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Research Paper

Phytotoxicity and cytotoxicity of *Citrus aurantiifolia* essential oil and its major constituents: Limonene and citral

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ABSTRACT

The essential oils are fast emerging as the source of natural herbicides owing to their environmentally benign properties. The focus of the present study, thus, was to investigate the phytotoxicity and cytotoxicity of *Citrus aurantiifolia* oil, and its major constituents-citral and limonene. *C. aurantiifolia* oil was selected due to its extreme commercialisation and safe nature. GC–MS analysis revealed that *C. aurantiifolia* oil is rich in monoterpenes (83.93%), with limonene (40.92%) and citral (27.46%) as the major compounds. Phytotoxicity was assessed against three agricultural weeds, *Avena fatua, Echinochloa crus-galli* and *Phalaris minor*, at concentration ranging from 0.10–1.50 mg/ml. Percent germination, IC_{50} value and seedling growth (root and coleoptile length) were significantly reduced in a dose-response manner. *C. aurantiifolia* oil, citral and limonene caused alteration in the cell cycle of *Allium cepa* root meristematic cells as evidenced by decrease in mitotic index (MI) and increase in chromosomal aberrations at progressive concentrations (0.01–0.10 mg/ml) and time periods (3 h and 24 h). Cytotoxic evaluation confirmed mitodepressive effect of the tested volatiles though the intensity was variable. Overall, citral was the most toxic followed by *C. aurantiifolia* oil and limonene. The significant phytotoxic activity of *C. aurantiifolia* oil and citral suggests the possibility of being developed into eco-friendly and acceptable products for weed management in agriculture system.

1. Introduction

The extensive use of synthetic herbicides has increased environmental pollution, toxicological implications to human health and the risk of development of resistance/cross-resistance among weed species (Dayan and Duke, 2014). From the agricultural perspective, increasing resistance among weeds is a matter of serious concern. Of late, due to increased consumer concerns and need for safe food production, more emphasis is laid on the search for environment-friendly and safer herbicides (Dayan et al., 2009). Alternative green measures such as natural plant products have, therefore, been evaluated as next-generation herbicides and crop protectants (Duke, 2014). These products exhibit great structural diversity, generally lack halogens, possess short environmental half-lives and novel molecular target sites (Dayan et al., 2009). Their use not only offers a solution for sustainable agriculture but also safeguard human health and the ecosystem (Dayan and Duke, 2014). Among these, essential oils and their main components can be exploited as valuable tools for integrated pest management in organic agriculture (Batish et al., 2008; Dayan et al., 2009; Kordali et al., 2015; Isman, 2016). These possess environmentally benign properties, and are generally regarded as safe (GRAS) chemicals (Isman, 2000; Tworkoski, 2002). Some of the essential oils like citronellal-rich eucalypt oil of *Eucalyptus citriodora* (Singh et al., 2005), eugenol-rich clove oil of *Eugenia caryophyllus* (Stokłosa et al., 2012) and manuka oil of *Leptospermum scoparium* rich in leptospermone (Dayan et al., 2011) possess good weed suppressing potential. Since their use for weed management seems to be promising, it is worthwhile to screen essential oils from commonly gown plants.

Citrus aurantiifolia (Christm.) Swingle, commonly known as key lime, is a shrubby tree cultivated extensively in hot subtropical and tropical regions of the world including India (Morton, 1987). Its essential oil is one of the major commercial products and has a long history of human consumption. It is widely used as a source of antioxidant, flavouring agent in food products and pharmaceutical industry (Chamblee et al., 1997). In fact, the citric industry is one of the world's largest agro-industry and the essential oils obtained from different varieties of citrus are gaining interest, largely due to their relatively safe nature and worldwide acceptance by consumers (Sawamura, 2000). *C. aurantiifolia* oil possesses antimicrobial, fungicidal and insecticidal property that suggests its potential in the development of broad

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spectrum pesticide (Aibinu et al., 2007; Ezeonu et al., 2001; Razzaghi-Abyaneh et al., 2009). However, nothing has been done to explore the herbicidal potential of *C. aurantiifolia* oil, although it may prove as a safe and effective option for weed management. A study was therefore conducted with the following objectives: (a) to assess the phytotoxicity of *C. aurantiifolia* essential oil against three grassy agricultural weeds (*Avena fatua* L., *Echinochloa crus-galli* (L.) Beauv, and *Phalaris minor* Retz.), (b) to determine the chemical composition of the oil, and (c) to determine whether the phytotoxicity is manifested through cytotoxicity (taking *Allium cepa* L. as model plant).

2. Materials and methods

2.1. Materials

Fresh leaves of *C. aurantiifolia* were collected from the plants growing in the Botanical Garden of Panjab University, Chandigarh (30°45'34" N 76°45'59" E), India. The voucher specimen (PAN #20579) has been deposited in the Herbarium of Botany department, Panjab University, Chandigarh, India. D-Limonene (purity: 97%) and Citral (mixture of *cis*- and *trans*- isomers, purity: 95%) of technical grade was purchased from Sigma-Aldrich Co., St. Louis, MO. The purity of the compounds was re-checked by GC analysis. Seeds of *A. fatua*, *E. crus-galli*, and *P. minor* were collected from the local agricultural fields. Seeds of all the test weeds were surface cleaned, disinfected with sodium hypochlorite (0.1%, *w/v*) for 2 min (followed by washing with distilled water) and stored at 25 °C for further use.

2.2. Extraction of oil and its physical characterization

Essential oil was extracted from freshly chopped leaves of *C. aurantiifolia* by hydrodistillation using Clevenger's apparatus. One kg of plant material and 5 l of distilled water was taken in a round bottom flask fitted with condenser. The contents were boiled for 4 h and pale-yellow colored oil was collected through the nozzle of condenser. It was dehydrated over anhydrous sodium sulphate and stored in airtight amber colored glass vial in refrigerator at 4 °C prior to the analysis and bioassay studies. The extraction procedure was repeated three times. Yield was calculated (%, *v/w*) based on the dried weight of the sample (mean of three replications). The color, odour and solubility of the essential oil in the organic solvents were checked manually. Refractive index and density was determined using a refractometer and a numeric densimeter, respectively.

2.3. Chemical characterization of the oil

The chemical composition of the extracted oil was determined by GC–MS (Gas Chromatography-Mass Spectrometry) using Thermo Scientific Trace 1300 gas chromatograph coupled with TSQ 8000 mass spectrometer (triple quadruple) and fitted with Thermo TG 5MS non-polar fused silica capillary column (30 m length × 0.25 mm diameter × 0.25 µm thickness). The initial oven temperature was maintained at 60 °C for 2 min and then increased to 250 °C at the rate of 3 °C/min and held for 5 min. Helium was used as the carrier gas at a flow rate of 1 ml/min. Injection volume was 1 µl of the oil. Injector and transfer line temperature were set at 250 °C and 280 °C, respectively. The mass spectra were recorded with ionization energy of 70 eV over a scan mass range of *m/z* 30 – 400 amu and MS source temperature of 240 °C with split injection ratio of 1:150.

The components of essential oil were identified by comparing their retention indices (RI) and their mass fragmentation pattern with those of Wiley 275, NIST 05, and present in the literature (Adams, 2007). The retention times of a homologous series of *n*-alkanes (C_8-C_{32}) were determined under the same operating conditions and used for the calculation of RI as per Hérent et al. (2007). Relative percentage of each component was obtained from% peak area based upon three injections of the oil.

2.4. Phytotoxicity: dose-response assay

The phytotoxicity of *C. aurantiifolia* oil and its major constituents – limonene and citral (concentration ranging from 0.10 – 1.50 mg/ml) – was evaluated against the selected agricultural weed species in a laboratory bioassay. The effect was determined in terms of germination and seedling growth. The emulsion of all volatiles was prepared using Tween-20 (final concentration 0.1%, *v/v*) as a surfactant and the final volume of each treatment was made with distilled water. Distilled water with the same amount of Tween-20 served as a parallel control. Seeds were imbibed in distilled water for 24 h prior to the treatment. 15 seeds of *A. fatua* and 25 each of *P. minor* and *E. crus-galli* were placed in 15 cm diameter Petri dishes lined with a thin layer of cotton wad and Whatman #1 filter paper moistened with 10 ml of respective emulsions. Petri dishes were then sealed with an adhesive tape to prevent escaping of volatile compounds. All the Petri dishes were kept in a growth chamber at 15 ± 2 °C temperature for *A. fatua* and *P. minor* and 25 ± 2 °C for *E. crus-galli,* and 16/8 h light/dark photoperiod of 240 µ mol photons $m^{-2} s^{-1}$ photon flux density provided with fluorescent tubes and lamps. After a week, number of seeds germinated was counted in each Petri dish, and the root and coleoptile length of the seedlings was measured with the help of a scale. Based on growth studies, IC_{50} value (concentrations that caused 50% inhibition in seed germination) of all three volatiles was also determined.

2.5. Cytotoxicity: Allium cepa assay

Small bulbs (1.5–2.0 cm in diameter) of the common onion, *A. cepa* (2n = 16), were purchased from a local market. Prior to the treatment, the outer scales of the bulbs were removed without destroying the root primordia. Bulbs were placed in the beakers with their basal ends dipped in distilled water at room temperature (25 ± 2 °C). Freshly emerged roots (1–2 cm in length) were used for evaluating cytotoxicity of *C. aurantiifolia* oil, limonene and citral (0.01, 0.05 and 0.10 mg/ml, emulsion prepared in distilled water using Tween-20). Distilled water with the same amount of Tween-20 served as a negative control and methyl methane sulfonate (MMS) (0.01 mg/ml) as a positive control. After 3 h and 24 h of exposure, several root tips were removed from the bulbs, fixed in 3:1 (*v/v*) ethanol: glacial acetic acid and stored overnight at 4 °C. Next day, these were rinsed in distilled water and stained in acetocarmine. Squash technique was applied for the study of mitotic index (MI) and chromosomal aberrations (CA). The aberrations were determined under the light microscope (Getner, India; model 66475) using the 40 × and 100 × objective lens. To determine MI and phase index, approximately 6000 cells (2000 cells in each of the three slides) were observed per treatment. The number of chromosomal aberrations was recorded in approximately 300 dividing cells (preferably 100 per slide). Cytotoxicity parameters were calculated according to the following equations:

$$\text{Miotic Index}\,(\%) = \frac{\text{Number of dividing cells}}{\text{Total number of cells counted}} \times 100$$

$$\text{Phase Index}\,(\%) = \frac{\text{Number of cells in a particular mitotic phase}}{\text{Total number of dividing cells}} \times 100$$

$$\text{Chromosomal Aberration}\,(\%) = \frac{\text{Number of Aberrant cells}}{\text{Number of dividing cells counted}} \times 100$$

2.6. Statistical analysis and graphical representation

The data analyses were carried out by one-way ANOVA followed by