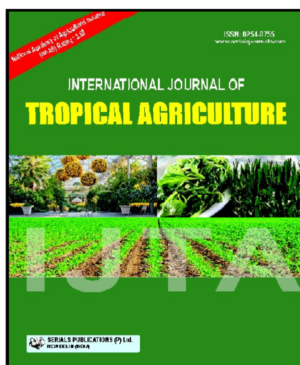

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Comparative Evaluation of Herbicidal Activity of *Citrus aurantiifolia* Essential Oil and Limonene towards Some Agricultural Weeds

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Abstract: The present study investigated the phytotoxicity of *C. aurantiifolia* oil and its major component (limonene), towards three agricultural weeds (*Echinochloa crus-galli*, *Phalaris minor* and *Avena fatua*). The effect of *C. aurantiifolia* oil and limonene (0.10-1.50 mg/ml) on the growth and development of seedlings after 7 days of treatment was studied in terms of percent germination, seedling length, dry weight, total chlorophyll content and cellular respiration. *C. aurantiifolia* oil significantly inhibited per cent germination in *P. minor* at ≥ 0.75 mg/ml, *A. fatua* at ≥ 1.0 mg/ml and *E. crus-galli* at ≥ 1.50 mg/ml, whereas limonene had no significant effect on all the weed species at all the tested concentrations. Likewise, other tested parameters in all weed species also inhibited more strongly by *C. aurantiifolia* oil exposure in comparison to limonene. Both volatiles were most effective inhibitors of *P. minor* followed by *A. fatua* and least for *E. crus-galli*. The results from this study suggest that *C. aurantiifolia* oil possess higher phytotoxic potential then limonene and can thus serve as lead molecule for the synthesis of environmentally safe herbicide in replacement to the harmful chemical herbicides.

Key words: Percent germination, Phytotoxicity, Cellular respiration, Chlorophyll content.

INTRODUCTION

Weeds are one of the serious threats to natural ecosystem. They cause severe yield losses in arable and horticultural crops which when combined with environment and health concerns, labour and energy costs make weeds the most important issue in agriculture. They also support population of non-native animals, microbes and hybridize with native

species subsequently altering gene pool [1]. Commonly, synthetic herbicides have been used in agriculture system for effective weed control. However, the excessive use of synthetic pesticides has resulted in an increased risk of development of resistant/cross-resistant among weed biotypes, toxicological implications to human health and increased environmental pollution [2, 3]. Alternative "green" measures have therefore been evaluated for crop protection, including mineral salts, biological agents and plant extracts, that are very promising because they are expected to have a narrow target range and a highly-specific mode of action. They show brief field persistence and also have a shorter shelf life. So, these types of organic products not only offer a solution for sustainable agricultural growth but are also safeguard human health and the ecosystem. Among biological plant products, essential oils and their main components, *i.e.* monoterpenes and sesquiterpenes, could be valuable tools for integrated pest management in organic agriculture because of their phytotoxic properties [4, 5, 6, 7].

Therefore, it is a need of time to explore more essential oils for their phytotoxic effects on weeds with the purpose of using them as active ingredient in herbicide formulations. *Citrus aurantiifolia* (Christm.) Swingle, commonly known as key lime, a potential source of essential oil, was selected for the present study. It is a shrubby tree widely grown in tropical and subtropical areas of the world. Its essential oil has been observed to possess antimicrobial, fungicidal and insecticidal properties [8, 9]. Analysis of essential oil from *C. aurantiifolia* leaves showed that limonene is its major component [8, 10]. Limonene is used as a leading compound in several commercialised herbicide formulations like GreenMatch O, GreenMatch EX and Avenger® [6]. Essential oils containing limonene as major compound will also have phytotoxic potential to some extent. So, *C. aurantiifolia* oil should be explored for herbicidal activity which may offer a useful pest management option in organic crop production system. To the best of our knowledge, no study has been reported on the phytotoxic activities of *C. aurantiifolia* essential oil. The aim of present study was to assess and compare phytotoxic potential of *C. aurantiifolia* oil and limonene against three agricultural weeds species, *viz. Avena fatua* L., *Echinochloa crus-galli* (L.) Beauv, *Phalaris minor* Retz. Further, investigations were also made to determine physiological causes (in terms of chlorophyll content and cellular respiration) for the inhibitory effects of *C. aurantiifolia* essential oil.

MATERIALS AND METHODS

Collection of Material

The fresh leaves of *C. aurantiifolia* were collected from plants growing in the botanical garden of Panjab University, Chandigarh, India. Limonene of technical grade, purity (97%), was purchased from Lancaster, U.K. Seeds of weed species *viz. E. crus-galli*, *A. fatua* and *P. minor* were collected locally from the agricultural fields on the outskirts of Chandigarh, India. Seeds of test weeds were surface cleaned, disinfested with sodium hypochlorite (followed by washing with distilled water) and stored at room temperature (25°C) until further use.

Extraction of Oil

Essential oil was extracted from leaves of *C. aurantiifolia* by hydro-distillation using Clevenger's apparatus. For this, 2.5 kg of freshly collected material was mixed with 10 L of water in a container of Clevenger's apparatus fitted with a condenser. Leaves were boiled for 4 h and after cooling, oil was collected from the nozzle. The oil thus obtained was dried over anhydrous sodium sulphate and stored at 4 °C for further use.

Growth Bioassay

The phytotoxicity of *C. aurantiifolia* oil and limonene (concentration ranging from 0.10-1.50 mg/ml) was evaluated against the selected agricultural weed

species in a laboratory bioassay. The solutions of oil and limonene were prepared using Tween-20 (final concentration 0.1 %) as a surfactant and the final volume was made with distilled water. Distilled water with the same amount of Tween-20 served as a parallel control. The pre-imbibed weed seeds (15 of *A. fatua*, 20 each of *P. minor*, *E. crus-galli*) were placed in Petri dishes (15 cm in diameter) lined with a thin layer of cotton wad and Whatman No. 1 filter paper. Each Petri dish was moistened with 10 ml of respective solutions. The Petri dishes were then sealed with an adhesive tape to prevent escaping of volatile compounds. The entire set up was placed in an environmentally controlled germinating chamber (15±2°C temperature for *A. fatua* and *P. minor* and 25±2°C for *E. crus-galli*, 75 ± 2 % relative humidity with a 16 h/8 h light/dark photoperiod and a photon flux density of approximately 240 µmol/m^2/s). After 7th day of treatment, number of seeds germinated was counted in each Petri dish, length of emerged seedlings was measured and their dry biomass determined. Seedling length was measured with the help of a scale, whereas dry weight of seedlings was measured after oven drying at 70°C for 48 h.

Chlorophyll Content

Chlorophyll was extracted from the leaves of the test plant species in dimethyl sulfoxide as per Hiscox and Israelstam [11]. Its amount was determined spectrophotometrically at dual wavelength of 645 and 663 nm and the chlorophyll content was calculated using equation of Arnon [12] and expressed on dry weight basis as suggested by Rani and Kohli [13].

Cellular Respiration

Respiration was not measured per se as CO_2 exchange, but rather determined indirectly in terms of cellular survival using 2,3,5-triphenyl tetrazolium chloride (TTC) [14]. TTC salt traps electrons released in electron transport chain and thus provides an indirect method for the measurement of cellular respiration [15]. Red formazan was extracted in ethanol, its absorbance read at 530 nm and the values expressed with respect to the control.

Statistical Analysis

The experiment was conducted in a completely randomized design with five independent (Petri dish) replicates for each treatment including control to ensure the reproducibility of results and the data presented is a mean of two experiments. Statistical analysis of data was done using one-way ANOVA followed by the comparison of mean values using post hoc Tukey's test at $p \leq 0.05$ and finally presented as mean ± SE (Standard Error), using software program SPSS (Version 16.0). Graphical representations were made on software programme sigma plot (Version 8.0).

RESULTS

C. aurantiifolia oil at ≥ 0.25 mg/ml caused a significant reduction in the emergence of test weed species, except *E. crus-galli* (reduction significant at ≥ 0.50 mg/ml). At 0.50 mg/ml *C. aurantiifolia* oil reduce seedling emergence approximately by 82%, 52% and 27 % in *P. minor*, *A. fatua* and *E. crus-galli*, respectively. *C. aurantiifolia* oil cause complete germination inhibition in *P. minor* at ≥ 0.75 mg/ml, *A. fatua* at ≥ 1.0 mg/ml and *E. crus-galli* at ≥ 1.50 mg/ml. However, limonene was not much effective as its influence was not significant at all the tested concentrations for all the weed species (Table 1).

Regarding the growth of seedlings, *C. aurantiifolia* oil and limonene showed variable response in a dose-dependent manner. In *E. crus-galli, C. aurantiifolia* oil caused significant reduction in the seedling growth at ≥ 0.25 mg/ml, whereas limonene showed significant reduction at ≥ 0.50 mg/ml. In *A. fatua*, *C. aurantiifolia* oil significantly inhibited the seedling growth at ≥ 0.10 mg/ml and limonene cause significant reduction at ≥ 0.50 mg/ml. *P. minor* was the most sensitive among all the tested weeds, both oil and limonene exhibited significant reduction in its growth at the lowest concentration, *i.e.* ≥ 0.10

Table 1
Effect of *C. aurantiifolia* oil and limonene on germination (%) of test weeds measured 7 days after the treatment. Different alphabets along each mean value represent significant differences from their respective control at $p \leq 0.05$; ± represent standard error.

	E. crus-galli		*A. fatua*		*P. minor*	
Conc. (mg/ml)	*C. aurantiifolia oil*	Limonene	*C. aurantiifolia oil*	Limonene	*C. aurantiifolia oil*	Limonene
0.0	100.0 ± 0.0a	100.0 ± 1.6a	100.0 ± 2.1a	100.0 ± 0.0a	100.0 ± 3.8a	100.0 ± 0.0a
0.10	90.6 ± 1.3ab	99.0 ± 2.5a	100.0 ± 2.8a	100.0 ± 0.0a	92.4 ± 4.0a	100.0 ± 0.0a
0.25	86.6 ± 1.3ab	99.0 ± 1.9a	74.2 ± 5.7b	100.0 ± 0.0a	79.6 ± 2.4b	100.0 ± 0.0a
0.50	73.3 ± 3.5bc	97.1 ± 4.3a	48.5 ± 2.8c	100.0 ± 0.0a	18.3 ± 0.5c	100.0 ± 2.7a
0.75	73.3 ± 1.3bc	94.2 ± 1.6a	8.5 ± 0.0d	100.0 ± 0.0a	0.0 ± 0.0d	95.8 ± 2.7a
1.00	63.6 ± 5.6c	89.5 ± 4.1a	0.0 ± 0.0d	92.8 ± 4.1a	–	–
1.25	30.6 ± 3.5d	88.5 ± 5.9a	–	–	–	–
1.50	0.0 ± 0.0e	85.7 ± 4.9a	–	–	–	–

mg/ml. At 0.50 mg/ml, *C. aurantiifolia* oil inhibited *E. crus-galli* growth by ~42%, *A. fatua* by ~ 45% and *P. minor* by ~86%. Likewise, limonene at 0.50 mg/ml showed growth reduction by approximately 7%, 26% and 27% in *E. crus-galli*, *A. fatua* and *P. minor*, respectively, over control seedlings (Figure 1).

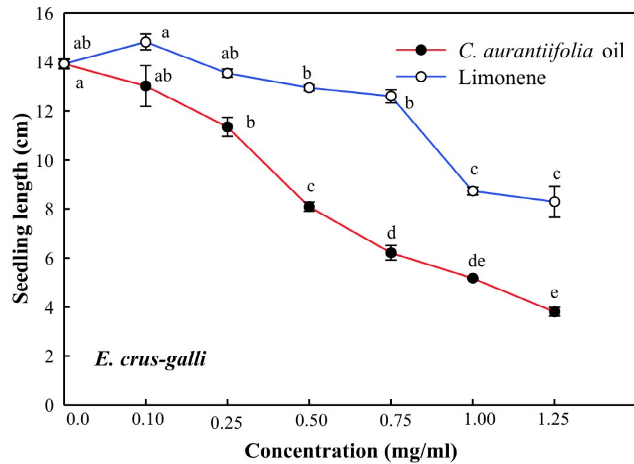
Seedling dry weight of tested weeds was also reduced by the exposure of these volatiles. All the tested weeds showed significant decline in dry weight on the exposure of ≥ 0.25 mg/ml *C. aurantiifolia* oil and ≥ 0.50 mg/ml limonene. At 0.50 mg/ml, *C. aurantiifolia* oil reduces seedling dry weight of *E. crus-galli* by ~12%, *A. fatua* by ~18% and *P. minor* by ~34%. Limonene at 50 mg/ml reduces dry weight of *E. crus-galli* seedling by ~5%, *A. fatua* by ~8% and *P. minor* by ~17% (Figure 2).

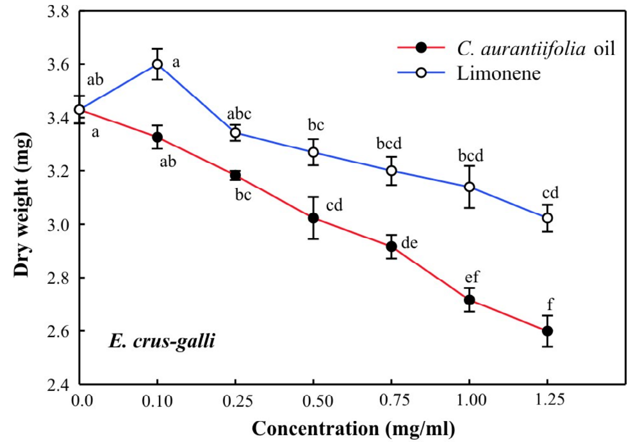
In addition to the measurement of growth and dry biomass of the test weeds, the physiological parameters, *i.e.* total chlorophyll content and cellular respiration, were also measured in response to *C. aurantiifolia* oil and limonene. Generally, a decrease in chlorophyll content was observed with an increase in the concentration of volatiles. At ≥ 0.10 mg/ml *C. aurantiifolia* oil exposure, all tested weed showed significant reduction in chlorophyll content. Limonene showed significant decline in chlorophyll content of *E. crus-galli* at ≥ 0.10 mg/ml, *A. fatua* at ≥ 0.75 mg/ml and *P. minor* at ≥ 0.25. At 0.50 mg/ml *C. aurantiifolia* oil exposure, the chlorophyll content reduced by nearly 35%, 23% and 53% in *E. crus-galli*, *A. fatua* and *P. minor*, respectively. Likewise, 0.50 mg/ml limonene declined chlorophyll content by ~ 21% in *E. crus-galli*, ~ 13% in *A. fatua* and ~38% in *P. minor* (Figure 3).

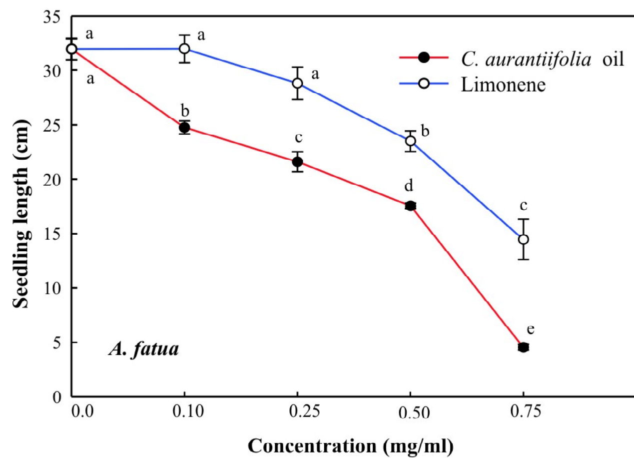
The percent respiration declined significantly at ≥ 0.10 mg/ml *C. aurantiifolia* oil exposure in *A. fatua* and *P. minor* but at ≥ 0.25 mg/ml in *E. crus-galli*. At 0.50 mg/ml *C. aurantiifolia* oil exposure, cellular respiration declined by approximately 36%, 32% and 54% over the control in *E. crus-galli*, *A. fatua* and *P. minor*, respectively. In all the tested weed species, ≥ 0.50 mg/ml limonene exposure cause significant decline. Limonene at 0.50 mg/ml, reduces cellular respiration in *E. crus-galli* by ~ 22%, *A. fatua* by ~ 14% and *P. minor* by 20% (Figure 4).

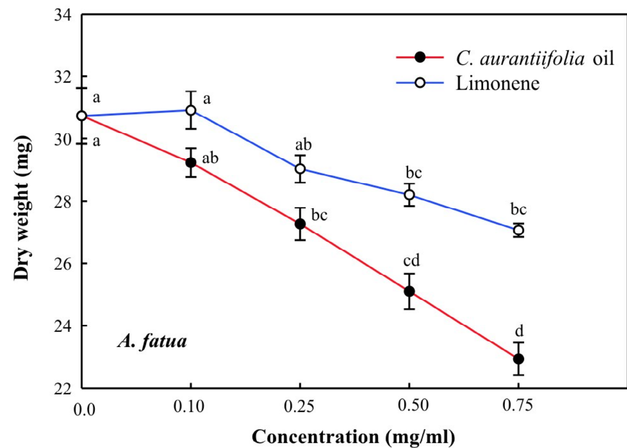
DISCUSSION

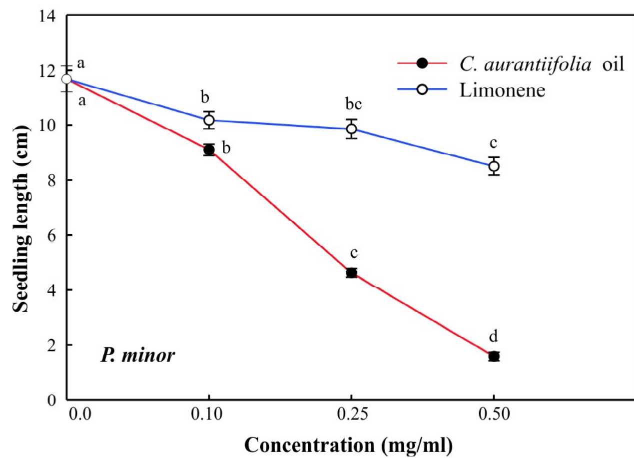
The interest in exploring essential oils for weed control has increased tremendously because of their eco-friendly nature. In this context, *Citrus aurantiifolia* oil was explored for its herbicidal potential. Chemical composition of *C. aurantiifolia* oil was subject of











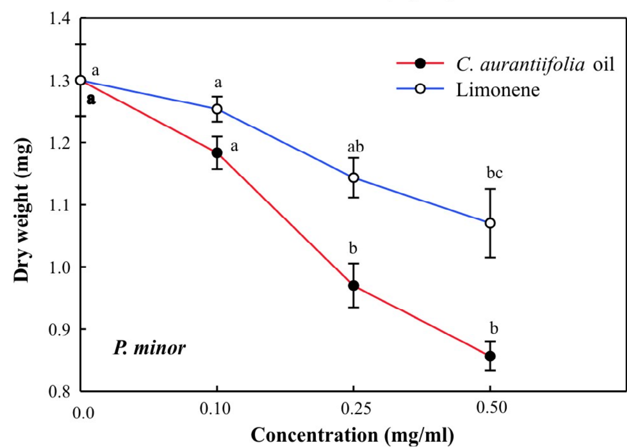
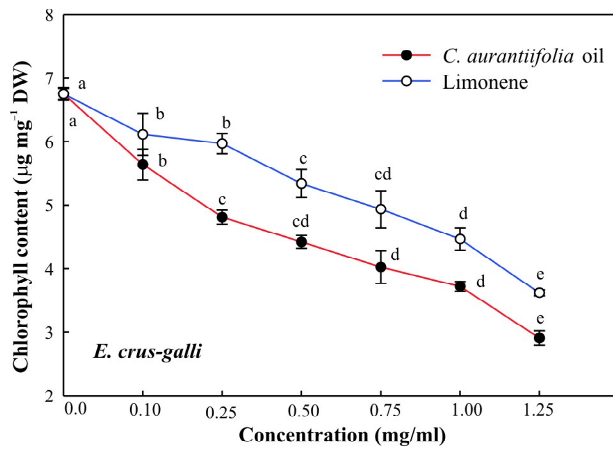
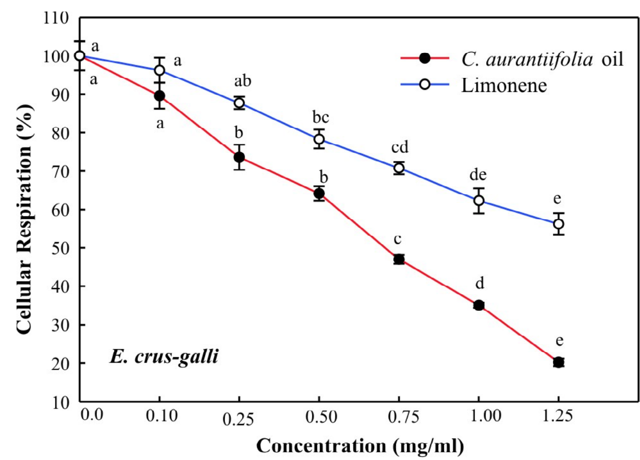
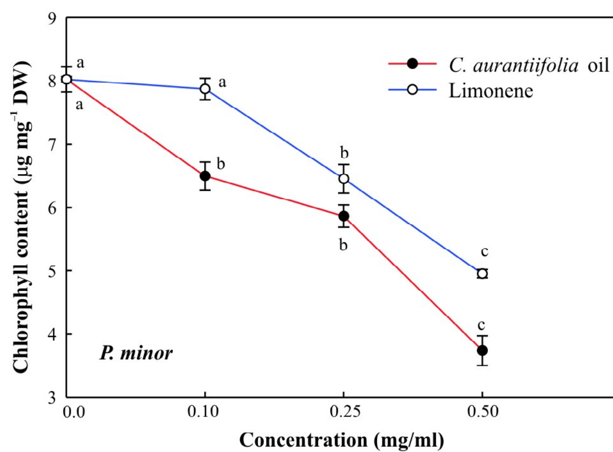


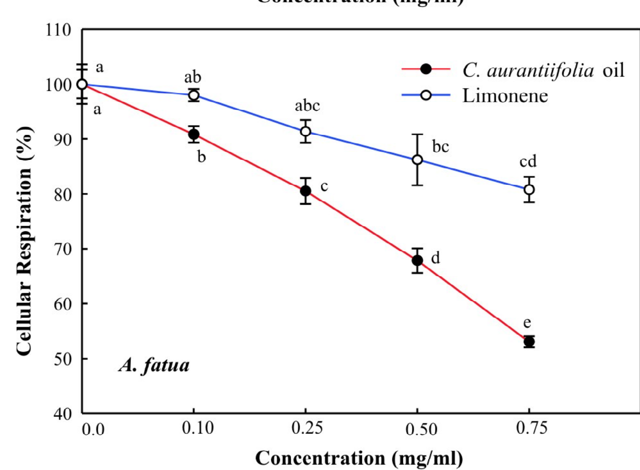
Figure 1: Effect of *C. aurantiifolia* oil and limonene on seedling length of test weeds measured 7 days after the treatment. Vertical bars along each data point represent the standard error of the mean and different alphabets along each value represent significant differences from their respective control at $p \leq 0.05$.

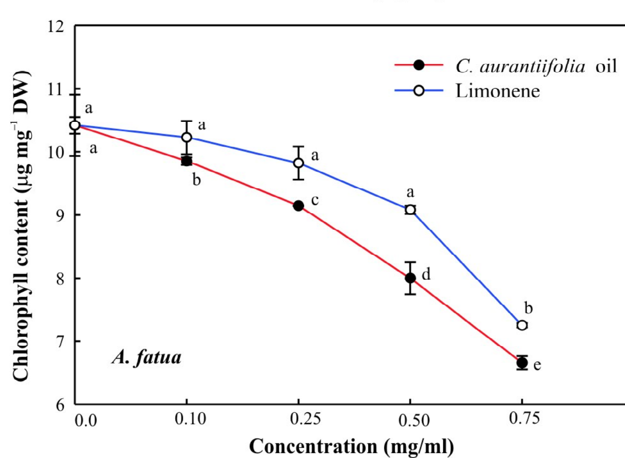
Figure 2. Effect of *C. aurantiifolia* oil and limonene on seedling dry weight of test weeds measured 7 days after the treatment. Vertical bars along each data point represent the standard error of the mean and different alphabets along each value represent significant differences from their respective control at $p \leq 0.05$.











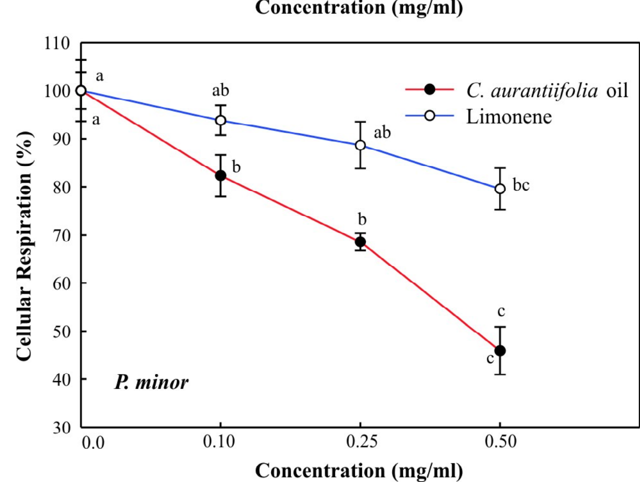


Figure 3: Effect of *C. aurantiifolia* oil and limonene on total chlorophyll content in leaves of test weeds measured 7 days after the treatment. Vertical bars along each data point represent the standard error of the mean and different alphabets along each value represent significant differences from their respective control at $p \leq 0.05$

Figure 4: Effect of *C. aurantiifolia* oil and limonene on cellular respiration activity of test weeds measured 7 days after the treatment. Vertical bars along each data point represent the standard error of the mean and different alphabets along each value represent significant differences from their respective control at $p \leq 0.05$

several studies, indicating that the major component of its oil is a limonene, which is a cyclic monoterpene [8, 10, 16]. In literature, a number of studies reported the phytotoxic activity of limonene [17, 18, 19, 20]. Limonene has also been successfully used as a lead compound in the development of commercial herbicides, Avenger® weed killer, AvengerAG® burndown herbicide (Cutting Edge Formulations, Buford, Georgia). In the present study, comparative phytotoxicity of *C. aurantiifolia* oil and its major constituent limonene in terms of their relationships between concentration and retardation in emergence and seedling growth of common agricultural weed species of rice and wheat were shown. It has been previously known that essential oils/monoterpenes inhibit germination and growth causing morphological and physiological change in the plant seedlings [7, 15, 21, 22, 23]. This study suggested that grassy weeds can be successfully controlled with *C. aurantiifolia* oil but all the tested weeds showed differential inhibition in response to oil and limonene. In general, inhibitory response of weed by oil exposure did not depend on the size of seeds as has been reported earlier [24]. Overall, *C. aurantiifolia* oil has almost equal efficiency to reduce germination and growth of weeds, whereas limonene showed inhibitory effect towards growth but was not effective against germination inhibition.

According to [18, 24, 25] hydrocarbon monoterpenes are less phytotoxic, which is in agreement with our results and justifies the lower performance of limonene. Essential oils are the mixture of both hydrocarbon and oxygenated monoterpenes and synergy among constituents improves their phytotoxicity [26]. In the literature, there are diverse suggestions on the physiological mechanisms behind the inhibitory effect of essential oils/monoterpenes on germination and seedling growth. Loss/disruption of mitotic activity might be responsible for the reduction/inhibition of germination and seedling growth of tested plant [27]. Essential oils/monoterpenes caused strong growth inhibition in plants due to an increase in the reactive oxygen species and lipoxygenase activity [28]. Essential oils caused accumulation of lipid globules in the cytoplasm and reduced size of cell organelles possibly due to inhibition in DNA synthesis or membrane disruption resulting in anatomical and physiological changes [29].

In the present investigation, *C. aurantiifolia* oil and limonene caused a decrease in total chlorophyll content in the weeds. The reduction in chlorophyll content indicates that oils/monoterpenes affects the photosynthetic apparatus of weed plants, thereby causing a possible breakdown in the food manufacturing machinery of the plants [30]. A decreased level of chlorophyll directly results in less productivity and thus weaker seedlings, as observed. This observation is in parallel to some other reports where essential oils/monoterpenes were found to decrease the chlorophyll content in the plants [15, 30, 31]. In addition to a decrease in the chlorophyll content, diminishing respiratory activity of plant cells could also be a possible reason for the inhibition in the weed growth.

A decrease in cellular respiration in response to *C. aurantiifolia* oil and limonene suggests a decline in the energy metabolism of the plant and its inability to cope with the stress. The loss of respiratory activity may affect the energy metabolism of the plants, which may in turn affect the synthesis of macromolecules and subsequent plant growth [32]. Such an observation is also supported by earlier studies demonstrating that essential oils/monoterpenes act as uncouplers of oxidative photophosphorylation [17], cause a reduction in the cellular respiration [15, 17, 30, 33] and might lead to a fluctuation in the ATP production, thereby disturbing the physiological processes in the plant [34].

CONCLUSION

The present study concludes that *C. aurantiifolia* oil and its major constituent (limonene) reduce growth of target weed species. However, the inhibitory effect of the limonene was comparatively lesser than that

of the *C. aurantiifolia* oil. So, *C. aurantiifolia* oil can be used as a constituent of various bioherbicide formulations as it is more potential inhibitor then limonene. Further, *C. aurantiifolia* oil can be extracted naturally in large quantity and no further distillation is needed like limonene. Due to all these reasons, *C. aurantiifolia* oil can act as both dose and cost-effective bioherbicide. It is worthwhile to make use of *C. aurantiifolia* oil for non-chemical weed management in organic farming systems. However, further field studies are required to determine the cost, applicability, safety, and phytotoxicity of *C. aurantiifolia* oil against crop plants for its practical implication as potential bio-herbicide.

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