Study effects the biological activity of Citrus sinensis against some pathogens

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Abstract
The current study aimed to investigate the biological activity of the orang plant extract against some pathogens. The results of the inhibitory activity of the aqueous extract showed orange plant that the concentration of 100% gave the highest inhibitory activity against Klebsiella pneumoniae and Streptococcus pyogenes. The value of the inhibition diameter was 9 mm ($P<0.05$) and at the same concentration the diameter of inhibition was 18 mm for Enterococcus faecalis and 19 mm for Staphylococcus saprophyticus ($P<0.01$). The results of the aqueous extract of orange plant showed that the concentration of 100% gave the highest inhibitory activity against Candida albicans and Candida glabrata yeasts. The value of inhibition diameter was 22 and 18 mm, respectively ($P<0.01$).The results of the alcoholic extract of the orange plant showed that the 100% concentration gave the highest inhibitory activity against Enterococcus faecalis, the value of inhibition was 20 mm and the least significant difference was 6.35 , and the inhibition diameters were 17 mm for the alcoholic extract against Staphylococcus saprophyticus and Streptococcus pyogenes ($P<0.01$).Also, the results of the alcoholic extract of the orange plant showed that the concentration of 100% gave the highest inhibitory activity against Candida albicans and Candida glabrata yeasts. The value of inhibition diameter was 23 and 20 mm, respectively($P<0.01$).
Keywords: Citrus sinensis, biological activity

1. Introduction

The problem of bacterial resistance to many drugs has increased, especially in the developed countries of the world. The most important microorganisms with increased numbers are methicillin-resistant Staphylococcus aureus, vancomycin-resistant Enterococcus faecium, and penicillin-resistant Streptococcus pneumoniae. New drugs are necessary to address this growing problem, and can be obtained from nature itself, so endophytic fungi are an important store of therapeutically active compounds (Deshmukh et al., 2015). Several studies were conducted to isolate several secondary compounds in endophytic fungi with antibacterial activity, including phenolic compounds that are effective against Bacillus subtilis and Klebsiella pneumoniae and Pseudomonas aeruginosa (Subban et al., 2013).

Citrus sinensis is a rich source of secondary compounds that contribute to pharmacological activities. Several types of chemical compounds have been identified in the fruit, peel, leaves, juice and roots of C. sinensis, which include the following groups: flavonoids and sterols, hydroxy amides, alkanes, and fatty acids, coumarins, peptides, carbohydrates, carbamates, alkylamines, carotenoids, volatile compounds, and nutrients such as potassium, magnesium, calcium, and sodium (Favela-Hernández et al.,2016)

Several studies showed the antibacterial activity of the orange plant, where one study used cold terpene oil of orange plant against Listeria monocytogenes, the lowest inhibitory concentration was 0.3% and 0.25%, and for Salmonella typhimurium, the lowest inhibitory concentration was 1 % (Chalova et al., 2010).

The essential oil containing cineole and hydrocarbons at a concentration of 10% showed the lowest inhibitory concentration of 90% against P. aeruginosa (Mayaud et al., 2008). Another study showed that C. sinensis oil exhibited varied inhibition diameters using 0.1 mL of oil against E. coli (18 ± 2) mm, Listeria monocytogenes (27 ± 2) mm, Bacillus cereus (19 ± 2) mm, and S. aureus (14 ±3) mm (Fisher and Phillips, 2006).Other studies showed the antifungal activity of the orange plant. The aqueous extracts, ethanol and petroleum ether of the orange plant C. sinensis showed activity against Candida albicans (Favela-Hernández et al., 2016). This study aimed to detection the activity of endophytic fungi and orange plant on some pathogenic bacteria and yeast.

2. Methods

Prepare aqueous extract of orange plant

The extract of the leaves of the orange plant was prepared with a weight of 40 g of the plant in 160 ml of distilled water in a ratio of 4: 1 (w/v), and the mixture was crushed with a Blender in an ice bath and the mixture was stirred using an electric magnetic stirrer for an hour until the cell wall was disintegrated and ruptured. The mixture was then left to infuse in the refrigerator for 24 hours, after which the mixture was filtered with layers of gauze and again filtered through a funnel in a Buechner container with a Whatman No1 filter paper and using

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a vacuum device to remove the remaining fibers and the uncrushed parts. The extract was lyophilized by a Lypholizer, and then the samples were placed after drying in airtight glass bottles under moisture-free conditions, and then the extract was kept by freezing until use (Riose et al, 1987). The concentrations of 25%, 50%, 75%, and 100% were prepared by dilution with distilled water. 100% concentration was prepared by taking 1 g of the dried filtrate and dissolving it in 9 ml distilled water, and the rest of the concentrations were prepared from the stock solution.

Preparation of alcoholic extracts of orange plant

Alcoholic extracts (20 g) of plant leaves were prepared in 200 ml of 95% ethanol in an ice water bath, the mixture was shaken well with an electric magnetic stirrer, and left in the refrigerator for 24 hours, then the mixture was filtered by layers of gauze, and to remove alcohol the mixture was placed in a rotary evaporator, which works on evaporation under vacuum pressure and a temperature of not more than 40%, and after alcohol evaporated from the mixture, a thick layer of the extract was formed and it was freeze-dried under vacuum pressure in a lyophilizer, and then the extract was kept in freezing until it was used (Riose et al, 1987). The concentrations of 25%, 50%, 75%, and 100% were prepared by dilution with distilled water. 100% concentration was prepared by taking 1 g of the dried filtrate and dissolving it in 9 ml distilled water, and the rest of the concentrations were prepared from the stock solution.

Preparing the fungal suspension

Fungi cultured in Potato Dextrose broth after preparing it in 250ml flask, sterilizing it, and adding chloramphenicol. Inoculate each flask with several colonies of fungi with a diameter of 0.5 mm. Then the flasks are incubated in the incubator at a temperature of 25°C for 28 days, taking into account the shaking of the flasks every 2-3 days. Also, it is placed in a shaker incubator for the purpose of distributing the fungal growth and then filtering is done with filter paper (Whatman no.1) using a vacuum pump (Chambers and Scott, 1995). The filtering was repeated using the filter (0.22 microns) and the concentrations of 25%, 50%, 75%, and 100% were prepared by dilution with distilled water. 100% concentration was prepared by taking 1 g of the dried filtrate and dissolving it in 9 ml distilled water, and the rest of the concentrations were prepared from the stock solution.

Determination of phenol content in endophytic fungal and plant extracts

The sample was dried in the shade at room temperature for 24 hours, then ground to a fine powder by an electric mixer, 5 g of it was taken and put in a Soxhlet and extracted with 300 ml of ethanol at 50-55 C within 3-4 hours this method conducted according to (Laouini and Ouahrani, 2017). Where the titration curve of gallic acid was calculated in units (mg/g dry weight).

Detection of alkaloids and determination of the total content

20 g of the sample was ground and extracted with methanol for 24 h in a Soxholet extractor. The extract was filtered and the methanol was evaporated by a rotary evaporator at 45°C until dry. The method was followed by (Evan,2009) to detect alkaloids using the Dragendroff reagent. A calibration curve was prepared from Atropine by preparing several concentrations and the measurement was done at a wavelength of 470 nm.

Bacteria and yeast isolates preparation

Bacterial isolates and yeasts were obtained from the private clinics of surgeons in Tikrit city/Iraq and the bacteria were grown on the nutrient agar, and the yeasts were grown on potato dextrose agar.

Inhibitory activity of plant extracts

The method of wells diffusion was used, where the of Muller-Hinton was inoculated with sterile cotton swabs of suspension bacteria and yeast, and wells were made on agar using a cork borer (diameter 0.6 mm) and 0.15 mm was placed in each wells of the concentrations from extracts and suspension of endophytic fungi. The sterile extract solution was used alone as control, then the petri dishes were incubated at room temperature for 20 minutes and the bacteria petri dishes were incubated for 24 hours at 37°C while the yeast petri dishes were for 48 hours, and the activity of the extracts and suspension of endophytic fungi were determined by measuring the diameters of inhibition around each well.

3. Statistical Analysis

The statistical analysis program - SAS (2018) was used to detect significant differences within the study parameters. The least significant difference (LSD) test and the Analysis of Variance (ANOVA) test were used .

Estimation of the total content of phenols and alkaloids in the orange plant

The results showed as in table (1) the total content of phenols in the orange plant was 57.5% whereas the total content of alkaloids in the plant was 2.36%,

Table (1) the total content of phenols and alkaloids in plant and fungus

Total content of alkaloids %	Total content of phenol (mg Gallic / 100mg)%	Name
2.36	57.5	Orange plant

One of the studies showed the variation in the levels of the total content of phenols in the orange plant according to the different varieties and the plant part used from leaves and peels, where there were significant differences (Lagha-Benamrouche and

Madani, the 2013). Another study also showed that the orange plant has a higher content of phenols and flavonoids than tannins (Oikeh et al., 2020). Also, Reddy *et al.* (2018) showed that orange seeds had a higher content of alkaloids and flavonoids than the rest of the active substances. In addition, He *et al.* (2011) appeared in their study the total content of phenolic acids and alkaloids in the peels of citrus fruits, including oranges, was higher from those in the citrus pulp.

Inhibitory activity of the aqueous extract of the orange plant

The results of the aqueous extract of the orange plant showed that the concentration of 100% appeared the highest inhibitory activity against Klebsiella pneumoniae and Streptococcus pyogenes bacteria. The value of the inhibition diameter was 9 mm. The value of the least significant difference was 4.92 and 4.85, respectively (P<0.05) and at the same concentration, the diameter of inhibition was 18 mm. for Enterococcus faecalis and 19 mm for Staphylococcus saprophyticus, the least significant difference value was 6.41 and 5.07 (P<0.01) and (P<0.05), respectively. The rest of the concentrations showed inhibitory activity against bacterial species with significant differences, and the lowest inhibition was in the concentration 25%, followed by the highest concentrations of 50% and 75% for against the bacteria species used in the study (Table 2).

Table (2) Average diameters of inhibition (mm) of aqueous extract of Citrus sinensis leaves on some bacteria

LSD	Conc.25% Diameter mm	Conc.50% Diameter mm	Conc.75% Diameter mm	Conc.100% Diameter mm	Bacteria species
4.92 *	4	5	7	9	Klebsiella pneumoniae
4.85 *	3	5	8	9	Streptococcus pyogenes
6.41 **	11	13	16	18	Enterococcus faecalis
5.07 *	13	15	17	19	Staphylococcus saprophyticus
	6.37 **	7.04 **	6.20 **	6.15 **	LSD
* P≤0.05 **P≤0.01					

Table (3) shows the results of the aqueous extract of orange plant that the 100% concentration showed the highest inhibitory activity against *Candida albicans* and *Candida glabrata* yeasts. The inhibition diameter was 22 and 18 mm, respectively, and the least significant difference was 6.08 and 5.58, respectively, (P<0.01). The rest of the concentrations showed inhibitory activity against the yeasts with significant differences, and the lowest inhibition was in concentration 25%, followed by the highest concentrations of 50% and 75% for against the yeasts used in the study.

Table (3) Average diameters of inhibition (mm) of aqueous extract of Citrus sinensis leaves on some yeast

LSD	Conc.25% Diameter mm	Conc.50% Diameter mm	Conc.75% Diameter mm	Conc.100% Diameter mm	Yeast species
6.08 **	11	17	21	22	Candida albicans
5.68 **	11	14	16	18	Candida glabrata
---	6.72 **	5.69 **	6.01 **	7.13 **	LSD
**P≤0.01					

The results of the alcoholic extract of orange plant showed that the concentration of 100% appeared the highest inhibitory activity against Enterococcus faecalis, the value of inhibition was 20 mm and the least significant difference was 6.35 (P<0.01), and the inhibition diameters were 17 mm for the alcoholic extract against Staphylococcus saprophyticus and Streptococcus pyogenes, the least significant difference value was 5.85 and 5.78, respectively (P<0.01), also at the same concentration, the inhibition diameter was 13 mm for Klebsiella pneumoniae , and the least significant difference value was 5.06 (P<0.01). The rest of the concentrations showed inhibitory activity against bacteria species with significant differences, and the lowest inhibition was in the concentration 25%, followed by the highest concentrations 50% and 75% of against the bacteria species used in the study (Table 4).

Table (5) Average diameters of inhibition (mm) of alcoholic extract of *Citrus sinensis* leaves on some yeast

LSD	Conc.25 Diameter mm	Conc.50% Diameter mm	Conc.75% Diameter mm	Conc.100% Diameter mm	Bacteria species
5.06 *	7	10	11	13	Klebsiella pneumoniae
5.78 *	10	13	15	17	Streptococcus pyogenes
6.35 *	12	15	17	20	Enterococcus faecalis
5.85 **	8	10	15	17	Staphylococcus saprophyticus
	6.05 **	7.41 **	6.38 **	6.98 **	LSD
*P≤0.05 **P≤0.01					

Table (5) shows the results of the alcoholic extract of orange plant that the 100% concentration showed the highest inhibitory activity against Candida albicans and Candida glabrata yeasts. The value of inhibition diameter was 23 and 20 mm, respectively, and the value of the least significant difference was 6.03 and 7.01, respectively (P<0.01). The rest of the concentrations showed inhibitory activity against the yeasts with significant differences, and the lowest inhibition was in the concentration 25%, followed by the highest concentrations of 50% and 75% for against the yeasts used in the study.

Table (4) Average diameters of inhibition (mm) of alcoholic extract of *Citrus sinensis* leaves on some bacteria

LSD	Conc.25% Diameter mm	Conc.50% Diameter mm	Conc.75% Diameter mm	Conc.100% Diameter mm	Yeast species
6.03 **	8	11	15	23	Candida albicans
7.01 **	7	11	17	20	*Candida glabrata*
	5.63 **	6.81 **	6.97 **	6.43 **	LSD
**P≤0.01					

The results of this study agreed with other studies in the activity of the aqueous and alcoholic extract of the orange plant against some species of bacteria and yeasts . One study showed that the aqueous extract of orange peel at a concentration of 10 mg/mm had anti-bacterial activity against S.aureus, P. aeruginosa and E. coli and the diameters of inhibition were 18, 10 and 16 mm, respectively, while the alcoholic extract had diameters of 20, 18 and 22 ml, respectively, as the inhibitory activity was due to the plant's containing secondary compounds of alkaloids, flavonoids and terpenes with antimicrobial activity (Baba et al., 2018). In addition, the alcoholic extract of orange peels was effective against Streptococcus mutans and Lactobacillus acidophilus, as the concentration of 25 mg/ml of cold and hot alcoholic extract gave the highest effectiveness, as it recorded the highest inhibition diameter of 11.34 and 12.9 mm, respectively (Shetty et al., 2016).Akdemir Evrendilek (2015) demonstrated the efficacy of orange extract against Klebsiella pneumoniae . Another study also showed the effectiveness of aqueous, ethanolic and methanolic extracts of orange peels against bacteria with inhibition diameters of 29, 24 and 25 mm, respectively, against Klebsiella pneumoniae , as well as these extracts were effective against Enterococcus faecalis bacteria, the diameters of inhibition for Staphylococcus aureus were 14, 12, and 13 mm, respectively, for Streptococcus pyogenes, the diameters of inhibition were 20, 18, and 18 mm, respectively, and for Candida albicans, the diameters of inhibition were 14, 13, and 14 mm, respectively, as the presence of phenols and flavonoids had antimicrobial effects (Salem and Saeed, 2020). Another study showed the inhibitory activity of the alcoholic extract of orange peel extract against yeasts and molds, including Candida albicans and Aspergillus flavus (Egbuonu and Amadi, 2016). Favela-Hernández et al. (2016) also showed the efficacy of aqueous and alcoholic extract of orange plant against Candida albicans.

Inhibitory activity of *Trichoderma* against species of bacteria and yeasts

Table (6) shows the results of *Trichoderma harzianum* extract that the concentration of 100% gave the highest inhibitory activity against *Staphylococcus saprophyticus* and Enterococcus faecali. The value of inhibition diameter was 20 and 15 mm, respectively, and the least significant difference value was 6.77 and 6.28, respectively (P<0.01). The same concentration, the diameter of inhibition was 13.5 mm for *Klebsiella pneumoniae*, and for *Streptococcus pyogenes* it was 9 mm, and the least significant difference value was 6.73 and 5.48 respectively(P<0.01) .The rest of the concentrations showed inhibitory activity against bacterial species with significant differences, and the lowest inhibition was in the concentration 25%, followed by the highest concentrations of 50% and 75% against of the bacteria species used in the study.

Table (7) Average diameters of inhibition (mm) of Trichoderma harzianum extract on some yeast

LSD	Conc.25% Diameter mm	Conc.50% Diameter mm	Conc.75% Diameter mm	Conc.100% Diameter mm	Bacteria species
6.73 **	10	15.5	11.8	13.5	*Klebsiella pneumoniae*
5.48 **	3	6	7	9	*Streptococcus pyogenes*
6.28 **	15	20	24	26	*Enterococcus faecalis*
6.77 **	5	15	19	20	*Staphylococcus saprophyticus*
	7.13 **	6.59 **	6.84 **	6.49 **	LSD
*P≤0.05 **P≤0.01					

Table (7) shows the results of Trichoderma harzianum extract that the concentration of 100% gave the highest inhibitory activity against Candida albicans and Candida glabrata. The value of inhibition diameter was 20 and 11 mm, respectively, and the least significant difference value was 6.87 and 6.75, respectively(P<0.01). The rest of the concentrations showed inhibitory activity against the yeasts with

significant differences, and the lowest inhibition was in the concentration 25%, followed by the highest concentrations 50% and 75% against of for the yeasts used in the study.

Table (6) Average diameters of inhibition (mm) of Trichoderma harzianum extract on some bacteria

LSD	Conc.25% Diameter mm	Conc.50% Diameter mm	Conc.75% Diameter mm	Conc.100% Diameter mm	Yeast species
6.87 **	14	16	25	20	Candida albicans
6.75 **	7	11	11	21	Candida glabrata
	6.02 **	6.77 **	6.62 **	7.18 **	LSD
**P≤0.01					

The results of the current study showed the effectiveness of fungal extracts against bacteria and yeasts, and this study agreed with previous studies. One of the studies showed the effectiveness of alcoholic extract of some types of Trichoderma fungi against Staphylococcus aureus and Escherichia coli, as this fungus is characterized by the presence of secondary substances effective against bacteria and yeasts from these substances, terpenes and aromatic compounds such as polyketides (Leylaie and Zafari, 2018). Baazeem et al. (2021) demonstrated the efficacy of Trichoderma hamatum as an antibacterial against Acidovorax avenae Erutimacarafavora, Xanthomonas campestris and against Rhizoctonia solani, Alternaria radicina, Alternaria citri, Alternaria dauci.

Another study used the extract of Trichoderma harzianum against some types of bacteria, including Eschericia coli, Pseudomonas aeruginosa Staphylococcus aureus, and the extract showed activity against these bacteria, where the fungi have the ability to secrete secondary metabolites from terpenes and flavonoids, which have anti-microbial, anti-fungal and anti-inflammatory for tumors (Anwar and Iqbal, 2017). So, the active secondary compounds work to break down the bacterial cell wall and cause an imbalance in the cytoplasm, thus causing the formation of pores in the cell wall membranes (Subban et al., 2013).

4. Conclusion

The alcoholic extract of the orange plant showed a higher inhibition effect than the aqueous extract on the microorganisms used in the study. Also, the fungal extract Trichoderma harzianum showed an inhibitory effect at a higher concentration on these microorganisms.

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