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Evaluating the biological activity of lemongrass and rosemary essential oils against some fungi isolated from vegetables and fruits

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ABSTRACT

Five dominant species of fungi were identified by morphological and molecular methods (Aspergillus niger, Penicillium fimorum , Penicllium digtaum, Sclerotinia sclerotiorum , Alternaria alternate. our study showed the antifungal effects of lemongrass(Cymbopogon citratus L) and rosemary(Rosmarinus officinalis L), essential oils (E.O) against the identified fungi which were determined in vitro conditions. Different concentrations were tested (500, 1000.1500) ppm. 1500ppm of lemongrass E.O showed 100% inhibitory effect against the tested fungi, whereas Rosemary (E.O) were less effective at the same concentration. The essential oils of lemongrass showed significant inhibitory effects against Aspergillus niger , Alternaria alternate when compared with rosemary oil. While the effect of rosemary oil had a clear effect on both Penicillium fimorum and Penicllium digtaum. In general, all tested essential oils showed. Inhibitory effect against all selected fungi.

Keywords: *lemongrass , rosemary, antifungals, fungal growth, essential oils, vegetables, fruits*

INTRODUCTION

Material losses in crops or agricultural products have always been an important material factor due to various fungi such as food-borne fungi and plant pathogenic fungi that cause various post-harvest diseases and animal and human disturbances. In order to reduce the hazardous effects of synthetic fungicide products, increased interest in the potential application of essential oils for pathogen management has led to the search for new sources of bioactive natural products in antifungal therapy (1).

Fungi cause human illness in different ways. Mycoses are the best-known diseases of fungal etiology, but toxic secondary metabolites produced by saprophytic species are also an important health hazard. The term mycotoxin is an artificial rubric used to describe pharmacologically active mold metabolites characterized by vertebrate toxicity. They fall into several chemically unrelated classes, are produced in a strain-specific way, and elicit some complicated and overlapping toxigenic activities in sensitive species that include carcinogenicity, inhibition of protein synthesis, immunosuppression, dermal irritation, and other metabolic perturbations (26).

Essential oils are complex mixtures of odorous principles stored in different parts of special plants that play an important role in plant defense mechanisms against pathogenic fungi. They are composed of different chemical compounds which have various biological activities and therapeutic effects that can be used in different industries as well as control molds and various fungi such as food-borne and phytopathogenic fungi which cause different postharvest diseases and animal and human disorders. The antifungal activity of essential oil is associated with phyto-chemical components. In addition, percentage inhibition of mycelia growth depends on some other factors such as the antifungal activity method, the day of observation, oil concentration, and examined fungal species. In order to reduce the hazardous effects of synthetic fungicidal products, the increasing interest in the possible application of essential oils for pathogen management has induced to investigate new sources of biologically active natural products in antifungal therapy (3). Rosemary containing more than 240 potent medicinal and nutritional compounds is important from a botanical point of view (4). little attention has been paid to assess the activity of (lemon grass and rosemary) E.O to inhibit fungi causing spoilage. In the current study was aimed to evaluate the essential oils of lemongrass and rosemary against some fungi causing spoilage of some vegetables and fruits stored in refrigerators.

MATERIALS AND METHODS

Identification of Fungi by Molecular methods

Isolation of fungi

The five pathogenic fungi were isolated from withered fruits taken from greenhouses in the governorates of Baghdad and Wasit, brought to the laboratory and parts of fruits that showed symptoms of rotting and ulcers were taken. Swabs were taken from the affected parts of the fruits using sterile cotton swabs, and the swabs were passed over the culture medium, where they were planted in Petri dishes with a diameter of 9 cm containing Nutritional medium PDA (Potato Dexstrose Agar) added to the antibiotic Ampicillin at a rate of 100 mg / liter to reduce bacterial growth at a rate of 4 pieces / plate. The dishes were incubated at a temperature of 25 ± 2 °C for two days, then they were examined to observe the success of the fungal growth, then they were re-examined after a week. Nutritional media PDA. The isolates were diagnosed after 4 days depending on the characteristics of the fungal colony and the nature of the branching of the modern mycelium and the structures that it forms using the taxonomic key Parmeter and Whitney (1970) and the fungi were diagnosed by microscopy and PCR.

DNA Extraction

Genomic DNA was isolated from fungal growth according to the protocol of ABIOpure manufactures. Quantus Fluorometer was used to detect the concentration of extracted DNA in order to detect the goodness of samples for downstream applications. For 1 µl of DNA, 199 µl of diluted Quantifluor Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected.

Polymerase chain reaction

The set of forward primer ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and reverse primer ITS4 (5' TCCTCCGCTTATTGATATGC 3') was used for the detection of fungal isolates at the gene level. In a total volume of 25 µl, the PCR mixture was prepared from 12.5µL of Taq PCR master Mix (Promega, USA), 1 µM of each primer and 2 ng/µL of template DNA, then the remaining volume was completed with nuclease-free water. The PCR protocol involved an initial denaturation for 5 min at 95ºC; 30 cycles of denaturation for 30 sec at 95ºC, annealing for 30 sec at 55ºC, extension for 30 sec at 72ºC then final extension for 7 min at 72ºC. The PCR products (5µl) were run on 1.5 % agarose gel stained with Ethidium bromide in Tris Acetate EDTA buffer (TAE, pH 8.4), electrical power was turned on at 100v/mAmp for 60min and observed under UV Transilluminator. The 100 pb DNA ladder (iNtRon, Korea) was used to determine the size of PCR products.

Sequencing

PCR products were sent for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation – Korea. The results were received by email then analyzed using geneious software.

Plant collection

The plants in question were collected from the local markets and cleaned to get rid of the dust that was suspended in them. The plant samples were dried in the shade, where they were spread in thin layers on a cardboard surface in a well-ventilated room and constantly turned to prevent the samples from rotting and to speed up their drying. Then the samples were ground after drying using an electric grinder of the Myer type - Australian origin, then both the powdered lemongrass and rosemary were placed in sealed glass bottles, where the name of the plant was fixed on the bottles and the weight of the plant was recorded. The plants stored at Laboratory temperature until use .

Plant extraction

The essential oil was extracted and isolated from Lemongrass and Rosemary after hydroistillation and Clevenger apparatus,three concentrations of them were prepared (500, 1000 and 1500 ppm). For sterilization, the solutions were passed throughout 0.45 filters then stored at -20 ºC .

Minimum inhibitory concentrations (MICs.)

The percent recoveries of the (cymbopogon and rosemary) E.O, as well as the MICs were determined for each month of the year. For the determination of MICs, the poisonous-medium technique was followed according to this technique. Different concentrations of E.O (500, 1000, and 1500 ppm), were prepared by supplementing the oil's requisite amount in petri dishes containing potato dextrose agar (PDA) medium. A mycelial disc (5-mm diameter) taken from the 7-day-old culture of the test fungus was inoculated to each petridish. Plates containing non-poisoned medium served as control. Fungal colony diameters in control as well as in treatment sets were recorded after incubation for 7 days at 25∓2oC.

Statistical analysis

SPSS v26 with nonparametric analysis was used in the present study include:

Descriptive statistics: These include the followings: (1) statistical tables, (2) arithmetic mean (M), (3) standard Error of mean (SEM), (3) standard deviation (SD), (5) and graphical representation by bar charts.

Inferential statistics: Paired t-test, at level of significance equal to 0.05 and 95% confidence interval, was used for comparison of significant differences between lemongrass and rosemary inhibition growth of some species of fungi.

RESULTS AND DISCUSSION

Isolation and identification of fungi by traditional methods

Thirteen isolates of the fungus Aspergillus niger were obtained from different plants showing symptoms of infection and from different regions, and they were given the symbols M.ph1, M.ph2 and M.ph3 to..5 M.ph.

The diameters of mushrooms ranged between 3.9 - 7.76. The same fungus was also seen on many other plants, where this mushroom is the most prominent among other fungi and explains the reason for the dominance of this genus as it is considered one of the saprophytic fungi with a wide spread in different soils, (5)as it has the ability to withstand drought and high humidity, (27) and its spores have the ability to withstand high temperatures. High temperature up to 50° (7).

The fungus possesses a wide enzymatic diversity through its production of various enzymes, as well as its ability to produce large numbers of spores (8).

Seven isolates of Penicillium fimorum were obtained from different plants showing symptoms of infection and from different regions and they were given the symbols M.ph1, M.ph2 and M.ph3 to 7 M.ph7. Species were identified by growth shape, color and diameter of the fungal

colony and based on the taxonomic key of the species belonging to the genus Penicillium (5). Most of the types of this fungus are found in all places, and they are opportunistic throwers and have the ability to grow on many materials and under different conditions. Most types of soil fungi are found on foodstuffs, and some types are pathogenic to fruits, while others can grow under conditions of low oxygen and resistance to preservatives (10)

The results of isolation from infected orange plants, and through the examination, showed that the colors of the fungal colonies start in white at the beginning of the isolation, then their color turns to varying degrees of brown and green when the colonies age, and these fungi produce an enzyme and it is possible to measure the amount of enzyme production based on Color halo diameter around fungal colonies after peroxidase addition.

The body of the fungus consists of fungal hyphae divided by barriers, and its growth is in the form of colonies on the culture media in different colors. When asexual reproduction occurs, the conidiophores arise branched ending with the formation of phialidas, which carry chains of conidia and the absence of a vesicle, while sexual reproduction is very rare. This species has been identified based on phenotypic and microscopic characteristics using international taxonomic keys such as: Barnett.etal (1965).(11)

In general, some of the studied penicillium species had some distinctive characteristics, but the distinction between these traits was very difficult by microscopy. On the other hand, PCR identification techniques have provided very useful information about the molecular identification and characterization of fungi, especially closely related fungi (12).

The fungus Penicillium digtaum was isolated by transferring its conidia using a sterile dissection needle from the infected fruit samples to dishes containing the sterile PDA culture medium prepared above, with three replicates. The dishes were closed tightly and placed in an incubator at room temperature of 25°C. It was examined after six to seven days (6)The mycelium was initially white in color and produced asexual conidia with medium to high density. The colony was greenish-gray to olive on top and light brown. From the bottom when the colony age, and the growth rate of the colony diameter ranged between 2-3 mm on the culture medium. After obtaining the mushroom, it was purified by transferring its conidia to other dishes containing sterilized PDA medium and incubating it under the same conditions. The process was repeated until the mushrooms were obtained pure, specifications recorded For three replicates and a person mushroom depending on the source. The pure isolated mushrooms were identified by using phenotypic features according to most documented keys, where the stipes were of thin, smooth walls, bearing at their ends a pincilli of the type Biverticillate and some of the type Terverticillate on the nutrient medium, and Phialides cylindrical in shape with a pointed end bearing in Its ends are ellipsoidal to cylindrical conidia with smooth walls, 8-7 µm in length, whitish-green to light green in irregular chains, and these characteristics are consistent with the taxonomic key that he put (14).

The frequent presence of this fungus, especially Alternate alteraria, on pepper fruits indicates that it possesses many virulence factors that enable it to invade the fruits and cause infestations that appear in the form of black spots. Various mycotoxins that cause damage in fruits and mushrooms multiply in their tissues (13).

This fungus is considered one of the fungi that produce the enzyme, as it is one of the factors of its virulence, disease and infecting fruits, as it was the producer of the enzyme Protease, in addition to its production of the enzyme Lipase, which is able to break down fats. its ailments because it is the first line for the fungus to invade the fruits and analyze the cell walls, thus enabling it to enter the internal tissues and absorb nutrients from them, analyze and spoil them, and thus become unsuitable for the consumer and cause economic losses due to these fungi, which may be sourced from the field and after harvest during transportation and storage, especially for imported species (27). (Barth, et al. The results showed that the fungus Alternata alternata on the medium used led to the appearance of a

transparent halo around the colony with a diameter of 3 mm (13).

Identification of dominant fungi by molecular methods

The PCR results for the most dominant studied fungal isolates were analyzed using gel electrophoresis that showed the amplicons size of ITS were between 350- 600 bp as shown in Figure 1.

The ITS1-5.8S-ITS2 nuclear ribosomal region was used for amplification of this region in the isolating fungi of this study. A DNA barcode is a short piece of DNA sequence used for species determination and discovery. The internal transcribed spacer (ITS/ITS2) region has been proposed as the standard DNA barcode for fungi and seed plants and has been widely used in DNA barcoding analyses for other biological groups, for example algae, protists and animals (28).

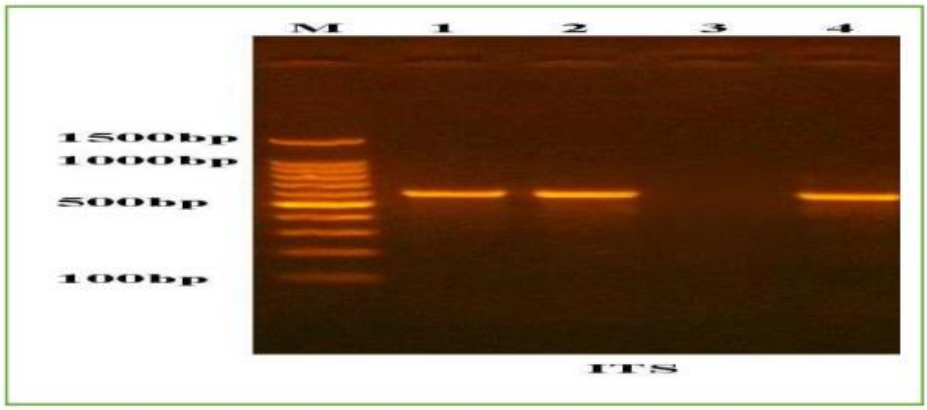


FIGURE 1: Results of the amplification of ITS gene of unknown fungal species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-5 resemble PCR products.

Only five fungal isolates were successful after sequencing. The isolates 1, 4 and 5 were identified as Penicillium fimorum after sequencing and compared with GenBank accession number MT558942. The isolate 2 and 3were identified as Penicillium digitatum and Sclerotinia sclerotiorum , after alignment with GenBank accession number MK450692 and MT534186 respectively. The ITS region was used classification and phylogenetic of Sclerotinia sclerotiorum , (15).

Effect of Rosmary and Lemograss E.O on studied fungi

Different concentration of Rosmary and Lemongrass E.Os (500, 1000 and 1500 ppm) were used to determine the effect of these E.Os on viability of fungi isolated from fruit and vegetables. The results showed no significant differences between the two plants in general, but lemongrass is more effective than rosemary with rate of 49.93 compared to 41.07 respectively as shown in Figure 2.

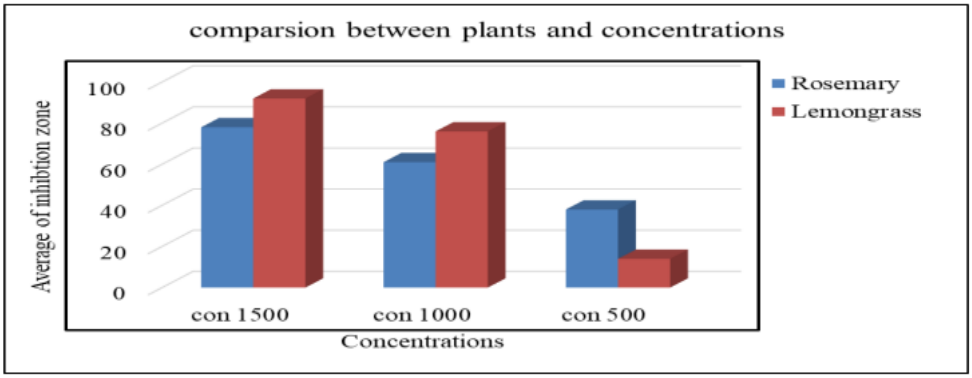


FIGURE 2: Comparisons between Rosmary and Lemongrass E.Os at different concentrations (500, 1000 and 1500 ppm) on isolated fungi from fruit and vegetables.

When used different concentration of two E.Os, found that there were significant differences (P≤ 0.01) between the concentrations 1500ppm and 1000ppm with standard rate 37.88 for 1500ppm compared to 1000 ppm 23.12. As well as showed significant differences (P≤ 0.01) between the concentrations 500 ppm and 1500ppm in favor of 1500 ppm with a standard rate of 45.47 compared to 15.53. There are significant differences (P≤ 0.01) that observed a standard rate of 43.10 for 1000ppm compared to 17.90 for 500ppm.

In general, Lemongrass was more effective at inhibiting fungal growth than Rosemary (figure 3). The percentages of fungal growth inhibition at concentration 500 were the lowest, then they increased with increasing concentration to 1000, and this in turn also increased with increasing concentration to 1500. We conclude from the above that the inhibition of fungal growth is concentration dependant.

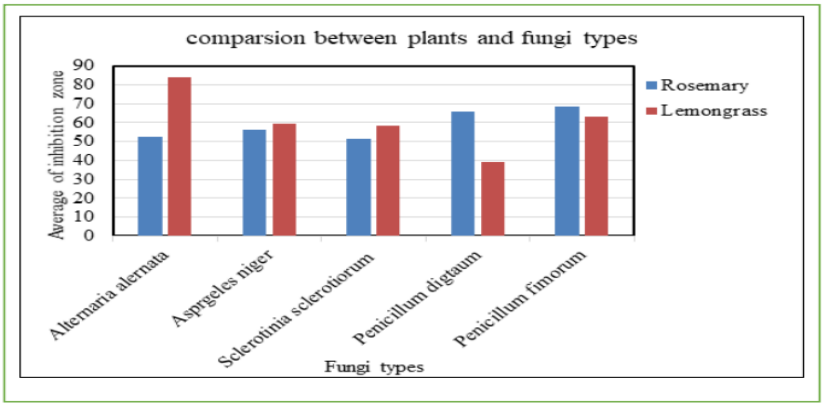


FIGURE 3: Comparisons between Average of plants and the fungi 8kik

The figure shows that lemongrass inhibited more fungi than rosemary. Alternaria alernata was more sensitive than the other species, while P. digtaum was the least sensitive among the studied species, while the rest of the three species were close in response to the extracts of the two plants under study.

Lemongrass E.O is active against such dermatophytes such as Trichophyton mentagrophytes, T. rubrum, Epidermophyton

floccosum and Microsporum gypseum,(18) and is among the most active agents against human dermatophytes. Other studies reported that lemon grass oil is active against keratinophilic fungi, ringworm fungi (16), (17) and food storage fungi.(19). Lemongrass oil is also effective as a herbicide and as an insecticide because of these naturally occurring antimicrobial effects.Our result showed that Lemon grass is highly inhibition at concentration of: 1500,1000ppm,but its lower than rosemary in concentration of 500ppm.

During screening of essential oils for their antifungal activities against Aspergillus flavus, the essential oil of Cymbopogon citratus was found to exhibit fungitoxicity. The MIC of the oil was found to be 1,000 ppm, at which it showed its fungistatic nature, wide fungitoxic spectrum, nonphytotoxic nature, and superiority over synthetic fungicides, i.e., (Agrosan etal., Thiride, Ceresan, Dithane M-45, Agrozim, Bavistin, Emison, Thiovit, wettable sulfur, and copper oxychloride. The fungitoxic potency of the oil remained unaltered for 7 months of storage and upon introduction of high doses of inoculum of the test fungus. It was thermostable in nature with treatment at 5 to 100 degrees C. These findings thus indicate the possibility of exploitation of the essential oil of C. citratus as an effective inhibitor of storage fungi (19).

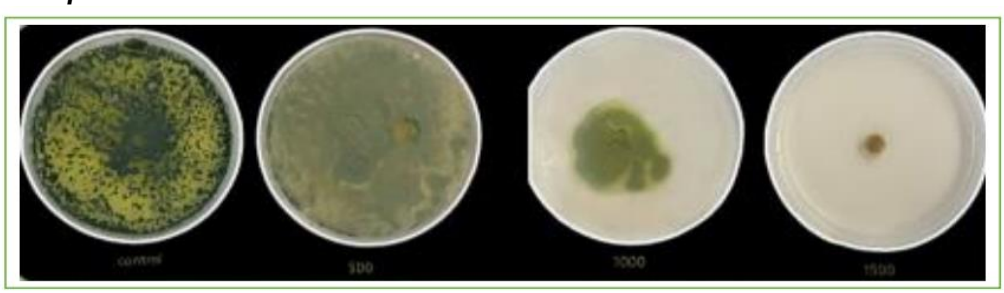
Rosemary shown a high antifungal effect on Penicillium fimorum , Penicllium digtaum. Study of the antifungal effects of the tested oil sample against Aspergillus flavus strain (PTCC = 5004) by disc diffusion through the intermediate inhibition zone, The results showed that rosemary essential oil with 1, 1/2 and 1/4 dilution of the oil showed strong antifungal activity compared to gentamicin antibiotic on A. flavus. The fungicide Binomial at 10% dilution had no inhibitory effect on A. flavus. A large proportion of the antifungal activities of rosemary oil are associated with α-pinene of monoterpenes as the main compound.`

Rosemary oil showed promising antifungal effects against C. albicans and Aspergillus niger (20).

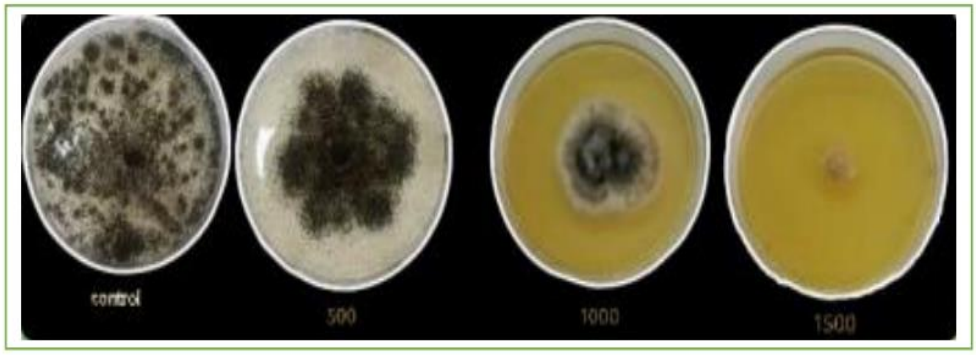
In other study, Rosemary had the highest activity against Candida albicans biofilm Phospholipase inhibition using sub-MIC of EOs against six potent phospholipase-producing isolates. Rosemary had the highest activity, with three strains showing complete inhibition of phospholipase, and three strains becoming weak producers. Also in the same study Hemolysin Inhibition (21) For rosemary oil exposure, all the tested strains showed moderate hemolysin activity in the presence of rosemary oil at ¼ MIC new agents that can inhibit biofilm formation and would enhance therapeutic efficacy are urgently needed. Results of another study showed that rosemary oil has strong anti-biofilm and anti-adhesion activity. This oil was able to prevent or reduce biofilm formation when used at sub-inhibitory concentrations.(22).

Antifungal effect of essential oils can be probably due to the phenolic components of essential oils (23), (24). It is considered to be a relationship between the chemical structure of the most abundant compounds in the essential oil and the antimicrobial activity (25).this study in general, all tested essential oils showed Inhibitory effect against all selected fungi.

1-Figures of lemmon Grass Inhibation
Penicillium fimorum



Aspergillus niger



Penicllium Digatum constration

CONCLUSION

The antifungal effects of lemongrass essential oil with concentration 1500ppm showed 100% inhibitory effect against the tested fungi: Alternaria alernata, Aspergillus niger, Penicillium fimorum, Rosemary were less effective at the same concentration. The essential oils of lemongrass showed significant inhibitory effects against Aspergillus niger, Alternaria alternate when compared with rosemary oil.

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