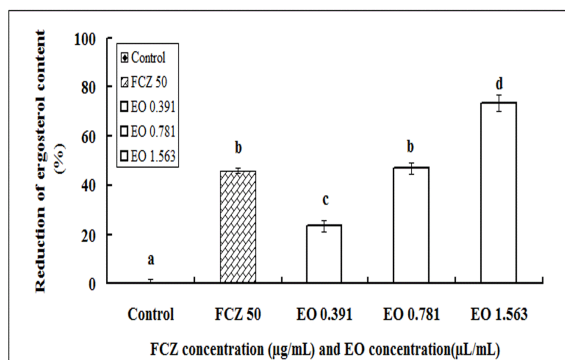


Figure 3: Ultraviolet spectrophotometric profiles of sterol in *C. neoformans* treated with EO from *C. tinctoria* flower (0.391-1.563 $\mu\text{L/mL}$) and FCZ 50 $\mu\text{g/mL}$. Bars with different letters (a-d) differ significantly ($P < 0.05$). The values plotted are means ($n = 3$) $\pm$ SD.

The effect of EO on $\text{mt}\Delta\psi$ of the cells was first measured by flow cytometry using Rh123, a potential-dependent distributional probe. The results are shown in Figure 4. The $\text{mt}\Delta\psi$ values (calculated from the mean fluorescence of all measured cells) of the cells untreated or treated with EO at concentrations of 0.391, 0.781, and 1.563 $\mu\text{L/mL}$ were 84.03 ± 1.24 , 183.3 ± 5.13 , and 278.76 ± 6.64 , respectively (Figure 4C-4E). The $\text{mt}\Delta\psi$ value of FCZ (50 $\mu\text{g/mL}$) (Figure 4B) without the increased intensity of fluorescent cells was similar to the control (Figure 4A). The results showed that the EO from *C. tinctoria* flower increased the intensity of fluorescent cells in a dose-dependent manner. In addition, EO increased $\text{mt}\Delta\psi$ in *C. neoformans* in a dose-dependent manner.]

The effect of the EO from *C. tinctoria* flower on the mitochondria of *C. neoformans* was assessed. The activities of the total mitochondrial dehydrogenases (Figure 5A), MDH (Figure 5B), SDH (Figure 5C), and A-TPase (Figure 5D) of *C. neoformans* were significantly reduced ($P < 0.05$) when treated with the EO at all concentrations.

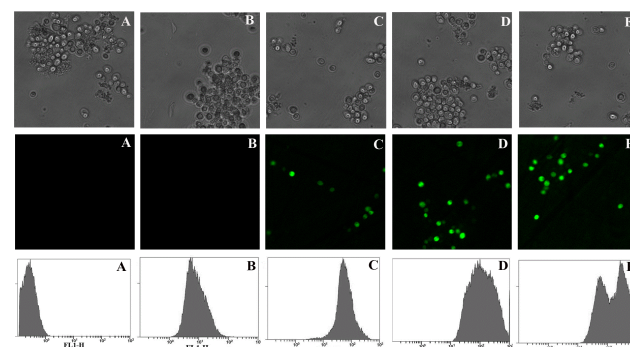


Figure 4: Efficacy of EO from *C. tinctoria* flower and FCZ on $\text{mt}\Delta\psi$ of *C. neoformans* cells stained with Rh123.

All of these reductions in the EO activity were dose dependent, and the positive control FCZ (50 $\mu\text{g/mL}$) without reduction was similar to the control.

The fluorescent dye DCFH-DA, a well-established compound used to assay and quantify ROS production, was used to investigate the changes in the intracellular ROS level of *C. neoformans* cells.

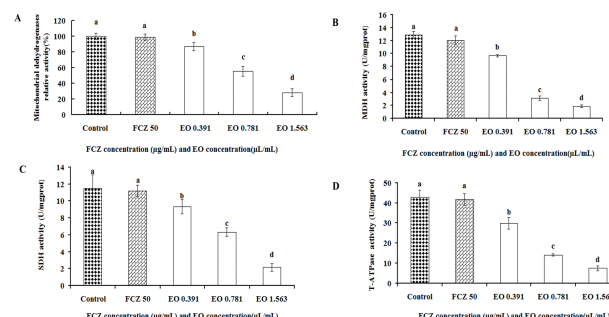


Figure 5: Effects of EO from *C. tinctoria* flower on the activities of the mitochondrial dehydrogenases of *C. neoformans*, A: Effects of EO on the total mitochondrial dehydrogenases activities of *C. neoformans*, B: Effects of EO on the MDH activity of *C. neoformans*, C: Effects of EO on the SDH activity of *C. neoformans*, D: Effects of EO on the A-TPase activity of *C. neoformans*. Bars with different letters (a-d) differ significantly ($P < 0.05$). The values plotted are means ($n = 3$) $\pm$ SD.

As shown in Figure 6C-6E, an increase in fluorescence intensity was detected in *C. neoformans* cells incubated with different concentrations of the EO for 12 h in a dose-dependent manner. Compared with that in the control (Figure 6A) and the positive control FCZ (Figure 6B), the promotion of the fluorescence intensity of the EO corresponded to 7.19, 9.06, and 12.03 times at a concentration of 0.391, 0.781 and 1.563 $\mu\text{L/mL}$, respectively. The percentage of ROS-positive cells clearly increased with the concentration of EO (Figure 6F). Compared with the control ($2.89 \pm 1.45\%$) and positive control FCZ ($3.51 \pm 2.03\%$), the ROS-positive cells of the EO was $82.8 \pm 5.45\%$, $49.20 \pm 3.64\%$, and $24.41 \pm 2.87\%$, respectively. The EO was observed to induce ROS accumulation in *C. neoformans* cells.

To investigate whether ROS production is directly involved in the antifungal effect of the EO and is not merely a secondary effect of the EO action, the effect of the antioxidant Cys on the net level of ROS production and on the antifungal activity in EO-treated *C. neoformans* cells was measured. As shown in Figure 6G, the net ROS production in cells induced by EO treatment was evidently reduced by the addition of the antioxidant Cys. With the presence of Cys, the quantity of ROS in cells increased only slightly with the MIC concentration (0.781 $\mu\text{L}/\text{mL}$). This finding suggests that Cys can arrest the induced ROS production by the EO. Thus, ROS formation plays a critical role in dill oil-induced killing of *C. neoformans* cells.

Discussion

EO, which is aromatic volatile products of the secondary metabolism of plants, can be applied in every aspect of daily living [8,31]. EOs has a great antifungal activity due to its synergistic effect with some active components, and thus they are more promising in commercial application than single compounds.

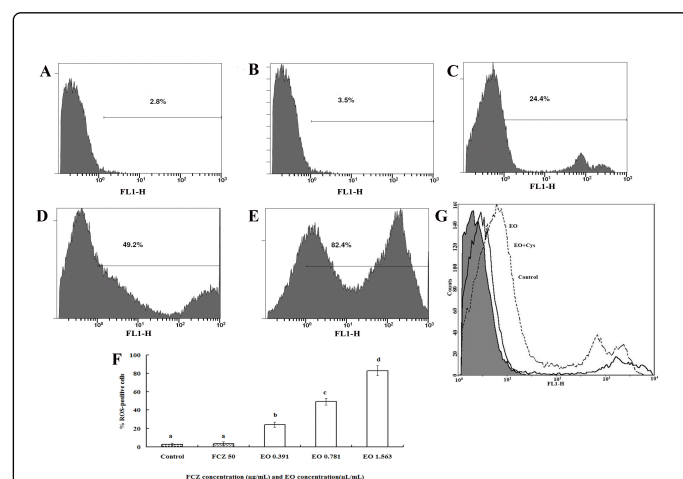


Figure 6: Effects of EO from *C. tinctoria* flower on the generation of endogenous ROS in *C. neoformans* by flow cytometry (excitation at 488 nm and emission at 520 nm). A: Fluorescence of non-treated cells stained with DCFH-DA for 30 min, B: Cells treated with FCZ 50 $\mu\text{g}/\text{mL}$, C-E: Cells treated with EO from *C. tinctoria* flower at 0.391 (C) 0.781, (D) and 1.563 $\mu\text{L}/\text{mL}$, F Effects of the EO and FCZ on the percentages of stained *C. neoformans* cells analyzed by flow cytometry for 12 h compared with untreated control. Bars with different letters (a-d) differ significantly (P0.05). The values plotted are means (n = 3) $\pm$ SD, G: Effect of the antioxidant 60 mM Cys on EO-induced ROS generation in *C. neoformans* cells analyzed using flow cytometry (excitation at 488 nm and emission at 520 nm).

The chemical compositions of the EO from the *C. tinctoria* flower used in the present study were previously determined by our laboratory. Using GC-MS, the major components detected in the EO from *C. tinctoria* flower are limonene (52.56%), pinene (11.70%), and zingiberene (7.63%). Compared with the reported composition of the EO from *C. tinctoria* flower by other researchers, the limonene and pinene were in agreement but had different contents. Pinene was detected but 9-fluorenemethanol and longifolene-(V4) were not in the EO presented in this paper. A literature search revealed limonene (63.5%), 3-carene (7.0%), p-cymene (5.2%), and zingiberene (2.4%) to

be the main constituents of EO from the wild-growing *C. tinctoria* flower native to the Kunlun Mountain area, Xinjiang, China [19]. Gaspar et al. [12] reported that the major components of *C. tinctoria* flower EO were α -pinene (45.2%) and trans-carve (28.6 %). These variations in the chemical constituents of the EO may be attributed to the various agro climatic (geographical, climatic, and seasonal) conditions of the regions, the adaptive metabolism of plants, and the stage of maturity.

The anti-cryptococcus activity of the EO from *C. tinctoria* flower was studied in vitro. In sum, the EO from *C. tinctoria* flower significantly restricted the germ tube formation and the biomass of the tested *C. neoformans* in a dose-dependent manner. In vitro studies on EO indicated its potential as an antifungal agent against *C. neoformans*. The EO exhibited antifungal activities that could be attributed to the presence of limonene, pinene, and zingiberene [32,33]. To the best of our knowledge, this report is the first on the anti-*C. neoformans* activity of the EO from *C. tinctoria* flower.

The plasma membrane plays a vital role in maintaining a homeostatic environment, transferring energy, and exchanging materials and information in the cell to keep cells healthy and alive. PI is one of the most popular fluorescent probes used in assessing the effect of drugs on plasma membranes [34]. Ergosterol is specific to fungi and is the major sterol component of the fungal cell membrane. It is also responsible for maintaining cell function and integrity [35]. The observed dose-dependent antifungal activity of the EO from *C. tinctoria* flower on *C. neoformans* cells with a severe lesion of the plasma membrane significantly reduced the ergosterol content. Our results indicated that the plasma membrane is a major target of the EO from *C. tinctoria* flower, and ergosterol is known to be the major sterol component of the fungal cell membrane and it maintains the cell function and integrity [36]. Other studies have also shown that plant-derived EO can cause a considerable reduction in the quantity of ergosterol [34,37], consistent with this study. Thus, the plasma membrane of *C. neoformans* should be an important antifungal target of the EO from *C. tinctoria* flower.

Furthermore, the mitochondria play a vital role in the cell, such as in apoptosis, pH homeostasis, redox [38], and ATP production. In the mitochondria, ATP is derived from mitochondrial oxidative phosphorylation, in which $\text{mt}\Delta\psi$ plays an essential role [39]. $\text{mt}\Delta\psi$ is a sensitive indicator of the energetic state of the mitochondria and the cell [40]. This indicator can also be used to assess the activity of mitochondrial proton pumps, electrogenic transport systems, and mitochondrial permeability transition [41]. The mitochondria of healthy cells maintain $\text{mt}\Delta\psi$ across their inner membranes, and this maintenance is the result of an electrochemical gradient maintained through the electron transport chain [42]. Inhibitors of mitochondrial electron transport, such as antimycin A and potassium cyanide, decrease $\text{mt}\Delta\psi$ by inhibiting the proton-pumping function of the respiratory chain that leads to decreased ATP production and cell death. A previous study showed that the antifungal action of EO is involved in $\text{mt}\Delta\psi$ hyperpolarization in yeast and *Aspergillus* [43,44]. To the best of our knowledge, the present study is the first to show that the antifungal activity of EO from *C. tinctoria* flower is causally linked to $\text{mt}\Delta\psi$ hyperpolarization in *C. neoformans*. In the present research, Rh123 was used to examine the effect of EO on mitochondrial potentials. Rh123 is a cationic and lipophilic dye that permeates the negatively charged mitochondria and reflects $\text{mt}\Delta\psi$. Exposure to different concentrations of EO for 12 h showed a significant

hyperpolarization of *C. neoformans* mt $\Delta\psi$ in a dose-dependent manner, thus suggesting mitochondrial dysfunction.

Mitochondria are also the main organelles for ATP synthesis. Mitochondrial dehydrogenases, including MDH and SDH, are important enzymes in the biosynthesis of ATP [45]. MDH catalyzes the interconversion of malate and oxaloacetate in the TCA cycle, and SDH catalyzes the oxidation of succinate to fumarate in the TCA cycle and transfers the electrons from succinate to ubiquinol [46]. In this study, the mitochondrial MDH and SDH activities were measured using assay kits, and the results showed that the mitochondrial MDH and SDH activities of *C. neoformans* were reduced after the treatment with EO from *C. tinctoria* flower. This result suggests that the mitochondria of *C. neoformans* are another important antifungal target of EO from *C. tinctoria* flower. Moreover, EO could damage the plasma membrane of *C. neoformans* and affect the enzyme activities of mitochondria; this process could be the antifungal mechanism of EO from *C. tinctoria* flower.

Mitochondria are organelles inside the cells of higher organisms that represent a major source of ROS. ROS lead to enzyme inactivation, cell dysfunction, and then cell death, and they also play physiological roles in the regulation of apoptosis [47,48]. In the assay for the detection of ROS, an increase in the amount of ROS was apparent after treatment with the EO from *C. tinctoria* flower. The addition of antioxidant Cys readily prevented ROS production, thus indicating that ROS may be an important mediator in the exhibition of the antifungal effects of the EO from *C. tinctoria* flower. Recent research has demonstrated that the antifungal action of many antifungal agents is involved in the induction of ROS formation in *C. neoformans* [49,50]. To the best of our knowledge, the present study is the first to show that the antifungal activity of the EO from *C. tinctoria* flower is causally linked to an intracellular process in *C. neoformans*, which is the induction of endogenous ROS. ROS accumulation is a common pathway underlying cellular damage induced by different types of stresses including exposure to EO. Normally, higher mt $\Delta\psi$ is accompanied by a more efficient electron transportation process, which in turn leads to the leakage of more electrons to generate ROS. Previous studies have indicated that ROS generation is exponentially dependent on mt $\Delta\psi$ [51], consistent with the results of our study. That is, both mt $\Delta\psi$ and endogenous ROS were obviously augmented in *C. neoformans* after treatment with the EO from *C. tinctoria* flower.

Conclusion

In summary, EO from *C. tinctoria* flower was successfully extracted and analysed. Its major components are limonene, pinene, and zingiberene. The EO from *C. tinctoria* flower showed anti-cryptococcus activity in vitro. It could inhibit the germ tube formation and biomass increase in the *C. neoformans*. Its mechanism of action was to disrupt the permeability barrier of the plasma membrane and the mitochondrial dysfunction-induced ROS accumulation in *C. neoformans*. With better antifungal activity than the commonly used antifungal agents and less likelihood of inducing drug resistance, the EO from *C. tinctoria* flower is a potential natural anti-cryptococcus agent.

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Author Contributions

Conceived and designed the experiments: HZ JT. Performed the experiments: TL HP. Analyzed the data: JT TL. Contributed reagents/materials/analysis tools: TL HP HZ. Wrote the paper: HZ JT.

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