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ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL SCREENING OF *CITRUS LIMON L.* PEELS

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ABSTRACT

To evaluate the antimicrobial activity of the peels of *Citrus limon Linn* (CL) against some microorganisms - bacteria and fungus were *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia, Pseudomonas spp, Aspergillus niger, Aspergillus fumigates, Mucor spp* and *Pencillium*. 100 µL of 10 mg CL were assessed against eight test microorganisms by agar well Diffusion Method. Gentamicin and Ketoconazole 10 mg/mL were used as standards. A different solvent was used to obtain CL peel extract by using maceration technique. %yield obtained for dried peel extract of CL with chloroform, ethanol, acetone, petroleum ether and aqueous ethanol was approximately 15%, 18%, 09%, 11% and 24% respectively. Due to its high yield value hydroalcoholic extract of CL was used for estimating the antimicrobial activity and its phytochemical screening. Phytochemical screening of CL plant reveals the presence of alkaloids, flavonoids, steroids and tannins. The study demonstrates that the hydroalcoholic extract of CL peel exhibit antibacterial activity on *Klebsiella pneumonia, Pseudomonas sp, Staphylococcus aureus* and antifungal activity among *Aspergillus niger, Aspergillus fumigates, Mucor species.* These recognized a good support to the use of this plant in herbal medicine and as base for the development of new drugs and phytomedicine.

KEYWORDS: Antimicrobial activity, Citrus lemon peels, agar well diffusion method, phytochemical screening, herbal medicine, phytomedicine.

INTRODUCTION

Throughout the ages, plants have been used by humans as a source of food, cosmetics, medicine, clothing and even shelter. Plant products also play an important role in the health

care systems of the remaining 20 percent of the population who mainly reside in developed countries.[1]

Fruits and vegetables had conferred to be capable of delivering health benefits besides fulfilling physiological needs.[2] Among various fruits that are consumed, citrus fruits are widely used in almost all countries. Citrus fruits are rich sources of bioactive compounds having beneficial effect on human health such as vitamin C, carotenoids, flavonoids, limonoids, essential oils, alkaloids, minerals and vitamin B complex.[3]

The peel of citrus fruits is an important byproduct of citrus processing industries. A large amount of peel is produced and is considered as waste. The citrus peels contain high quantity of phenolic compounds including several flavonoid compounds. The citrus peel extracts and essential oils are known to exhibit various biological activities such as antimicrobial and antioxidant activities.[3]

Citrus fruits make up the largest sector of the world's fruit production, with more than 100 million tons produced each season. About 34% of citrus fruits are made into juices; therefore, large amounts of residues are formed every year[3]. Citrus peels, which comprise the dominant residue, exhibit potent antioxidant, antimicrobial and anti-inflammatory activities, and are considered potential sources of functional components[3]. Except for ascorbic acid, citrus peels contain more bioactive compounds, such as phenolic acids, flavonoids, limonoids, and fibre than do juices.[3]

Among the well-known citrus bioactive compounds, flavonoids, especially the citrus unique polymethoxy flavones and flavanone glycosides, attract considerable attention for their significant biological activities.[4]

Due to their high flavonoid content, citrus peels could be exploited by both pharmaceutical and food industries. In spite of this, the compounds present in citrus peel are usually processed as by-products or wasted, resulting in environmental pollution. One of the main reasons for this is the absence of effective extraction procedures to obtain the flavonoids from the citrus peels.[4]

Lemon fruit [*C. limon* (L.) Burm. f.] contains many important natural chemical components, including phenolic compounds (mainly flavonoids) and other nutrients and non-nutrients (vitamins, minerals, dietary fiber, essential oils and carotenoids). Their health-promoting

effects and properties have been associated with their contents, namely vitamin C and flavonoids, due to their natural antioxidant characteristics. Overall, lemon fruits, rich in flavonoids, are a very important part of a balanced diet, particularly for their role in prevention of diseases, such as obesity, diabetes, blood lipid lowering, cardiovascular diseases and certain types of cancer.[5]

The lemon tree is an evergreen, growing to over 6 m in height. The plant is cultivated in Mediterranean and subtropical climates worldwid.[6] Lemon juice has long been used as an astringent, diaphoretic, diuretic, gargle, lotion, and tonic.[6] Lemon juice has been shown to increase citrate levels in patients with hypocitraturic calcium nephrolithiasis in a small, long-term trial (mean duration, 44.4 months); 120 mL diluted lemon juice containing 5.9 g consumed daily resulted in a clinically important reduction in stone formation.[7] Lemon juice and lemon oil have been evaluated for antimicrobial action. The oil shows some bacteriostatic and antiviral action thought to be due to citral and linalool content.[6] Lemon has been shown to inhibit the growth of Aspergillus mold.[8] and has been used to disinfect drinking water and to inactivate rabies virus.[9]

MATERIALS AND METHODS

PLANT MATERIALS

The fresh local lemon fruits(*Citrus limon* L.) were collected from the city of Erbil (Iraq), in November 2015. The Lemons fruits were washed several times with clean water, peels were separated, cut into small spieces, air-dried under shade and ground into uniform powder using a Thomas-Willey milling machine.

PREPARATION OF EXTRACTS

Air-dried peels of *Citrus limon* (15 g) were powdered and extracted with different solvents (50 mL) individually for 5 days at a room temperature. The solvents used were chloroform, ethanol, acetone, petroleum ether and aqueous ethanol. The extracts were filtered and the solvents were removed under reduced pressure at relatively low temperature (<35^{o}C) to leave a dark yellow solids and the weight of each residue was recorded and percentage yield was calculated.

PHYTOCHEMICAL SCREENING

Hydroalcoholic extract of *Citrus limon* L. peels were subjected to preliminary phytochemical screening for the presence or absence of various active metabolites.[10,11]

Test for Tannins About 0.2 g of ethanolic extract of *Citrus limon* peels was boiled in 5 ml of water in a test tube. A few drops of ferric chloride solution were added and a blue-black coloration observed in each extract indicated the presence of tannins.

Test for Steroids

Two ml of acetic anhydride were added to 0.5 g ethanolic extract of the sample with 2 ml concentrated H_2S0_4. The color changed from violet to green in *Citrus limon* peels indicating the presence of steroids

Test for Flavonoids

Three methods were used to determine the presence of flavonoids in the plant sample. Five ml of dilute ammonia solution were added to a portion of the ethanolic filtrate plant extract followed by addition of concentrated H_2S0_4. A yellow coloration was observed in each extract indicated the presence of flavonoids.

Few drops of 1% aluminium solution were added to a portion of the ethanolic filtrate plant extract. A dark yellow colouration was observed in the extract indicating the presence of flavonoids.

A portion of the powdered plant sample was heated with 10 ml of ethanol over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colouration was observed in each sample indicating a positive test for flavonoids.

Test for Alkoloids

Five ml of 2N hydrochloric acid was added to 0.5 g ethanolic extract of the sample and the solutions were heated with stirring in a water barh for 10 minutes. The cooled solutions were filtered and a few drops of Dragendorff's reagent were added to a portion of these solutions. A formation of a reddish-brown precipitates in *Citrus limon* peels were considered as a positive test for alkaloids.

Test for Terpenoids (Salkowski Test)

Five ml of the extract was mixed with 2 ml of chloroform, and concentrated H_2S0_4 (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface was not formed in *citrus limon* L. peels to show negative results for the presence of terpenoids. Terpenoids were absent in *citrus limon* L. peels.

Test for Saponins

About 2 g of the dried powdered samples of *citrus limon* L. peels were boiled in 20 ml of water in a test tube and then filtered. Ten ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion to show a positive results for the presence of saponins. Sponins were absent in *Citrus limon* L. peels.

BIOLOGICAL ACTIVITIES

Micro-Organism

The test microorganisms used in this study were Bacteria: *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas* spp. and fungus: *Aspergillus niger*, *Aspergillus fumigates*, *mucor* spp and Pencillium. The test organisms were clinical isolates and obtained from the laboratory of Microbiology, Rezgari Hospital, Erbil, Iraq.

Antibacterial Activity

The antibacterial activity of the hydroalcoholic extract was determined in accordance with the agar-well diffusion method. The bacteria was first isolated and grown in a nutrient broth for 18 h before use and standardize the culture to 106 cfu/mL. Mueller-Hinton agar (OXOID) was prepared and bored the wells into the agar using a sterile 4 mm diameter cork borer. 200 µL of the standardized cell culture was spread on a MH agar. Approximately 100 µL of the hydroalcoholic extract at 10 mg/mL were introduced into the wells, allowed to stand at room temperature for about 2 h and then incubated at 37^{o}C. After 24 h the plates were observed for zones of inhibition. The zone of inhibition was compared with that of control and standard Gentamicin at a concentration of 10 mg/mL.[12,13]

Antifungal Activity

The fungal organisms were first isolated and allowed to grow on a rose bengal agar (RBA) (OXOID) at 25^{o}C for 72 h. The fungi were harvested after sporulation by pouring distilled water on the surface of the plate and later scraped the spores with a sterile glass rod. 100 µL of the standardized fungal spore suspension was spread on the Potato Dextrose Agar (PDA) using a glass spreader. Sterile 4 mm diameter of cork borer was used to bored wells into the PDA. Approximately 100 µL of 10 mg Citrus limon L. peel extract were introduced into the wells and allowed to stand (1h) for proper diffusion of the extract into the media. The plates

were observed for zones of inhibition after 72 h at 25^{o}C and compared with ketocanazole at a concentration of 10 mg/mL.[14,15]

RESULTS

PHYTOCHEMICAL SCREENING

Preliminary phytochemical screening of Hydroalcoholic extracts of *Citrus limon* L. peel was shown in Table 1.

Table 1 Phytochemical screening of ethanolic extract of *Citrus limon L.* peels.

Phytochemical Test	lemon peels	Inference
Tannins (Firric chloride test)	+	Tannins Present
Steroids (Libermann-burchard test	+	Steroids Present
Flavonoids (Shinoda test)	+	Flavonoids Present
Alkoloids (Dragendorff's test)	+	Alkoloids Present
Terpenoids (Salkawski test)	-	Terpenoids Absent
Saponins test	-	Saponins Absent

EXTRACTION BY USING DIFFERENT SOLVENTS

The peels was extracted by using different solvents such like Chloroform, Ethanol, Acetone, Petroleum ether and hydroalcohol and the obtained %yield value was 15%, 18%, 09%, 11% and 24% respectively. In this study hydroalcoholic peel extract of *Citrus limon* L. posses high solubility property and high % yield value hence it is used for evaluating its antimicrobial activity.

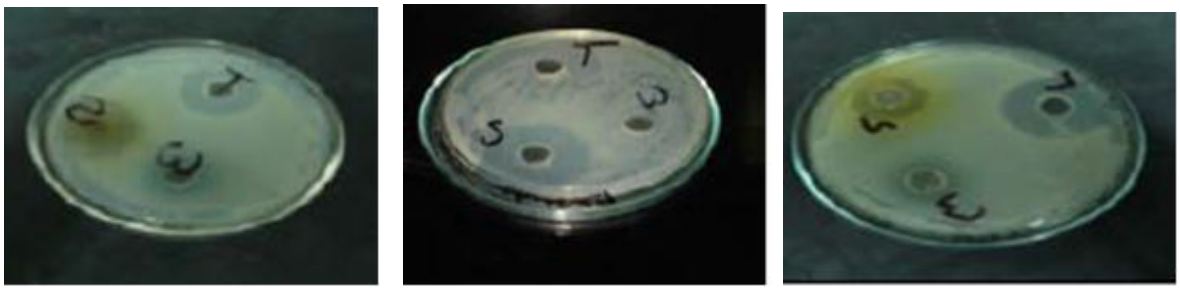
BIOLOGICAL ACTIVITIES OF EXTRACT

ANTIBACTERIAL ACTIVITY

The obtained zone of inhibition indicates that hydro CL peels exhibited in-vitro antibacterial activity against Gram-positive and Gram-negative organisms. Control group represents the diameter of sterile cork borer of 4 mm without any zone of inhibition. All the bacterial strains established some degree of sensitivity to the plant. Among the four organisms, the CL extract showed a higher activity on *Klebsiella pneumonia* and *Staphylococcus aureus*, (Table-2).

Table 2 Antibacterial activity of Hydroalcoholic extract of Citrus limon L. peel.

Microorganism	Zone of Inhibition (mm)		
Gram +ve/ Gram –ve	Test Samples		
	Control	Lemon peels	Gentamicin
Staphylococcus aureus	04	12	14
Escherichia coli	04	-	10
Klebsiella pneumonia	04	12	15
Pseudomonas spp	04	10	16



Staphylococcus aureus ***Klebsiella pneumonia*** ***Pseudomonas spp***

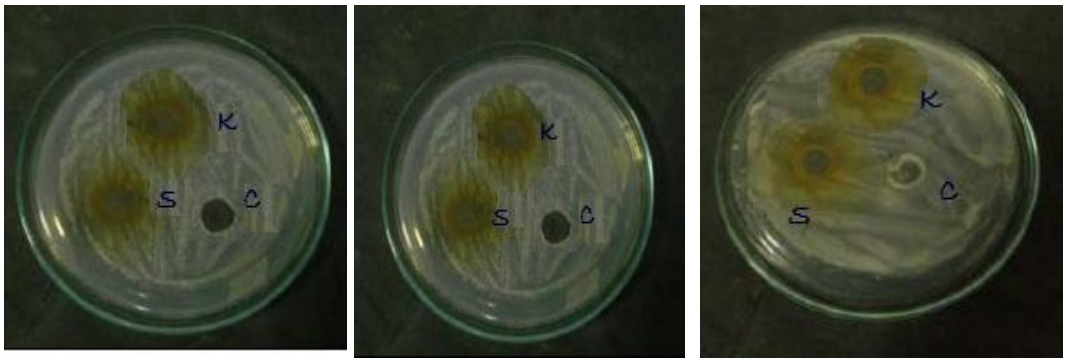
Figure 2 Antibacterial activity of Hydroalcoholic extract of Citrus limon L. peel.

ANTIFUNGAL ACTIVIT

The obtained zone of inhibition indicates that CL peels exhibited in-vitro antifungal activity against *Aspergillus niger*, *Aspergillus fumigates* and *Mucor spp*. Control group represents the diameter of sterile cork borer of 4 mm without any zone of inhibition. Hydro alcoholic extract of CL show antifungal activity on *Aspergillus niger*, *Aspergillus fumigates* and *Mucor spp* (Table 3). The results reveal that extracts of*Citus limon* L. were significantly effective against *Mucor spp*.

Table 3 Antifungal activity of Hydroalcoholic extract of *Citrus limon* L. peel.

	Zone of Inhibition (mm)		
Microorganism	**Test Samples**		
	Control	Lemon peels	Ketoconazole
Aspergillus niger	04	10	15
Aspergillus fumigates	04	12	17
Mucor spp	04	16	21
Pencillium	04	-	18



Aspergillus niger ***Aspergillus fumigate*** ***Mucor spp***

Figure 2 Antifungal activity of Hydroalcoholic extract of *Citrus limon* L. peel.

DISCUSSION

Now a day's many research are focused on herbal medicines and their natural compounds. In traditional systems of medicines Citrus fruits having its own importance to treat various human alignments. Citrus juice is used as antidepressant, promoting resistance against various infections and famously used for scurvy disease which is caused due to the lack of Vitamin C.

In the present study we evaluate the antibacterial and antifungal efficacy of *Citrus limon* L. peels. A different solvents was used to prepare CL extract by using maceration technique. Each solvent having its own capability to soluble various active components in it. Preliminary phytochemical screening of Hydro alcoholic peel extract of *Citrus limon* L. reveals the presence of Alkaloids, Flavonoids, Steroids and Tannins. This is well known, since flavonoids are important plant metabolites which is majorly responsible for antimicrobial activity.[23] Our results shows that CL peel extract has antibacterial activity against *Klebsiella pneumonia*, *Pseudomonas sp, Staphylococcus aureus* and antifungal activity amongst *Aspergillus niger, Aspergillus fumigates,* Mucor species. The percentage bacterial inhibition of CL peel extract was obtained as *Staphylococcus aureus* (85.7%), *Klebsiella pneumonia* (80%) and *Pseudomonas* spp (62.5%) when compared to standard Gentamicin. The percentage fungal inhibition of CL peel extract was obtained as *Aspergillus niger* (66.6%), *Aspergillus fumigates* (70.5%) and Mucor spp (76.1%) when compared to standard Ketoconazole. Our result indicates that Hydroalcoholic peel extract posse's strongest antibacterial activity specifically against *Staphylococcus aureus*.

CONCLUSIONS

In the present study the results indicates that the hydro alcoholic extracts of *Citrus limon* L. peels possess good antibacterial and antifungal activity, confirming the great possible of bioactive compounds and are useful for rationalizing the use of this plant in primary health care. The results suggest that the extract of *Citrus limon* L. were significantly effective against Mucor spp in case of fungi and showed a higher activity on *Klebsiella pneumonia* and *Staphylococcus aureus* amongst bacteria. In vivo information may be helpful in determining the actual potential usefulness of this plant for the handling of causal organisms of infectious diseases. Thus further work can be carried on the isolation procedure for finding out the exact moiety responsible for the biological activity.

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