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Chemical Composition and Antifungal Activity of Essential Oil of *Cymbopogon citratus* (DC.) Stapf. Against Three Phytophthora Species

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ABSTRACT

Many fungi including *Phytophthora* species cause severe pre- and post-harvest damage to agricultural crops. Research focused on plant-derived fungicides and their possible application in agriculture is being intensified as these are having enormous potential to inspire and influence modern agro-chemical research. In this study, we survey the effectiveness of essential oil of *Cymbopogon citratus* (DC.) Stapf. on the mycelium growth of three species of *Phytophthora* including *P. capsici*, *P. drechsleri* and *P. melonis*. The essential oil was extracted from fresh leaves of *C. citratus* that were subjected to hydro-distillation through Clevenger's apparatus and its chemical composition was analyzed with Gas Chromatography-Mass Spectrometry (GC-MS). α- citral (39.16%), Z- citral (30.95%), limonene (5.83%), and caryophyllene (3.44%) were identified as main components. The rate of growth inhibition were measured after placing 7 days active mycelial plugs of each fungus on petri dishes containing CMA amended with specific concentration of essential oil and incubated at 25 ± 2 °C. Since growth inhibition of studied essential oil was evident in this study, they have potential to control of Phytophthora species and could be considered for developing new fungicides.

INTRODUCTION

Plant pathogens that include fungi, nematodes, bacteria and viruses can cause diseases or damages in plants. Among these, fungi are the main pathogen and cause many diseases in plants. They cause yield losses in numerous economically important crops (Fletcher and Bender, 2006). *Phytophthora* is a genus of Oomycetes that includes over 60 species of destructive plant pathogens (Gan et al., 2009). *Phytophthora* crown and root rot is particularly severe, resulting in permanent wilting and rapid plant death (Meyer and Hausbeck, 2013). In Iran, cantaloupes, melon and cucumber occupy more than 250,000 irrigated hectares that are often affected by a severe wilt disease, known locally as "green death", caused by *Phytophthora capsici* Leonian, *P. drechsleri* Tucker, and *p. melonis* Tucker. These pathogens causes damping-off, crown and root rot, stem lesion, foliar blight and fruit rot (Mansoori and Banihashemi, 1982). All plant tissues, including roots, crows, fruits, and leaves, are susceptible to *phytophrhora* species (Meyer and Hausbeck, 2013). For many years, a variety of different chemical and synthetic compounds have been used as antimicrobial agents to inhibit the plant pathogenic fungi. Antimicrobial chemicals such as benzimidazoles, aromatic hydrocarbons and sterol biosynthesis inhibitors are often used in control of plant disease in agriculture (Zhang et al., 2009). However, there is a series of problems against the effective use of these chemicals in areas where the fungi have developed resistance (Barnard et al., 1997; Isman, 2000). The application of higher concentration of chemicals in an attempt to overcome this problem increases the risk of high level toxic residues in the product, which is particularly serious because fruit and vegetables are consumed in a relatively short time after harvest (Mansoori and Banihashemi, 1982; Maleki et al., 2011).

Essential oils are the steam-distillable fraction of plant tissues and are often responsible for a plants distinctive scent or taste. These oils are of rather complex composition, with component compounds generally consisting of low-molecular-weight monoterpenes (10-carbon) and related phenols (Toloza et al., 2006). The main characteristics of the essential oils are that they are easily extractable, eco-friendly, biodegradable , possess low or no toxicity against mammals and are very effective against wide spectra of pests (Isman, 2000; Lucia et al., 2012). Results of several studies have shown that some of essential oils are able to control plant pathogenic pests or at least used as a model for construction of new pesticide compound (Deferera et al., 2003; Amini et al., 2012; Ebadollahi, 2013).

Cymbopogon citratus (DC.) Stapf. (Lemongrass) is a plant in the Poaceae family that contains 1 to 2% essential oil on a dry basis. *C. citratus* is of West Indian origin and yields an essential oil with high content of citral (>70%) (Paranagama, 2003). Lemongrass oil was non-phytotoxic in nature, since it did not exhibit any adverse effects on germination and seedling growth of wheat and rice (Tzortzakis and Economakies, 2007).

Our interest is focused on the effectiveness of essential oils against serious plant pathogens. The specific objectives in the present work were to determine the effectiveness of different concentration of essential oils from *Cymbopogon citratus* on three species of *Phytophthora* including *P. capssici*, *P. drechsleri* and *P. melonis*. This approach will allow us to identify natural and safer agents for the development of bio-rational fungicides to manage *Phytophthora* species.

MATERIAL AND METHODS

Extraction of Essential Oils

Fresh leaves of *Cymbopogon citratus* at flowering stage were harvested, air-dried under the shade and stored at room temperature in darkness until distillation. Extraction of essential oil from subjected plant was carried out using a Clevenger apparatus with the following conditions: 40-50 g of air-dried plant material, 500 ml distilled water and 2.5 h distillation. The essential oil was dried over anhydrous sodium sulfate to remove water after extraction. The resulting oil placed into sealed vials and was stored in refrigerator at 4 ºC.

GC-MS analysis of essential oil

GC-MS analysis was done at 250℃ on an Aglilent7890 a gas chromatograph at 70 eV. The GC column was as follows: HP-5MS; the size of fused silica capillary was 0.25 × 3000 µm with film thickness of 0.25 µl. In addition, the GC-MS was operated under the following condition: the initial temperature was 50℃ and was heated for 5 min, then it was heated up to 240℃ at the rate of 3℃ per min. Meanwhile, carrier gas (helium) flow was 0.8 ml/min. Identification of compounds was done based on the following explanation: the retention indices were calculated for all volatile constituents using a homologous series of n-alkanes C6 to C24. Moreover, the method of mass spectra was used for the identification of individual compounds with those of similar compounds from a database (Wiely/NBS library) or with authentic compounds and it was confirmed by the comparison of their retention indices with authentic compounds or with those reported in the literature (Ozcan et al., 2006).

Fungal isolate

Fungal were isolated directly from infected cucumber, cantaloupe, melon and pepper seedling diseased plants according to Akgül and Mirik (2008) from field of Ardabil province, Iran. Crown and root section were surface

disinfested with a 70% ethanol solution and blotted dry with paper toweling. Four pieces of tissue were excised from each crown and root section and plated on CMA (CMA, Merck, Germany) amended with PCNB (penta chloro nitrobenzene) at 100 ppm, ampicillin at 100 ppm, rifampicin at 30 ppm and hymexazol at 25 ppm (CMA-PARPH). Fungal were grown on media and identified using morphological characteristics and key developed by Waterhouse. Pathogenicity tests were carried out on pepper plants (*Capsicum annuum* L.). While the experiments were in progress, reisolated cultures were used for pathogen inoculation. The pathogenic fungal isolates were grown on CMA for 7 days at 25℃ then the plates were incubated under fluorescent light for 3 days at 25℃ to induce sporangial formation. One day before inoculation, the fungal colonies were covered with 20 ml of tap water, and incubated under light overnight. At the day of inoculation, petri dishes were placed for 30 min at 4℃, followed by 60 min at room temperature to enhance zoospore release from sporangia. The flooding water, containing zoospores and mycelium was filtered using two layers of cheesecloth, after which the concentration of the zoospore suspension was adjusted to approximately 106 zoospores ml-1 using a haemocytometer. The inoculum was used immediately.

Antifungal activity

The antifungal assay was carried out in the Petri dishes (8 cm in diameter) containing CMA. When temperature of the medium (CMA) reached about 40 ºC, specific initial concentration of plant essential oils (diluted in Tween 80%) were added to CMA and mixed thoroughly. This solvent also served as control. Requisite amounts of the essential oils were dissolved separately in 0.5 ml of 0.1% Tween-80 and then were mixed with CMA medium for even distribution of the oil in CMA media. For control sets, the requisite amount of sterilized water in place of the oil was added to the medium. The CMA with added oils was then poured into 8 cm Petri dishes. For inoculation, mycelium was taken from the periphery of 7-day old stock cultures. Plugs of mycelium were removed with a 6 mm diameter cork borer, inverted, and where placed in the center of each Petri dish. Plates were sealed with paraffin to prevent realization of volatile compounds. Three replicate plates were sited up for each concentration, and were plates incubated in the dark at 26 ± 2℃. The rate of mycelial growth inhibition was measured after placing an active seven days mycelia plug of fungi on petri dishes containing CMA. The observations were recorded after 2 days. The rate of mycelia growth inhibition (GI %) was calculated by the following formula:

$$GI\% = dc\text{-}dt/dc \times 100$$

Where dc is mean colony diameter of control sets and dt is mean colony diameter of treatment sets (Amini et al., 2012).

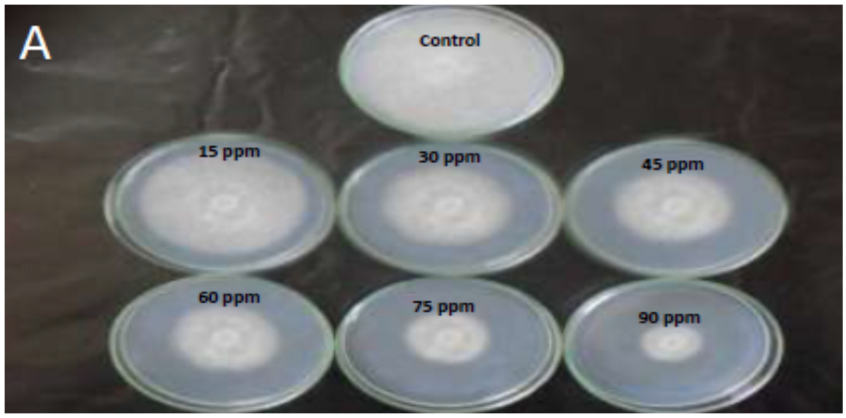
The initial experiments were conducted in a completely randomized design (CRD) with six concentrations and three replicate. Analysis of variance (ANOVA) was used to determine the effect of plant essential oils on mycelial growth inhibition of fungi and compare means were done using Tukeys' test. Statistical analysis was performed with SPSS statistic software.

RESULTS AND DSICUSSION

Table 1: Chemical composition of *Cymbopogon citratus* essential oil identified by GC-MS analysis

Number	Compound	RT	Percentage
1	Camphene	6.875	0.34
2	Methyheptenone	7.796	1.15
3	Limonene	8.980	5.83
4	1,3,6- Octatriene, 3,7-dimethyl	9.158	0.58
5	cis- β- Ocimene	9.444	0.39
6	Nonanone	10.125	0.87
7	β- Linalool	10.909	1.38
8	Trans- Chrysanthemal	12.345	0.32
9	6- Octenal, 3,7-dimethyl-, (R)	12.408	0.75
10	2-Cyclopenten- 1-one, 3,4,4-trimetnyl	12.728	0.72
11	Cyclohexane, ethenyl	13.243	1.43
12	Decanal	13.867	0.25
13	D- Citral	14.725	0.58
14	Z- Citral	15.172	**30.95**
15	Geraniol	15.292	0.47
16	α- Citral	16.081	**39.16**
17	2,7- Octadiene, 4- methyl	16.167	0.47
18	Eugenol	18.050	0.35
19	Geranyl acetate	18.725	3.10
20	β- Elemene	19.000	0.29
21	Caryophyllene	19.784	3.44
22	α- Bergamotene	20.081	0.39
23	Iso- Eugenol	20.390	0.43
24	α- Caryophyllene	20.608	0.42
25	Benzene,1-methyl-4-(1,2,2-trimethylcyclopentyl)-, (R)	21.884	0.30
26	Naphtalene	22.067	0.79
27	Vinyl dimethyl (1,3,3-triboromoprophyl) silane	22.393	0.22
28	Caryophyllene oxide	23.772	2.02
Total			**97.39**

α- citral (39.16 %), Z- citral (30.95 %), limonene (5.83 %), caryophyllene (3.44 %) and ceranyl acetate (3.1 %) identified as the main components in *Cymbopogon citratus* essential oil (Table 1). In the other research, Gupta et al. (2011) found that citral (77.8), limonene + traces of eucalyptol (4.0), geraniol (2.7), 6-methyl-5-hepten-2-one (2.4) and geranyl acetate (1.1) were main components in the *C. citratus* essential oil. These differences in the composition of mentioned essential oils might have been derived both from harvest time and local, climatic and seasonal factors, or it may be hypothesized that these samples belong to a different chemotype (Rahimi-Nasrabadi et al., 2013).



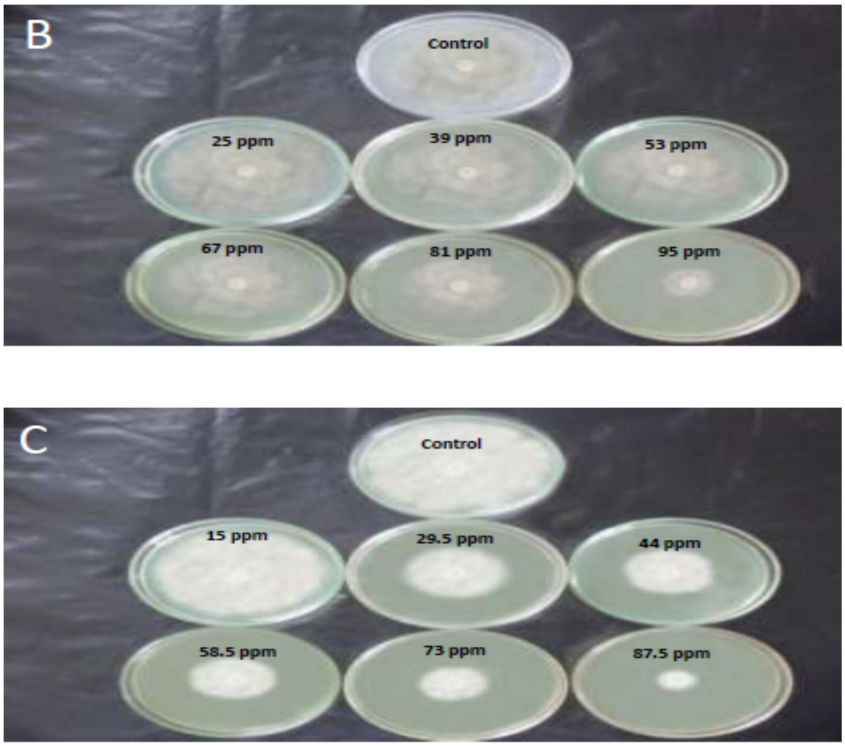
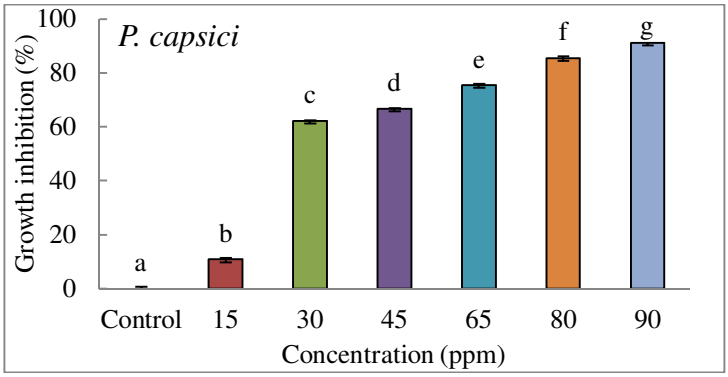
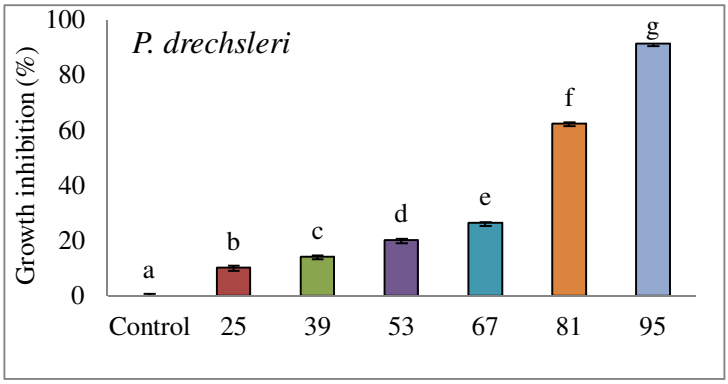


Figure 1: Growth inhibitions of essential oil of *Cymbopogon citratus* on *P. capsici* (A), *P. drechsleri* (B) and *P. melonis* (C) (Results of first replication of antifungal bioassay)





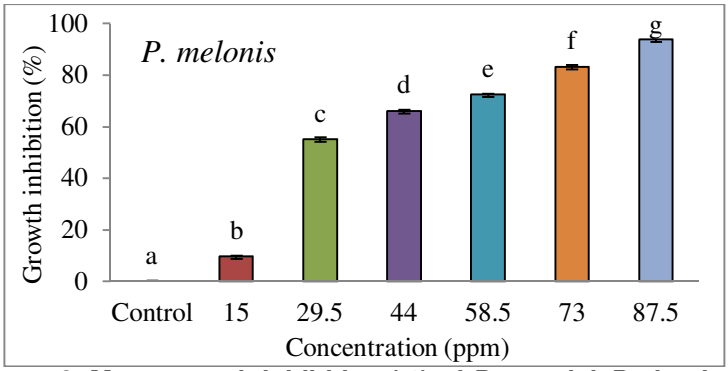


Figure 2: Mean growth inhibition (%) of *P. capsici*, *P. drechsleri* and *P. melonis* exposed to different concentrations of the essential oil from *Cymbopogon citratus*. Different letters on top of columns are significant differences according to Tukey's test at p = 0.05. Vertical bars indicate standard deviation of the mean (±); very small values are not represented

In-vitro antifungal activities of oils showed that *C. citranus* (for *P. capsici*: F = 8097.97; d.f. = 5, 12; $p < 0.0001$; for *P. drecshleri*: F = 10319.47; d.f. = 5, 12; $p < 0.0001$; and for *P. melonis*: F = 10304.25; d.f. = 5, 12; $p < 0.0001$) essential oil had significant growth inhibitory effect on tested isolates and it is dose dependent (Figure 1). According to Figure 2, the increasing doses of *C. citranus* caused a significant increase in the rate of mycelia growth inhibition when the all *Phytophthora* species were exposed to these oils.

Recent studies indicated that *C. citranus* essential oil has the potential for fungi control. For example, in the study of Kumar et al. (2009), *C. citratus* essential oils exhibited broad fungitoxic activity against *Aspergillus flavus*. The essential oils obtained from *Cymbopogon martini* (Roxb.) Wats. and *C. citratus* was studied against some fungi, viz. *Cladosporium* sp., *Aspergillus fumigatus, A. niger, Candida* sp., *Mucor, Trichophyton rubrum* and *T. viollacellium,* causing disease in human beings (Singatwadia and Katewa, 2001). *C. citratus* oil was found to be effective against *Cladosporium* sp., *Aspergillus niger,* and *Mucor* at lower concentrations, where as that of *C. martini* was more effective against *Candida* sp., *Aspergillus fumigates* and *Trichophyton rubrum* compared with the oil of *C. citratus*. The effects of *C. citratus* essential oil on the growth, lipid content and morphogenesis of *Aspergillus niger* ML2-strain were demonstrated by Helal et al. (2006). Results obtained by Tzortzakis and Economakis (2007) revealed that *C. citratus* oil inhibits the fungal spore production of *Colletotrichum coccodes*, *Botrytis cinerea*, *Cladosporium herbarum*, and *Rhizopus stolonifer*. *C. citratus* essential oil exhibited antifungal potency against various *Candida* species, including *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis* and *C. krusei* of which *C. albicans* appeared to be the most susceptible. The GC analysis identified citral as major component with a percentage of 76%. Since equal anticandidal results were obtained when citral was singularly used, it is obviously the most powerful constituent (Silva Cde et al., 2008). Gupta et al. (2011) adopted the disc diffusion method to test the anti-fungal activity of the *C. citratus* oil against *Fusarium oxysporum* and found its strong inhibition measured in terms of the average inhibition zone diameter.

The activity of the oils would be expected to relate to the respective composition of the plant volatile oils, the structural configuration of the constituent components of the volatile oils and their functional groups and possible synergistic interactions between components (Lee, 2007; Kim and Park, 2012). Citral as main components of *C. citratus* is an oxygenated terpenoid (aldehyde), which has been identified as a compound exhibiting antifungal properties (Paranagama et al., 2003). The US National Toxicology Program (NTP) reported that citral did not cause cancer in male or female rats receiving 4000 ppm citral in the feed for 2 years. The Acceptable Daily Intake is 5 mg citral/ kg body weight and it was given Generally Recognize As Safe (GRAS) status in the United States .Moreover, it is one of the most common used flavor compounds in the world and it is used in different concentrations, for example; 170 ppm for chewing gum and 9 ppm for beverages (National Toxicology Program, 2003). This monoterpene has proved effective in controlling mycelial growth and conidial germination of *C. gloeosporioides* (Palhano et al., 2003). The anti-fungal activity of the lemongrass oil may be due to the presence of its aldehyde containing the active constituent citral (Gupta et al., 2011).

From the present study, it can be concluded that *C. citratus* oil is a good alternative to the harmful chemical pesticides and can be effectively used as an efficient bio-pesticide. Further studies would still be required for better understanding of the structure activity relationship as the presence of the other constituents in the crude oil definitely modifies the influence of its major constituent alone. We believe that the present study

together with the previous studies on oils support the bio-pesticidal nature of the plant derived essential oils.

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