

Antimicrobial Activity for Crude Watery Extract of Seeds of *Citrus aurantifolia* (Lime fruit) against Gram Positive and Negative Bacteria In Vitro

Assist. Prof Bahaa A.L. Al-Rubai 1 ; Lec. Hind Hussein Obaid 1*;

Assist. Prof Tahrrer Hadi Saleh2

1 Department of Biology, College of Science, University of Baghdad, Baghdad – Iraq

2 Department of Biology, College of Science University of Al-Mustansyria , Baghdad – Iraq

Abstract:

The effect of crude watery extract for seeds of *Citrus aurantifolia* (Lime fruit) was tested against different clinical isolates including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Klebsiella pneumoniae* and *Proteus vulgaris*. The inhibitory effect for watery extract was carried out by agar well diffusion method and represented as inhibition zones around wells and measured with millimeter unite; the large zones are linked with concentration 10, 20, 40 % of seed extract, the antimicrobial activity of crude seeds extract may be attributed to bioactive compound present in it. The results obtained shown that the crude seeds watery extract of *C. aurantifolia* fruit can be considered as good source of antimicrobial compounds and can be used as alternative therapy for many infectious diseases and can be incorporated into different drug formulations.

الفعالية ضد مايكروبية للمستخلص المائي الخام لبذور النومي البصرة تجاه بعض أنواع البكتيريا الموجبة والسالبة لصبغة كرام خارج الجسم الحي

أ.م.د. تحرير هادي صالح
الجامعة المستنصرية - كلية العلوم - قسم علوم الحياة

أ.م.د. بهاء عيد الله لفته الربيعي
م.د. هند حسين عبيد
جامعة بغداد - كلية العلوم - قسم علوم الحياة

المستخلص:

أختبر تأثير المستخلص المائي الخام لبذور نومي بصرة ضد عزلات
سريرية مختلفة والتي تضمنت

Enterococcus faecalis و *Staphylococcus epidermidis* و
staphylococcus aureus . *Proteus vulgaris* و *Klebsiella*
pneumoniae .

أن التأثير التثبيطي للمستخلص المائي نفذ بطريقة الأنتشار في الحفر وتمثل
بمناطق تثبيط النمو حول الحفر مقاسة بوحدة القياس الملمتر ، ان كبر مناطق
التثبيط مرتبطة بتركيز مستخلص البذور (١٠ و ٢٠ و ٤٠) % .
ان الفعالية ضد مايكروبية لمستخلص البذور الخام قد تعزى لمركبات حيوية
موجودة فيه، النتائج التي تم الحصول عليها أظهرت ان المستخلص المائي الخام
لبذور ثمره النومي بصرة يمكن أن يعد مصدر أ جيداً للمركبات ضد مايكروبية
ويمكن استخدامها كعلاج بديل للعديد من الامراض المعدية ويمكن أن يهيئ بأشكال
علاجية مختلف.

الكلمات المفتاحية: بذور النومي بصرة ، البكتيريا الموجبة والسالبة ، صبغة كرام

Introduction:

Usage of plants in medicines for a wide variety of human ailments has deep roots in man's history (1, 2), Infectious diseases account for high proportion of health problems in the developing countries (3). As the World Health Organization (WHO) report, 80% of the world population presently uses herbal medicine for some aspect of primary health care (4) . Approximately 20% of the plants found in the world have been subjected to pharmacological or biological tests, and many new antibiotics introduced in the market are obtained from natural or semi-synthetic resources (5, 6) . Many researches and practical experiences have shown that using medicinal plants is better than chemical and semi synthetic drugs because of their fewer side effects, relative low cost and effectiveness besides having synergistic effect; Medical plants are used in the treatment of different disorders such as malaria, diarrhoea, burns, gonorrhoea, stomach disorders and other infectious diseases(7,8).

One of such plants is *Citrus aurantifolia* (Lime fruit), it is very much employed in herbal medicine and their bioactive activities for cold fevers, sore throats, sinusitis and bronchitis, as well as helping asthma, suppress stomachache and it has been found to be an excellent cough relieving (9). It can be helpful for rheumatism arthritis, obesity and cellulite and has an astringent and toning action to clear oily skin and acne, helps with herpes, cuts and insect bites (10). Lime is also used to overcome dysentery, constipation, dizziness body odor, increased appetite, tonsils and nose inflammation, prevent hair loss, dandruff, too fat, and to induce apoptosis in human pancreatic cells was studied (11). *C. aurantifolia* is known as West Indian lime, bartender's lime, Omani lime, Mexican lime, dayap and bilolo; it belongs to Rutaceae family, it is a shrubby tree, to 5 m (16 ft), with many thorns; the leaves are

ovate, 2.5–9 cm (1–3.5 in) long, resembling orange leaves (the scientific name *aurantiifolia* refers to this resemblance to the leaves of the orange, *C. aurantium*); the flowers are 2.5 cm (1 in) diameter, are yellowish white with a light purple tinge on the margins. Flowers and fruit appear throughout the year, but are most abundant from May to September in the Northern Hemisphere (12). The growing resistance of microorganisms to conventional antimicrobial agents is becoming a source of concern to clinical microbiologists all over the world. This study aims to investigate the potency of seed of *C. aurantifolia* against different pathogenic bacteria and may be used in traditional medicine as antimicrobial agent.

Material and methods

Lime fruits:

Lime (*C. aurantifolia*) fruits used in this study were brought from local markets in Baghdad and the seeds were collected to use in next steps.

Preparation of Seed powder:

The seeds of *C. aurantifolia* were washed by distilled water and dried rapidly by air current at room temperature, then milled by mortar until get fine powder and kept without sieving in clean flask.

Preparation of aqueous extract for seeds:

Watery extraction of seeds were prepared at 10, 20 and 40 % by mixing the required quantity of seeds powder with distilled water and kept for 2 hours at room temperature and then heated slowly at boiling degree 100 °C for at least 1 hour. The solution was cooled at room temperature for 2 hours and then centrifuged at 5.000 rpm; the supernatant was collected and used as crude extract and kept until testing their susceptibility against bacteria.

Bacterial strains and culture conditions:

The tested bacteria used in this study were three gram-positive (*Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis*) and two isolates belonging to gram negative (*Klebsiella pneumoniae* and *Proteus vulgaris*) were obtained from pathogenic bacteria laboratory in the Department of Biology, College of Science, University of Baghdad ,and were collected previously from different hospitals in Baghdad city. These isolates were cultured on brain heart infusion broth at 37°C for 24 h (Oxoid), the pure colonies were selected and streaked on blood agar plates (blood agar base –Oxoid– supplemented with 5% of human blood). These isolates were identified by biochemical tests (13). APIE- 20 test (bio- Merieux) was employed for the confirmation of identification.

Maintenance of strains:

Bacterial strains were maintained on deep Nutrient agar slant (Oxoid) for 5-6 weeks with periodic subculture and nutrient broth (Oxoid) with 35 % glycerol at -20°C (14).

Standardization of inoculum:

Test organisms were sub-cultured onto fresh plates of nutrient agar (Oxoid) at 37°C for 24 h. Colonies from these plates were suspended in Mueller-Hinton broth (Oxoid) to a turbidity matching 0.5 mc McFarland standard (108 cfu/ml) for bacteria; all plates were incubated at 37°C for 24 h (15).

Antimicrobial activity Assay:

The agar diffusion method is used for the antimicrobial evaluations. Suspension of the bacteria 1 X 10⁸ cfu/ml was made in sterile normal saline. Each labelled medium plate was uniformly seeded with a test organism by sterile swab rolled in the suspension and streaked on the plate surface. Wells of 5

mm in diameter were punched in the culture media with sterile cork borer. The various concentrations of watery extract for seeds of *Citrus aurantifolia* were transported 100 μ l by micropipette into each well to fullness (16). The plates were kept in room temperature for 1 h before incubating at 37°C for 24 h. Zones of inhibition around the wells, measured in millimeters, were used as positive bioactivity.

Results:

The results have shown that seeds watery extract of *C. aurantifolia* has inhibitory action when increased in concentration gradually against these bacteria; the inhibition zones 19, 20 and 18 mm in diameter for *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Klebsiella pneumoniae* at 10%, while for *Enterococcus faecalis* and *Proteus vulgaris* were shown 13 and 15 mm respectively; but at 20% the results have shown the same inhibition zone for *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Klebsiella pneumoniae* about 21 mm, while *Enterococcus faecalis* and *Proteus vulgaris* have shown 14 and 18 mm in their diameter of inhibition zone around wells. In contrast the sensitivity for *Staphylococcus aureus*, *staphylococcus epidermidis*, *Klebsiella pneumoniae*, and *Enterococcus faecalis* were recorded as inhibition zones 23, 22, 21 and 18 mm respectively, behavior of *Proteus vulgaris* showed higher sensitivity than other isolates reaching to 24 mm after treatment with 40% concentration of seeds watery extract of *C. aurantifolia*. (Figure 1 & table 1).

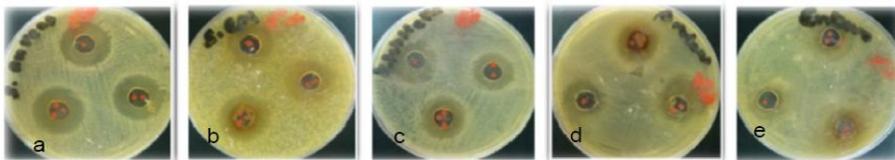


Figure 1:The agar diffusion method for the antimicrobial activity to crud watery extract of seeds of *C. aurantifolia* against pathogenic bacteria (a) *S. aureus*, (b)- *S. epidermidis*, (c) *K. pneumoniae*, (d) *P. vulgaris*, (e) *E. faecalis*

Table 1: Zones of inhibition (mm) of organisms to crud watery extract of seeds of *C. aurantifolia* .

Bacteria spp	Inhibition zone (mm)		
	10%	20%	40%
Gram positive			
<i>Staphylococcus aureus</i>	20	21	23
<i>staphylococcus epidermidis</i>	19	21	22
<i>Enterococcus faecalis</i>	13	14	17
Gram negaitive			
<i>Proteus vulgaris</i>	15	18	24
<i>Klebsiella pneumoniae,</i>	18	21	21

Discussion:

The clinical isolates in this study were diagnosed by biochemical tests and API 20. These bacteria will cause various diseases for human like *Staphylococcus aureus* (pneumonia, impetigo, cellulitis, scalded skin syndrome, mastitis, chorioamnionitis and neonatal sepsis), *Staphylococcus epidermidis* (endocarditis, nosocomial, biofilms to grow on catheters, acne) *Klebsiella pneumoniae* (pneumonia, thrombophlebitis, urinary tract infection, cholecystitis, diarrhea, upper respiratory tract infection, osteomyelitis, meningitis), *Proteus vulgaris* (urinary tract infections), and *Enterococcus faecalis* can causes endocarditis and bacteremia, urinary tract infections , meningitis, and other infections in humans (17, 18 , 19). Citrus health promoting properties have always been associated with their content of vitamin C and several bioactive compounds

that play a major role in preventing chronic diseases, specifically flavonoids and limonoids which give bitterness in taste for acidity of Citrus being after kept for long time (20, 21). Citrus limonoids appear in large amounts in citrus juices and citrus tissues as water-soluble limonoid glucosides or in seeds as water-insoluble limonoid aglycones (22, 23). Results of HPLC analysis and using GC-MS reported by Jaiprakash in 2009 revealed that limonin was the major compound in lime seeds, followed by Isolimonexic acid and L-limonexic acid, and there are other compounds like D-limonene, D-dihydrocarvone, verbena, β -linalool, α -terpinol, trans- α -bergamotene (24). All these chemical compounds being present in seeds or seed coats; probably function in the protection of seeds from microbial degradation until conditions are favorable for germination (25, 26). The protection role of chemical compounds in seeds was employed in this study to investigate their role in inhibition growth of different pathogenic bacteria in vitro that may threaten human life; The variety of bacterial inhibition may attribute to many reasons such as reaction the bioactive compounds in seed extract with different compounds found in bacterial cell wall structure; this reaction may form new compound having bactericidal or bacteriostatic effect; also the bioactive compound may invade into bacteria and attack the important active molecules in cytoplasm such as DNA, ribosomes and others. The available reports that deal with employed seeds of *C. aurantifolia* in medicine field were rare and not documented, but there are many literatures that clearly point to the importance of root, leaf, flower and fruit of *C. aurantifolia* as drug; Jaiprakash and his co-workers observed ability of the leaf extract and leaf essential oil of *Citrus aurantifolia* to inhibit the growth of *Staphylococcus aureus* and use it to overcome dysentery, constipation, diphtheria, acne, dizziness, cough, and inflammation of the nose (11); whereas Joji Reddy reported the antibacterial activities for leaf extract of *Citrus aurantifolia* was investigated against different pathogens such as *Bacillus cereus*, *Enterobacter faecalis*, *Salmonella paratyphi*,

Staphylococcus aureus, *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Serratia marcescens* (27, 28); also the antimicrobial activity of leaf extraction of *Citrus aurantifolia* by Chloroform, ethanol, acetone, petroleum ether and hydro alcohol inhibited the growth of *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas spp*, *Aspergillus niger*, *Aspergillus fumigates*, *Mucor spp* and *Pencillium* (29) . But the potency of *Citrus aurantifolia* fruit and the oil obtained from steam distillation of the fruit was investigated by Ibukun Aibinu and his colleagues in 2007 against many clinical isolates including Anaerobic facultative bacteria *Staphylococcus aureus*,, *Salmonella paratyphi*, *Shigella flexnerii*, *Streptococcus faecalis*, *Citrobacter spp*, *Serratia spp*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*; Fungi such as *Aspergillus niger* and *Candida albicans*; and Anaerobes which includes *Bacteroides spp*, *Porphyromonas spp*, and *Clostridium spp* (30).

There are many solvents used to extract the bioactive chemical compounds from plant parts like chloroform, ethanol, acetone, petroleum ether, hydroalcohol and water; but the extraction by water was used in this study because it has many advantages like being available, chapter, rapid and safe; whereas the disadvantages were summarized that bioactive compounds were not extracted totally with high purification or may be attributed to rigidity of seed structures than other soft parts of *C. aurantifolia*. Based on our results, it can be concluded that seeds of *C. aurantifolia* possess significant antibacterial activity recommending that their consumption could be useful in the prevention of diseases caused by different infectious microorganisms such as alternative for many synthetic antibiotics (31). Future researches are needed for the isolation and identification of active principles present in the extracts which could possibly be exploited for pharmaceutical use.

References:

- 1- Iqbal, A. and Arina, Z. B. (2001). Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. *J.Ethnopharmacol.*, 74: 113-123.
- 2- Jayana, B. L. ; Prasai, T. ; Singh, A. ; Yami, A. ,K. (2010). Study of Antimicrobial Activity of Lime Juice against *Vibrio cholera*. *Scientific World* , 8(8):44-46.
- 3-. Sashi, K.J., Ramya, M., Janardhan, K. (2003). Antimicrobial activity of ethnomedicinal plants of Nilgiri Biosphere reserve and Western Ghats. *Asian J.Microbiol. Biotechnol. Environ. Sci.*, 5: 183- 185.
- 4- Sujatha, S. (2005). Complementary and alternative therapies in palliative care: a transition from modern medicine to traditional medicine in India. *J. Cancer Pain and Symptom Palliation*. 1: 25-9.
- 5- Mothana, R.A., Lindequist, U. (2005). Antimicrobial activity of some medicinal plants of the island Soqotra. *J. Ethnopharmacol.*, 96(1-2): 177-181.
- 6- Pathan, R. k.; Gali, P.R. ; Pathan, P.; Gowtham, T.; Pasupuleti, S. (2012). In vitro Antimicrobial Activity of *Citrus aurantifolia* and its Phytochemical screening. *Asian Pacific Journal of Tropical Disease*, 2: s238- s331.
- 7- Ndukwe, K. C., Okeke, I. N., Lamikanra, A., Adesina, S. K. and Aboderin, O. (2005). Antibacterial Activity of Aqueous Extracts of Selected Chewing Sticks. *J.Contemp. Dent. Pract.*, (6)3:086-094.
- 8- Adedayo, O., Anderson, W. A., Moo-Young, Snieckus, V., Patil, P. A. and Kolawole, D. O. (2001). Phytochemistry and Antibacterial Activity of *Senna alata* flower. *J. Pharmaceu. Biol.*, 39:408- 412.
- 9- Onyeagba, R. A., Ugbogu, O. C., Okeke, C. U. and Iroakasi, O. (2004). Studies on the antimicrobial effects of garlic (*Allium sativum* Linn), ginger (*Zingiber officinale* Roscoe) and Lime (*Citrus aurantifolia* Linn). *Afri.J. Biotechnol.*, 3(10):552-554.
- 10- Trease and Evans. *Pharmacognosy*. 15 edition, Published by Elsevier, (2005); pg: 266-267.

- 11- Jaiprakash, R., Patil, K. N., Chidambara, M., Jayaprakasha, G. K., Mahadev, B.C. and Bhimanagouda, S.P. (2009). J. Agric. Food Chem., 57 (22): 10933.
- 12- Golob, P.(1999). Food and Agriculture Organization of the United Nations"Alphabetical List of Plant Families with Insecticidal and Fungicidal Properties". The use of spices and medicinals as bioactive protectants for grains. Food & Agriculture Org. pp. 13-. ISBN 978-92-5-104294-6. Retrieved 19 June 2011.
- 13- Atlas, R. M. and Snyder, J. W. (2006) . " Handbook of Media for Clinical Microbiology". 2 ed. Taylor and Francis group. CRC press. USA .
- 14- AL-Rubaii, B.A.L. (2009) ."Role of *Proteus mirabilis* Metalloprotease in degradation of different animal proteins and cloning of responsible gene in *E.coli* BL21. Ph.D thesis. Biology Department. College of Science .Baghdad University.
- 15- McFarland, J. (1907). Nephelometer an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. JAMA . (14): 1176-1178.
- 16- Shahidi Bonjar G. H. (2004). Evaluation of Antimicrobial Properties of Iranian Medicinal Plants against *Micrococcus luteus*, *Serratia marcescens*, *Klebsiella pneumoniae* and *Bordetella bronchiseptica*. Asian J. Plant Sci. , 3(1):82-86.
- 17- Rajpal bansal et al., Pharmaceuticle Microbiology.(2009) I edition, Birla Publications PVT.
- 18- Rosamund, M. Baird. Hand book of Microbiology quality control .(2006). CRC press, 1 edition.
- 19- Nwankwo, I.U; Osaro-Matthew; R C and Ekpe, I.N. (2015). Synergistic Antibacterial Potentials of *Citrus aurantifolia* (Lime) and Honey against Some Bacteria Isolated from Sputum of Patients Attending Federal Medical Center Umuahia. Int.J.Curr.Microbiol.App.Sci , 4(4): 534-544.

- 20- Tian, Q., Miller, E. G., Ahmad, H., Tang, L. and Patil, B. S. (2001). Differential inhibition of human cancer cell proliferation by citrus limonoids. *Nutr. Cancer.* , 40 (2): 180-184.
- 21- Emerson, O. H. (1948). The bitter principles of citrus fruit. I. Isolation of nomilin, a new bitter principle from the seeds of oranges and lemons. *J. Am. Chem. Soc.*, 70: 545.
- 22- Poulouse, S. M., Harris, E. D. and Patil, B. S. (2005). Citrus limonoids induce apoptosis in human neuroblastoma cells and have radical scavenging activity. *J.Nutr.*, 135 (4):870-877.
- 23- Wang, Y. C., Chuang, Y. C. and Ku, Y. H. (2007). Quantitation of bioactive compounds in citrus fruits cultivated in Taiwan. *Food Chem.*, 102 (4): 1163-1171.
- 24- Jaiprakash, R. P. (2009). Studies on isolation and characterization of bioactive compounds in lime [*Citrus aurantifolia* (Christm) Swingle], their antioxidant and anticancer properties. PH.D Thesis, University of Agricultural Sciences, Dharwad.
- 25- Cai, Y., Luo, Q., Sun, M., Corke, H. (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci.*, 74: 2157-2184.
- 26- Komutarin, T., Azadi, S., Butterworth, L., Keil, D., Chitsomboon, B., Suttajit, M. and Meade, B.J. (2004). Extract of the seed coat of *Tamarindus indica* inhibits nitric oxide production by murine macrophages in vitro and in vivo. *Food Chem. Toxicol.* 42:649-658.
- 27- Reddy, L. J., Jalli, R. D., Jose, B. and Gopu, S. (2012). Evaluation of Antibacterial & Antioxidant Activities of The Leaf Essential Oil & Leaf Extracts of *Citrus Aurantifolia* . *Asian J. of Biochemica. and Pharmaceutica. Res. Issue* , 2: (2) 2012.
- 28- Chakraborty , A. ; Chandran, A. ; Baht, G.K. (2015). Does Citrus Fruit Juice Have Antibacterial Effect Against Enteric Pathogenic Bacteria? . *INDIAN JOURNAL OF APPLIED RESEARCH*, 2: 23-25.

- 29- Pathan, R. k., Gali, P. R., Pathan, P., Gowtham, T and Pasupulet, S. (2012). In vitro Antimicrobial Activity of Citrus aurantifolia and its Phytochemical screening " Asi. Pacific J. Trop. Dis ., S328-S331.
- 30- Aibinu, I., Adenipekun, T., Adelowotan, T., Ogunsanya, T. and Odugbemi, T. (2007). Evaluation of the antimicrobial properties of different parts of Citrus aurantifolia (lime fruit) as used locally . Afr. J. Trad. CAM., 4 (2): 185 – 190.
- 31- Ouedrhira, W.; Bouhdid, S.; Balouiria, Lalami, A., E. ; Moja, S.; Chahdi, F. O.; Greche, (2015). Chemical composition of Citrus aurantium L. leaves and zest essential oils, their antioxidant, antibacterial single and combined effects. Journal of Chemical and Pharmaceutical Research, 2015, 7(1):78-84