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Synergistic Antifungal Potential of Essential Oils Extracted from Peels of Selected Citrus Fruits

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Abstract

Many medications and therapeutic agents found in nature can be produced from various plant parts. Different citrus plants have therapeutic qualities. Essential oils that are extracted from the peels of various citrus fruits are used to treat a variety of illnesses, including diabetic, fungal, bacterial, and insecticidal conditions. Using molded corn as a sample, the current study examined the antifungal qualities of essential oils extracted from the peels of Citrus reticulata, Citrus sinensis, and Citrus lemon and mixture containing all EOs. Their composition was checked by GC/MS which showed that limonene, manitol, pinene, aldehydes and ketones were dominant among others. Their antifungal activity was checked against Aspergillus flavus. Well diffusion assay was performed to check the antifungal activity. Minimum inhibitory concentration of mixture essential oil was checked by serial broth dilution method and it was found 8.96mg/ml for the strain.

Keywords

Citrus Peel, Antifungal, Gc/Ms, Lemon.

INTRODUCTION

The significant degradation and contamination of food caused by food contamination in agricultural goods resulted in 30% of food loss globally. Moreover, mycotoxin produced by fungi are also toxic to human health. Now-a-days use of synthetic additives in foods has also caused resistant problems [1]. Essential oils are the compounds that show antifungal activities. Their antifungal property is associated with presence of aldehydes, ketones, phenols and alcohols because of solubility of oil components in membranes of microbes and thus disrupted their function [2]. Citrus EOs are mixture of vaporous molecules mainly consisting of monoterpene hydrocarbons and are used extensively in the food and pharmaceutical sector because to their antifungal qualities. Multidisciplinary research efforts have turned their attention to studies involving natural bio-preservatives in order to address the increasing public knowledge and concern over the safety of food and health [3]. Essential oils can be extracted from leaves, shoots, flowers and peels of aromatic plants. In recent years, they are used as herbal medicines. They are regarded as 'essential' because they consist of essence of fragrance. Presence of aromatic compounds makes them as an alternative of wounds healing. They are used as natural compounds and alternatives of drugs and medicines. They are extracted from different parts of plants using steam distillation method. Their chemical composition is measured by GC/MS techniques. However, storage and handling process may affect its composition and properties [4]. They provide many components that are biologically active. They are used in food systems for aroma and as preservatives. Essential oils use in foods is one of the most significant things that have changed over time. The earliest application approach involved adding essential oils directly to the food matrix but this had some special disadvantages, mostly due to inherent difficulties including limited hydrophilic nature, more vaporization, less stability, low bioavailability, and powerful smell. Additionally, EOs are employed as supplements in coatings and sustainable films for useful food packaging depending on their content [5].

This study was emphasized on antifungal potential of essential oil from peels of *Citrus reticulata*, *Citrus sinensis* and *Citrus lemon* and their mixture against *Aspergillus flavus.* Synergistic potential was evaluated by using molded corn as sample (Figure 1).

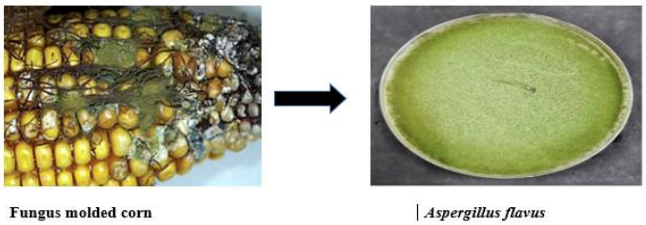

Figure 1. Molded corn used as sample

METHODOLOGY

Experimental work was conducted at Postgraduate Molecular Biochemistry laboratory at the Institute of Biochemistry and Biotechnology, University of Veterinary and Animal sciences, Lahore, Pakistan.

Essential oil extraction

Citrus peels of the selected plants were collected from the Corporation chowk and Madina market Raiwand, Lahore, Punjab, Pakistan. Essential oils were extracted from peels of selected citrus peel plants by using Clevenger apparatus. This apparatus uses hydro distillation principle. Sample was mixed with water and brought to boil until steam passed through sample. Two layers were formed. Distillate was then

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collected in Eppendorf tubes. Tubes were tightly closed by using parafilms. For mixture of samples, equal concentrations of sample were loaded in flask. Collected oil phase was centrifuged at 1500 rpm for 10 minutes to separate water from oil. Thus pure oil was extracted. Essential oils were preserved in amber bottles to avoid their evaporation.

Identification of compounds

GC/MS was used to analyze essential oil´s composition. This apparatus was used for checking the detailed structure of compounds present in the samples.

Antifungal Activity

Potato dextrose agar medium preparation

To prepare the Potato dextrose agar medium in 400ml distilled water. 200ml distilled water was taken in the beaker and then 9.6 grams of nutrient agar powder was added into the beaker and then the volume was made up to 400ml. This medium was transferred to the conical flasks. The flask was covered with cotton plug aluminum foil. Then autoclaving was performed for sterilization of medium at 121ºC and 15 psi for 15 minutes. This sterilized medium was used for the growth of microbial cultures.

Fungus strain isolation

Fungal strain that was employed in this investigation were extracted and recognized from naturally occurring corn with mold. Isolation and purification were accomplished using the plate scribe method. Following the selection of isolated fungal colonies from naturally infected corn, sterile saline was used to create a fungal suspension. This suspension was then spread out on petri dishes containing potato dextrose agar medium and incubated at 28 °C for three to five days, or until the fungi had finished growing. In order to get pure culture, the growing colonies were recultured, moved to medium containing potato dextrose agar, and maintained at 4°C [1].

Fungus strain identification

Fungal strains were identified by amplification of genomic DNA sequence.

Antifungal activity assay

Antifungal potential of selected essential oils was checked by using the well diffusion method. The fungal lawn was prepared by pouring the Potato dextrose agar into the plates. The fungal lawn was prepared by using the nutrient agar plates. 100 microliters of the fungal suspension were taken with the pipette and transferred on to the surface of the plate and then spread by using the sterilized spreader. Spreading was done in different directions by rotating the plate at 90º for uniform distribution of inoculum. Wells were made by a sterile well borer in the nutrient agar plates. Essential oils were added to each well by using the sterilized micropipette and then placed undisturbed for 25 to 30 minutes in the laminar air flow for homogenous diffusion. These plates were incubated at 37ºC for 18 to 24 hours.

Minimum Inhibitory Concentration

The minimum inhibitory concentration was checked by serial broth dilution method. The potato-dextrose broth was prepared in 300ml distilled. 100ml distilled water was added into the beaker and then 3.9 grams of potato-dextrose broth was added to the beaker and the final volume was made up to 300ml and then transferred into the conical flask and covered with the aluminum foil. The broth was autoclaved for sterility purposes. First, the 50 microliters of potato-dextrose broth were added to each well of 96 well microliter plate and then 50 microliters of the essential oil of mixture were added to the first well and diluted to the last well. The equal volume of inoculum was added to each well (Figure 2). The reading was taken in ELISA reader at 520nm before and after incubation [6].

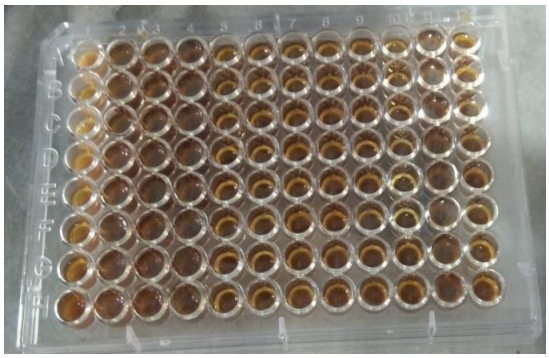

Figure 2. Fungal MIC kit

EO's antifungal impact on mycelial growth

With minor adjustments, previously published methodology was used to assess the impact of *Citrus reticulata*, *Citrus sinensis* and *Citrus lemon* and their mixture against *Aspergillus flavus* essential oils on mycelial growth of fungi. EOs were introduced to potato dextrose medium (which was at a temperature of 40–45 °C) right away after being dispersed as an emulsion in 0.1% Tween 80. In order to get EO concentrations of 0.07, 0.14, 0.28, 0.56, 1.12, 2.24, 4.48, and 8.96mg/mL, the mixed PDA medium was transferred to a petri plate. As a control, PDA medium was combined with sterile 0.1% Tween 80. Next, 5 mm-diameter mycelium plugs were removed from the edge of the examined fungus's actively growing colony, inverted, and placed on the surface of each PDA plate. For a period of three to five days, the treated plates were incubated at 28 °C. As soon as the control group's mycelial growth diameter reached 30 ± 2 mm, the other groups' growth was noticed. Every group received three treatments, and diameter (mm) of mycelium growth was determined from the colony's center to its edge. The following formula was used to quantify impact of EOs on growth of mycelium, which was expressed as a percent inhibition of mycelial growth (%) [7].

$$Inhibition\ \% = \frac{C - T}{C} \times 100$$

Where C = Control group diameter
T= Treated group diameter

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Antifungal activity of EO on germination of spores.

By using light microscope, the antifungal effects on spore germination were detected. In a nutshell, the spore concentration (spores/mL) and sterile distilled water were used to make fungal spore suspensions. All essential oils were dissolved in 1 milliliter of 0.1% Tween 80 to produce a range of concentrations, including 0, 0.07, 0.14, 0.28, 0.56, 1.12, 2.24, 4.48 and 8.96mg/ml. Each test tube containing 1ml of previously described EO solution was then filled with 100 µL of spore suspension. To monitor spore germination, concave slide was filled with 10 µL of solution and it was incubated for 20 hours at 28 °C. When the hypha's length was more than the spore's length, the spores were said to have germinated.

In vivo antifungal efficacy of EO

180 mm plates were fastened with filter paper discs (2 × 4 cm) that contained a solution of mixture´s essential oil. We bought fresh corn from the neighborhood market and arranged it on the dishes above. To see how the mold spread on the corn, the plates were sealed and maintained for 10 days at 28 °C in an incubator. When mold patches were seen on the corn's surface, it was deemed tainted. Three copies of this therapy were administered, with the control group receiving no EO treatment.

Statistical Analysis:

All experiments for antifungal activities were done in replicates so that the mean value was taken.

RESULTS AND DISCUSSION

Gass Chromatography- Mass Spectrometry analysis

The mixture's constituent compounds were determined using GC/MS, containing essential oil of all samples (Figure 3).

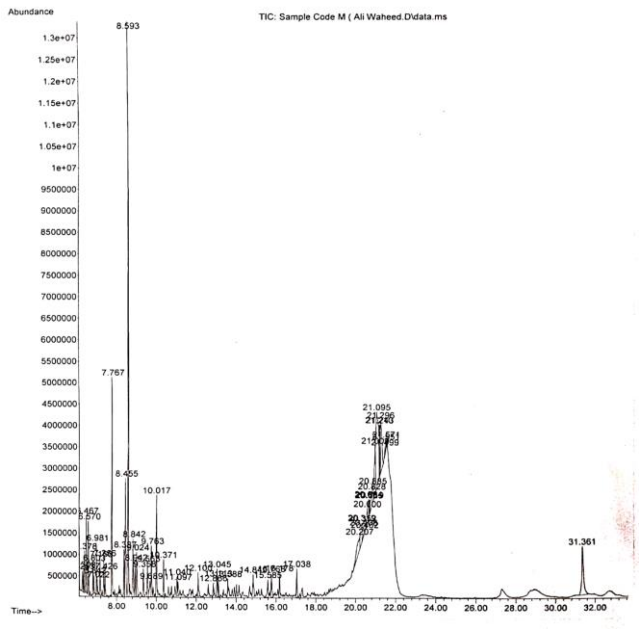
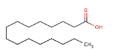


Figure 3. GC/MS analysis of essential oil of mixture

All samples were found to be consist of aldehydes, alcohol, ketones, fatty acids and some sulphur and nitrogen containing compounds. However, essential oil of mixture is found to have high concentration of manitol, hexadecanoic acid, pinene and limonene which made this sample as most effective agent against fungus (Table 1).

Table 1. Compounds identified in the GC/MS of mixture of essential oils

Compound name	Molecular formula	Molecular weight	Retention time	Structure
Limonene	$C_{10}H_{16}$	136.23g/mol	8.5	
Pyrazinoic acid	$C_5H_4N_2O_2$	124.10g/mol	19.5	
Hexadecanoic acid	$C_{16}H_{32}O_2$	256.4g/mol	21.21	

Fungal strains identification

Fungal culture was isolated, purified and characterized by PCR.

Antifungal activity

According to the gained results, the EOs of *Citrus reticulata*, *Citrus sinensis* and *Citrus lemon* and their mixture showed significant antifungal activity. Anti fungal activity was measured against *Aspergillus flavus* (Figure 4).

Figure 4. Antifungal activity of EOs from peels of *Citrus reticulata*, *Citrus sinensis*, *Citrus lemon* and their mixture against *Staphylococcus aureus* +ive control = Fluconazole

Essential oil of mixture was found to be more powerful antifungal agent as compared to other samples as it showed zone of inhibition(ZOI) of 13mm. *Citrus sinensis* showed ZOI of about 10mm while *Citrus lemon* have 8mm ZOI followed by *Citrus reticulata* of 7mm ZOI (Table 02).

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Table 2. Antifungal activity of EOs of *Citrus lemon*, *Citrus sinensis*, *Citrus reticulata* and their mixture in mm against *A. flavus*

Sample	Zone of inhibtion (mm) Fluconazole (Control)	Zone of inhibtion (mm) (Samples)
Citrus lemon	7 ± 0.26	8 ± 0.08
Citrus reticulata	15 ± 0.52	7 ± 0.79
Citrus sinensis	17 ± 0.61	10 ± 0.16
Mixture	19 ± 0.7	13 ± 0.19

Statistical analysis of antifungal analysis resulted as follow (Figure 5).

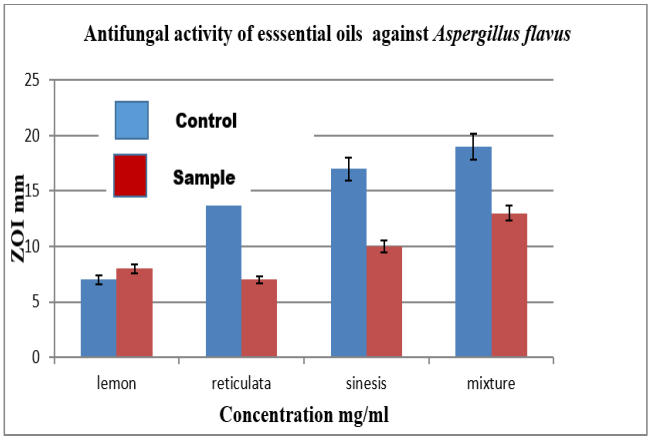

Figure 5. Mean value of zone of inhibition of *Citrus reticulata*, *Citrus sinensis*, *Citrus lemon* and their mixture against *Aspergillus flavus*

The findings showed that the chemical makeup of essential oils determines their effectiveness [8]. At room temperature, chemical components function as active inhibitors of several types of fungus [9]. Because EOs accumulate in the lipophilic hydrocarbon molecules of the cell lipid bi-layer, they are thought to have antifungal properties. This action also facilitates the easier transfer of other EO constituents to the interior of the cell. are considered to have antifungal properties due to their accumulation in the lipophilic hydrocarbon molecules of the cell lipid bi-layer; such action also allows the easier transfer of other EOs constituents to the interior of the cell [10]. Passive diffusion of the component molecules through the fungal cell wall is probably the primary interaction between an essential-oil component and a microbe cell. Because of the lipophilic character, numerous components of essential oils preferentially partition into cell membranes, changing the characteristics of those membranes [2].

Minimum inhibitory concentration

Minimum inhibitory concentration of essential oil of mixture was checked against *Aspergillus flavus* and it was found 8.96mg/ml for this strain. The results of MIC test were in accordance with the agar diffusion method depicting that synergistic essential oil containing mixture of all samples could be called as natural antifungal agent in food industry.

Mycelial growth inhibition

It was investigated how EOs altered the mycelial development of spoilage mold based on the MIC data mentioned above. In line with the MIC results, all samples combined with EO seemed to have inhibitory effects on the growth of mycelium in comparison to control group. Inhibition of fungus strain mycelial growth was facilitated by an increase in EO concentration. Complete inhibition of the fungus's mycelial growth was seen at a dosage of 8.96 mg/ml.

Spore germination inhibition

As synergistic potential of all sample essential oil was determined so only essential oil containing mixture of all samples was used for evaluating further activities. The effects of mixture´s EO on germination rate of corn fungi´s spore was determined. This EO may considerably prevent germination of spores. Fungi spore germination rates ranged from 9% to 100% when the EOs concentrations were in range between 0.56 and 8.96 mg/ml. Germination *A. flavus*. Spores could be totally suppressed by EO at a concentration of 8.96 mg/ml.

In vivo antifungal efficiency

Throughout a ten-day storage period at 28 °C, EOs may successfully decrease the occurrence and growth of natural fungus deterioration of the corn. In contrast to the control group, mixture EO was able to successfully manage fungal infection of corn during storage and extend the storage term. After storing corn for at least eight days, EO may totally prevent the naturally occurring mold from growing on its surface. However, as the length of storage period increased, inhibiting impact of EOs against corn mold deterioration was diminished. This could be due to the vapor phase of EO.

CONCLUSION

Fungicides and antifungal characteristics of EOs, expanding body of research on their mechanisms of action, and our understanding of both their traditional and innovative uses demonstrate the wide range of potential uses for these natural compounds in different fields such as agriculture, medicine, food technology and reduction of artificial drugs use. Their usage is in consistent with search of natural materials that are secure for environment and human health, even though additional study is required.

Following are areas that merit more in-depth research: (1) assessing potential synergistic effects between EOs and/or their components, (2) identifying potential components while taking into consideration the possibility of chemo- and ecotypes; (3) examining potential toxicity; and (4) examining the possibility of constructing bio-factories to produce EOs of desired chemical and biological characteristics. EOs play a crucial role in food and pharmaceutical chemistry and technology as additives and active ingredients. Essential oils' antifungal, antitoxic, and anti-biofilm characteristics can be operated as a bridge between their traditional applications and

their usefulness in alternative treatments.

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