

Original Research Article

Anticancer Effects of *Chenopodium ambrosioides* L. Essential Oil on Human Breast Cancer MCF-7 Cells *In vitro*

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Abstract

Purpose: To investigate the most effective compound of *C. ambrosioides* essential oil for the induction of cell death in human breast cancer cells (MCF-7), and the mechanism of induction.

Methods: MCF-7 cells were treated with essential oil and its two main components, 1-isopropyl-4-methylbenzene and α -terpinene, respectively, for 24 and 48 h *in vitro*. To determine their cytotoxicity on MCF-7 cells, *in vitro* cytotoxicity, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and live/dead cell fluorescent staining were used. MCF-7 cellular superoxide dismutase (SOD), catalase (CAT) vitality and malondialdehyde (MDA) content were also evaluated.

Results: MTT results showed that essential oil and its two main compositions significantly inhibited the growth of MCF-7 cells in 24 h ($p < 0.05$), which was consistent with the Live/dead cell fluorescent staining results. After 24 h incubation the average inhibition rate is 58.98 % for essential oil, 37.8 % for 1-isopropyl-4-methylbenzene and 32.09 % for α -terpinene. With increase in the concentration of essential oil and the two main components, the relative activity of SOD significantly decreased ($p < 0.05$), while the relative activity of CAT was gradually increased ($p < 0.05$), compared with control. MDA relative content significantly increased ($p < 0.05$) until the concentration was 1.25, 0.21 and 0.17 $\mu\text{g/ml}$ for essential oil, 1-isopropyl-4-methylbenzene and α -terpinene, and thereafter significantly decreased ($p < 0.05$), compared to control.

Conclusion: The data suggest that the essential oil of *C. ambrosioides* and its two main components inhibit MCF-7 cell proliferation cell death by inducing oxidative damage. However, the two main components are less effective in their anticancer activity than the essential oil

Keywords: *Chenopodium ambrosioides* L. Essential oil, 1-isopropyl-4-methylbenzene, α -Terpinene, Breast cancer MCF-7 cells, Antitumor activity

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INTRODUCTION

Secondary metabolites of plants have been applied in medicine and pharmacology for hundreds years in history. Over the past decades, anticancer substances of plant origin, particularly essential oils from plants have become one of the study focus in the field of cancer therapy [1]. Numerous studies suggest that essential oils may have good anticancer

activity [2-3]. *Chenopodium ambrosioides* L. is commonly called 'red Ze blue', 'Smelly grass' or 'Hookworm grass' in China, 'mastruz' in Brazil and 'epazote' and 'paico' in other countries of America. It is an annual or perennial herbaceous shrub, and its whole plant is rich in essential oils [4]. *C. ambrosioides* can kill insects such as hookworm and roundworm, and also it is commonly used as traditional chinese medicine for the treatment of rheumatoid arthritis, eczema,

dysmenorrhea, amenorrhea and snake bites [5]. Recent studies found that hydro alcoholic leaf extract of *C. ambrosioides* may be able to inhibit solid and ascitic Ehrlich tumor growth in mice, and increase cellular recruitment, phagocytosis ability and NO production in C₃H/HePas mice [6, 7]. More recently, our studies showed that essential oils from *C. ambrosioides* have a significant inhibitory effect on human breast cancer cells (MCF-7), and induces cancer cells apoptosis [8]. Essential oil from *C. ambrosioides* is a complex of many components. It mainly contains monoterpenes, sesquiterpenes and its oxygenated derivatives [9], but the proportion of these may be changed depending on the growth areas of *C. ambrosioides*. In our study, essential oil of *C. ambrosioides* has been analyzed and found to mainly contain 1-isopropyl-4-methylbenzene and α -terpinene. On the basis of our preliminary work, this study is aimed to explore the most effective ingredient for anticancer activity of essential oil from *C. ambrosioides*, and the potential anticancer mechanism.

EXPERIMENTAL

Materials

Materials and experimental instruments used in this study mainly includes *C. ambrosioides* (Chengdu, China), 1-isopropyl-4-methylbenzene (mass fraction 99.5 %, Ehrenstoefer Quality Company, Augsburg, Germany), α -terpinene standard (mass fraction 90 %, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), MTT (Sigma, U.S.), Live/dead viability/cytotoxicity dye (Molecular Probes, U.S.), Superoxide dismutase (SOD) WST-1 method assay kit, Catalase (CAT) assay kit, Malondialdehyde (MDA) and the BCA method protein quantification Kit (Jiancheng Bioengineering Institute, Nanjing, China).

Analysis of essential oils

The essential oil from *C. ambrosioides* was extracted by steam distillation method [10], then dried with anhydrous sodium and finally stored at

4 °C. The essential oil of *C. ambrosioides* consists of 16.9 % 1-isopropyl-4-methylbenzene analyzed by GC-MS and 13.5 % α -terpinene with meteorological chromatography.

Experimental procedures

The essential oil was dissolved in dimethyl sulfoxide (DMSO, 99.5 %) to obtain a concentration of 50 mg/mL as stock solution. In addition, the stock volume was further diluted with DMSO (99.5 %) to obtain various working solutions. The treatment concentration of 1-isopropyl-4-methylbenzene and α -terpinene was prepared similar to total essential oil. Details of samples treatment concentrations are presented in Table 1.

Cell lines and culture

Human breast cancer cell line MCF-7 (Chengdu Hali company, Chengdu, China) was cultured in RPMI-1640 medium supplemented with 10 % fetal calf serum, 100 units/ml penicillin, 100 μ g/ml streptomycin and 2 Mml-glutamine. Cells were maintained at 37 °C in a 5 % CO₂ atmosphere.

MTT assay

Cells in the concentration of 1×10^5 cell/ml were plated in 96-well plates with the amount of 100 μ l/well at 37 °C in a 5 % CO₂ atmosphere for 20 h.

The cells were cultivated for a further 24 or 48 h after different dilutions of essential oil, 1-isopropyl-4-methylbenzene, and α -terpinene were added to triplicate wells. Cell viability was evaluated by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. In brief, 20 μ L MTT (5 mg/ml) were added to each well and cultured for 4 h. The medium was then removed and 150 μ L of DMSO (Sigma) was further added to each well. The absorbance of the solutions was measured at 490 nm after 10 min shaking. Using cells treated with DMSO as control, the growth inhibition (GI) was calculated as in Eq 1.

$$GI (\%) = \{(1 - AT/AC)\}100 \dots\dots\dots (1)$$

Table 1: Treatment plan of the essential oil from *Chenopodium ambrosioides* L. and two main components

| Sample | Concentration (μ g/ml) | | | | | |
|-----------------------------|-----------------------------|------|------|-------|-------|-------|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| Essential oil | 0.75 | 1.25 | 5.00 | 12.50 | 25.00 | 50.00 |
| 1-isopropyl-4-methylbenzene | 0.12 | 0.21 | 0.82 | 2.06 | 4.12 | 8.23 |
| α -terpinene | 0.13 | 0.17 | 0.68 | 1.69 | 3.38 | 6.75 |

where AT and AC represent the absorbance of test sample and control, respectively.

Mean inhibition was calculated as in Eq 2.

Mean inhibition = $\frac{\sum \text{Inhibition of each treated group}}{6}$

Live/dead viability/cytotoxicity fluorescent dye assay

MCF-7 cells were treated for 24 h with 4 and 6 work solutions (Table 1) according to MTT results and then cultured for a further 24 h. These cells were stained according to manufacturers' instructions for Live/dead viability/cytotoxicity fluorescent dye. These samples were viewed under a fluorescence microscope (TE2000-U, Nikon, China).

Measure relative activity of SOD, CAT and MDA assay

After cultivation for (1×10^5 cell/mL, 3 ml/gals) 20 h in growth medium at 37 °C in a 5 % CO₂ atmosphere, MCF-7 cells were treated with work solution 2, 4, 5, and 6, respectively for 24 h. Then superoxide dismutase (SOD), catalase (CAT) activity and malondialdehyde (MDA) content of the treated MCF-7 cells were measured according to the manufacturers' instructions. In order to facilitate comparison and observable trends, activity and MDA levels were converted into relative value as in Eq 3.

Relative activity (or MDA) = $(Et/Ec)100$ (1)

where Et is the enzyme activity of treated group and Ec is the enzyme activity of control group.

Statistical analysis

Quantitative data were described as mean \pm standard deviation (SD) and analyzed using one way ANOVA. Statistical analysis was carried out using PASW software, version 18.0 (Chicago, IL, USA). $P < 0.05$ was considered significant unless stated otherwise.

Table 2: Comparison of mean inhibition rates of essential oil and its two main components from *C. ambrosioides* on MCF-7 cells

| Time (h) | Mean inhibition (%) | | |
|----------|---------------------|-----------------------------|---------------------|
| | Essential oil | 1-isopropyl-4-methylbenzene | α -terpinene |
| 24 | 58.98 | 37.80 | 32.09 |
| 48 | 53.52 | 34.76 | 33.04 |

RESULTS

MTT assay

Details of MTT results are presented in Fig.1. Generally, *C. ambrosioides* essential oil, 1-isopropyl-4-methylbenzene, and α -terpinene showed strong inhibition effects on proliferation of MCF-7 cells. Essential oils induced a dose-dependent inhibitory effect on MCF-7 cells that was statistically and significantly different at 24h and 48 h ($p < 0.05$). The inhibition rate of 1-isopropyl-4-methylbenzene was statistically and significantly higher (One-Way ANOVA, $p < 0.05$) than that of α -terpinene in the high concentration group (solution 4, 5 and 6). After 24 h incubation, mean inhibition rate was 58.98 % for essential oil, 37.8 % for 1-isopropyl-4-methylbenzene and 32.09 % for α -terpinene (Table 2). We thus selected 24h as treatment time for the later morphological observation and the subsequent antioxidant activity determination test due to its better inhibition effect.

Live/dead viability/cytotoxicity

The MCF-7 cells treated with essential oils, 1-isopropyl-4-methylbenzene and α -terpinene were stained using Live/dead viability/cytotoxicity fluorescent dye, and observed under fluorescence microscope. Living cells give green fluorescence, whereas dead cells give red (Fig 2). These results show that the increment of dead cells was highly consistent with the increased concentration of essential oil, 1-isopropyl-4-methylbenzene and α -terpinene. Dead cells increased (Fig 2-B) in the essential oil group 4 (12.5 μ g/mL) compared with control, of which almost all cells are living (Fig 2-A). There are almost all dead cells (Fig 2-C) in the group of 50 μ g/ml essential oil. In all essential oil treatment groups, dead cells were more than that in the corresponding treatment group of 1-isopropyl-4-methylbenzene (Fig 2, D-F) and α -terpinene (Fig 2, G-I). This result is consistent with that observed for MTT assay.

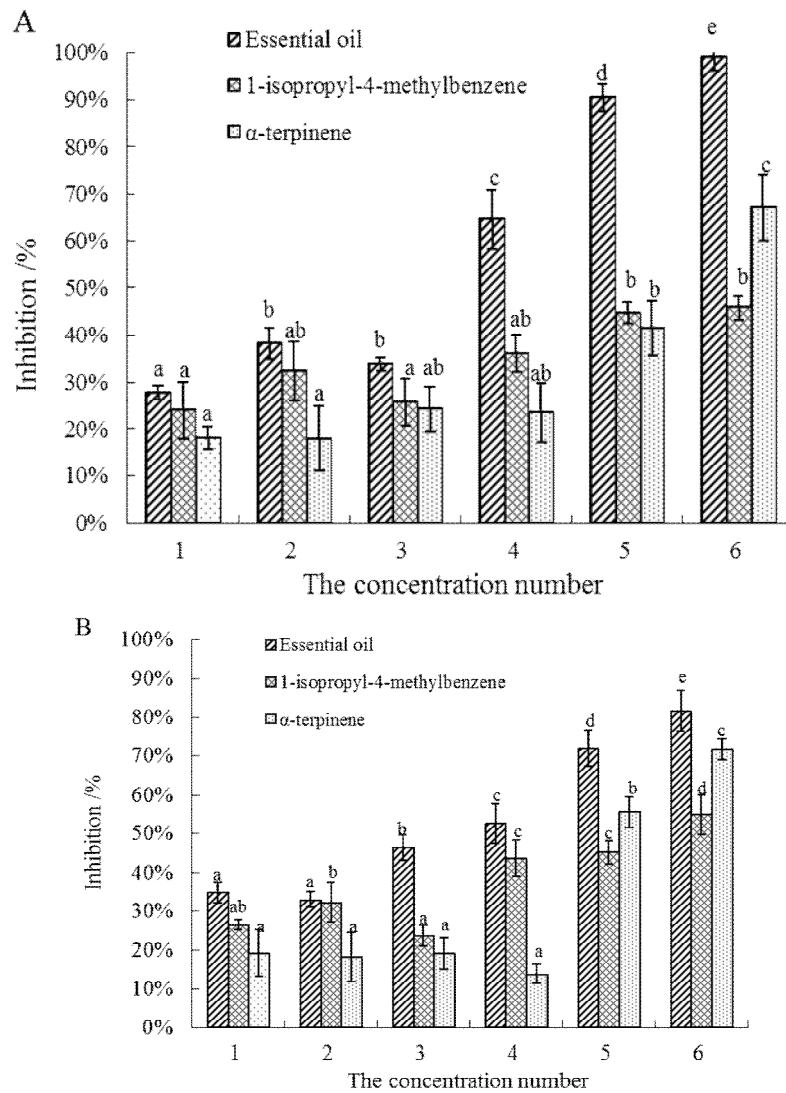


Fig 1: Effect of the essential oil and two main components from *C. ambrosioides* on proliferation of MCF-7 cells. **Note:** Different letters represent significant difference among treatments with the same sample ($p < 0.05$; A represents treatment group (after 24 h), while B represents treatment group after 48 h

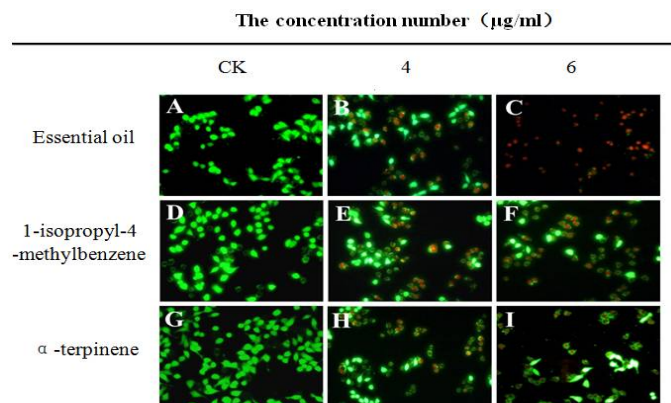


Fig 2: Morphology of MCF-7 cells treated with *C. ambrosioides* essential oil and two main components. **Note:** A, D and F: control (cells treated with 2.0 % DMSO); B, C: cells treated with 12.5 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ *C. ambrosioides* essential oil respectively; E and F: cells treated with 1-isopropyl-4-methylbenzene; H and I: cells treated with α -terpinene

SOD, CAT relative activity and MDA relative content of MCF-7 cells

Our data showed that essential oil, 1-isopropyl-4-methylbenzene and α -terpinene may affect antioxidant systems of MCF-7 cells (Fig. 3). SOD relative activity decreased and CAT relative activity gradually increased compared with control group ($p < 0.05$) as essential oil concentration increased. The MDA content increased initially and decreased later, with significant difference compared with the control group ($p < 0.05$), since the concentration of essential oil was higher than 1.25 $\mu\text{g/ml}$. Among the treatment groups of essential oil, 1-isopropyl-4-methylbenzene and α -terpinene treatment group, the average value (Table.3) of SOD relative activity treated with α -terpinene was the highest (72.3 %), while the average value of CAT relative activity treated with 1-isopropyl-4-methylbenzene was the highest; the average value of MDA relative content treated with 1-isopropyl-4-methylbenzene was the highest.

DISCUSSION

The composition of *C. ambrosioides* is very complex and the main components are monoterpenes, sesquiterpenes and their oxygenated derivatives [9]. Though the components of essential oil from various areas are different, they all contain 1-isopropyl-4-methylbenzene and α -terpinene [11-14]. Natural plant essential oil is found to have potential anticancer effects and may inhibit the proliferation of cancer cells [15-19]. In a previous work, α -terpinene inhibited cancer cells *in vitro* [20]. The results of this study showed that essential oil from *C. ambrosioides* and its two major components 1-isopropyl-4-methylbenzene and α -terpinene have inhibitory effects on the proliferation of human breast cancer cells MCF-7 with MTT assay.

The morphology of MCF-7 cells stained with Live/dead viability/cytotoxicity fluorescent dye showed essential oil and its main ingredients have lethal effect on MCF-7 cells activity with dose-response effect, consistent with that of MTT assay. Lethal and the inhibitory effect of the monomer components of 1-isopropyl-4-methylbenzene and α -terpinene on MCF-7 cells were significantly weaker than that of essential oil. This may be due to a potential synergy effect between these two components and other active ingredients in the essential oil. Our results are highly consistent with the study that investigated the cytotoxic activities of *Amomum tsaoko*

essential oils and its components, 1, 8-cineole and geraniol, on a series of human cell lines by the MTT assay [15]. Therefore, essential oil has anticancer activity and each component of the essential oil may have synergic inhibition effect on the proliferation of tumor cells, or even directly kill tumor cells.

There is a certain relationship between cytotoxicity and antioxidant activity [21,22]. An organism will generate excessive reactive oxygen species (ROS) when it is under all kinds of stress [23]. ROS reacts with lipids with relatively high affinity, causing membrane lipid peroxidation. The final product of lipid peroxidation is cytotoxic malondialdehyde (MDA), which cross-links with proteins, nucleic acids and other biological macromolecules in the cells [24], and then induces oxidative damage of proteins, lipids and DNA, finally leading to cell death [11,25].

Intracellular antioxidant enzymes such as SOD, POD and CAT are very important in protecting the integrity and regulating reactive oxygen metabolism of the cells, which can eliminate reactive oxygen species. The anticancer activity of essential oils often affects antioxidant system of cancer cells. *Abies balsamea* essential oil induces a dose- and time-dependent decrease in cellular glutathione (GSH) levels and an increase in ROS production [26].

The essential oil of *Lycopus lucidus* induces apoptosis of liver carcinoma Bel-7402 cells and reduces the intracellular GSH level, in which inhibition effect on cancer cell proliferation is relative with the antioxidant activity [27]. The present study showed that the relative activity of SOD gradually decreased while that of CAT increased compared to the control group when the concentration of essential oil, 1-isopropyl-4-methylbenzene and α -terpinene increased. MDA relative content showed increase in the beginning and then decreased afterwards.

These results indicate that essential oil and its main components affect the metabolic balance of ROS and antioxidant systems in MCF-7 cells. The increasing MDA content indicates accumulation of ROS in the cell. Meanwhile, the decreased SOD activity indicates cell oxidative damage. 1-isopropyl-4-methylbenzene caused the most serious oxidative damage on MCF-7 cells, compared with essential oil and α -terpinene.

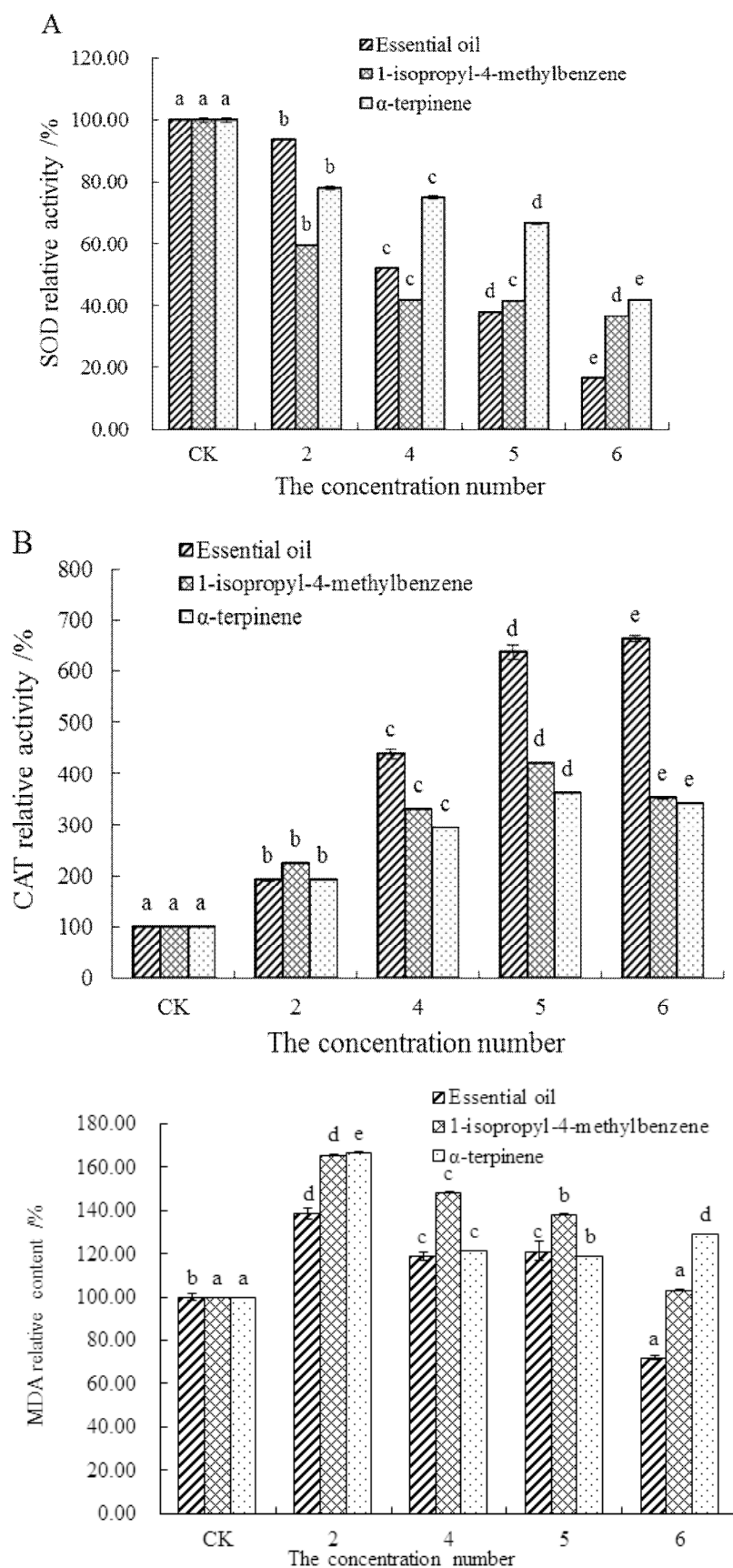


Fig 3: Effect of *C. ambrosioides* essential oil and two main components on MCF-7 cells antioxidant activity. **Note:** Different letters represent significant difference among treatments with the same sample ($P < 0.05$). A represents MCF-7 intracellular SOD relative vitality, B represents MCF-7 intracellular CAT relative vitality, C represents MCF-7 intracellular MDA relative content.

Table 3: Mean antioxidant activity of *C. ambrosioides* essential oil and two main components

| Treatment | Mean | | |
|-----------------------------|---------------------------|---------------------------|---------------------------|
| | SOD relative activity (%) | CAT relative activity (%) | MDA relative activity (%) |
| Essential oil | 59.95 | 406.59 | 110.18 |
| 1-isopropyl-4-methylbenzene | 55.86 | 286.40 | 131.23 |
| α -terpinene | 72.30 | 259.32 | 127.33 |

CONCLUSION

Essential oils and its major components, 1-isopropyl-4-methylbenzene and α -terpinene lower cell viability and proliferation of cultured human breast cancer cells MCF-7. Antioxidant enzyme system was also disrupted and MDA relative content rose in the cells. Thus, these findings suggest that oxidative damage may be implicated in the cytotoxicity of essential of *C. ambrosioides*. The anticancer activity of 1-isopropyl-4-methylbenzene is superior to that of α -terpinene.

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