

In vitro* and *In vivo* evaluation of antimicrobial effect of leaves *Ocimum basilicum* of ethanolic extract against *Staphylococcus aureus

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Abstract

The well diffusion method, Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) were implemented to evaluate the antibacterial activity of *Ocimum basilicum* against *Staphylococcus aureus*. The growth of *S.aureus* was inhibited after application of *Ocimum basilicum* at concentrations of 0.34 to 10.96 mg/ml in the test of MIC and MBC. The greater inhibition zones (12.2 ± 0.3 to 20.0 ± 0.2 mm) were observed due to *O. basilicum* at concentrations of 20 to 100 mg/ml in the well diffusion test respectively. *Ocimum basilicum*. Extract enhanced wound healing and disappearance of inflammation signs with regrowth of hair at the site of the experimentally made incision inoculated with *Staphylococcus aureus*. Antibacterial activity of *Ocimum basilicum* extract could be attributed to some active ingredients having the ability to complex with extra cellular and soluble protein and to complex with bacterial cell wall disrupting microbial membranes.

تقييم المستخلص الكحولي لأوراق نبات الريحان ضد جراثيم المكورات العنقودية الذهبية في الزجاج والحي

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الخلاصة

استخدمت تقنياً طريقة الانتشار في الحفر، وحساب التركيز المثبط الأدنى MIC، والتركيز القاتل الأدنى MBC لتقييم نشاط مستخلص نبات الريحان المضاد لبكتيريا المكورات العنقودية الذهبية *Staphylococcus aureus*. أظهرت النتائج تثبيط نمو جراثيم *Staphylococcus aureus* وبتركيز تراوح ما بين 0.34 إلى 10.96 ملغم/ مل من المستخلص الكحولي على التوالي لكل من اختبار MIC و MBC. وسجلت أكبر مناطق تثبيط 12.2 ± 0.3 إلى 20.0 ± 0.2 ملم من نبات الريحان بتركيز من 20 إلى 100 ملغم/ مل من المستخلص الكحولي في طريقة الانتشار من الحفر على التوالي. ساعد المستخلص الكحولي للريحان في الاسراع من التئام الجروح واختفاء العلامات الالتهابية وعودة ظهور الشعر في مناطق الجروح المحدثه والملوثة بجرثومة المكورات العنقودية الذهبية *Staphylococcus aureus* ان خاصية فعالية المستخلص لنبات الريحان المضادة للنمو الجرثومي قد تعزى الى وجود بعض المكونات الفعالة التي تمتلك خاصية تكوين معقد مع البروتينات خارج الخلية والبروتينات الذائبة وبالتالي أحداث معقد مع جدار الخلية مؤدية الى تهتكه.

Introduction

Some medicinal plants have been used for a wide variety of purposes such as food preservation, pharmaceutical alternative medicine, and natural therapies for many thousands of years. It is generally considered that compounds produced naturally, rather than synthetically, will be biodegraded more easily and therefore be more environmentally acceptable. Thus, natural antioxidants, antibacterial, cytotoxic, antiviral, fungicidal agents and nutrients have gained popularity in recent years, and their use and positive image among consumers are spreading. In recent years, multiple drug resistance in both human and plant pathogenic microorganisms have been developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases (1,2) In order to find new therapeutic agents, plants that have antimicrobial activity have attracted attention (3,4,5). *Ocimum basilicum*, commonly known as basil, is naturally distributed in all regions of Iraq. The leaves of this plant are used in traditional cuisine as spices. Basil, which is a popular culinary herb, and its essential oils have been used extensively for many years in flavoring of meats and sausages. Basil oil has also found a wide application in perfumery, as well as in dental and oral antimicrobial products. In addition, because the public nowadays prefers natural food additives, naturally derived antimicrobial agents such as basil, are becoming more important in antimicrobial packaging as they present a perceived lower risk to consumers (6). Leaves and flowering parts of *O. basilicum* are traditionally used externally, when applied for the treatment of acne, disguising of smell, insect stings, snake bites, and skin infections (7). The *Ocimum* oil has been described to be active against several species of bacteria and fungi. These include *Listeria monocytogenes*, *Shigella*, *Salmonella* and *Proteus*, for the fungi *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Cryptococcus neoformans*, *Penicillium islandicum*, and *Candida albicans* (8-12). From recent findings, *O. gratissimum* proved to be useful in the medication for people living with Human Immunodeficiency Virus (HIV), and Acquired Immune Deficiency Syndrome virus AIDs (13). The aim of the present study was to verify the registered benefit of leaves of *Ocimum basilicum* and to determine its antimicrobial activity against the test microorganisms.

Materials and Methods

Ocimum basilicum L. (voucher No. 1281) was selected as a test plant. Fresh plants of *O. basilicum* were collected from Baghdad during 7-8.2010. These plants were identified at Ministry of Agriculture State Board for Seed Testing and Certification (S.B.S.T.C.), All plants were air-dried.

- **Preparation of plant extract:** Dried leaves of the plant were mechanically ground. Plant powder was extracted with ethanol. Aliquots of extract were rinsed for 24 h at room temperature. The extracts were filtered using Whatman filter paper No. 1 and the filtrates were evaporated in incubator at 30 °C. (14). The resultant concentrates were stored in the refrigerator until used.
- **Test microorganisms:** *In vitro* antimicrobial studies were carried out on *Staphylococcus aureus* bacteria were stored on brain heart infusion slant agar in incubator at 37C^o these organisms were obtained from the department of Microbiology of Veterinary Medicine.
- **Part I: *In vitro*.**
- **Determination of antimicrobial activity:** Determination of antimicrobial activity was carried out by employing well diffusion method (15). Fresh cultures of test

microorganism incubated for 24h were used diluted 10^{-7} with sterile peptone water. One hundred microliters of test microorganism suspension containing 1.4×10^7 colony forming units (cfu/ml) of bacteria were used to inoculate the surface of Muller Hinton Agar (Merck) plates. Plant extract was dissolved in Dimethyl sulfoxide (DMSO) to prepare different concentrations (20,40,60,80 and 100) mg/ml. six concentration of Gentamicin (10,20,40,60,80 and 100 mg/ml) in distilled water were prepared to assess its “in –vitro” antibacterial activity. Triplicate samples (20 μ l) of each concentration of the plant extract and standard antibiotic Gentamicin were poured in 6-mm diameter wells in agar plates and incubated at 37 ± 0.1 C $^{\circ}$ for 24 hours. After incubation, the diameter of inhibition zones were measured in (millimeters) on all plates (16). Gentamicin was used as positive control and tested on the same microorganism under the same conditions.

- **Determination of Minimum Inhibitory Concentration (MIC) and (MBC) of *Ocimum basilicum*, Against *Staphylococcus aureus*:** MIC and MBC were determined by using broth dilution assay method (17). In the tube dilution assay. Standard bacterial suspension (1.4×10^7 cfu/ml) was added to tube containing 10ml Nutrient broth with different concentrations (0.0340, 0.680, 1.370, 2.740, 5.480, 10.960 mg/ml). Two sets of tubes containing plant extract (0.0340, 0.680, 1.370, 2.740, 5.480, 10.960 mg/ml) and nutrient broth served as negative control and positive control respectively 24 hr, after incubation at 37 C $^{\circ}$, the tubes were examined for growth. The MIC of the extract was taken as the lowest concentration that showed no growth (18). MBC was determined by culturing one standard loop of the tubes showing no apparent growth on Nutrient both and subsequent incubation at 37 C $^{\circ}$ for 24 hr. The least concentration that showed no colony formation on agar assumed as MBC for this extract.

- **Part II: *In vivo*.**

Nine adult rabbits of both genders were divided into (3) groups (3 rabbits/group), Thirty millimeter long incisions were made using sterile scalpel on one side of the flank area of the animals .The incisions were treated as follows:

- **Group A:** Infected with *S.aureus* suspension containing 1.4×10^7 cell /ml, and treated with Alcohol plant extract (80 mg/gm) (24) hr. after infection for (5) days.
- **Group B:** Infected with *S.aureus* suspension containing 1.4×10^7 cell/ ml, and treated with Gentamicin (24) hr. after infection. For (5) days.
- **Group C:** Infected with *S.aureus* suspension containing 1.4×10^7 cell/ml, and left without treatment.

For this part of the experiment the dried extract of *Ocimum basilicum* leaves was formulated in the form of cream with the aid of emulsified lanoline and the dose of 80mg/gm was chosen according to its reasonable activity shown in part I of the experiment which very closely approached the in-vitro antimicrobial activity of the highest concentration of the standard antibiotic Gentamicin. Gentamicin cream 0.3% was used for topical treatment of wound (standard antibiotic) in this part of the experiment.

Results and Discussion

Many investigations were carried out to discover plant products that inhibit the growth of bacteria like *S.aureus* This bacterial species causes common infections in humans and animals which are difficult to control effectively The pharmaceutical arsenal currently available against it is rather limited (19,20,21), hence, plant products that inhibit its growth without harming the host represent potential therapeutic agent.

- **Part I: *In vitro*.**
- **Well diffusion assay:** The antimicrobial activity of *O. basilicum* (ethanolic) extract against *S.aureus* was examined in the present study and its potency was assessed by the presence or absence of inhibition zones and zone diameter. The results are given in (Table 1 and Fig. 1). The minimum inhibitory concentration of *O. basilicum* against *S. aureus* is presented in (Table 2). Growth and no growth tubes were identified by comparison to the turbidity of the positive control. The inhibited growth was observed after using *O. basilicum* extract at concentrations of 0.34 to 10.96 mg/ml, the growth of *S. aureus*, was found at concentrations of 0.17 mg/ml and less concentrations (Table 2). TSA plates (spot plate) streaked from no growth (negative growth) tubes showed no colonies while those streaked from positive tubes showed colonies of *S. aureus* bacteria. The MBC value of the *O. basilicum* against *S. aureus* bacteria was exactly similar to its MIC value (Table 2), In the present study, the antimicrobial activity of the ethanolic extract of *O. basilicum* against *S. aureus* (Table.1 and Fig.1) was remarkably better than that of the standard antibiotic tested (Gentamicin) (Table.3 and Fig.2). The inhibitory effect of the extract was proportionally related to its concentration. These results, however, fall in agreement with those found by authors in previous studies who tested a variety of standard antibiotics to which *S. aureus* showed obvious resistance (22,23). Ethanolic extract of *O. basilicum* showed stronger antibacterial activity against *S. aureus*. This result was in agreement with (24) who reported that gram negative bacteria are more resistant than gram positive bacteria to the essential oils which have antimicrobial activity Nweze and his coworkers (25) reported in their phytochemical screening of *O. basilicum* the presence of alkaloids, tannins, glycoside, saponin, resins, cardiac glycoside, steroidal terpenes and flavonoids Flavonoids are reported to exhibit antioxidant activity (26) and are effective scavengers of superoxide anions (27). Thus this can significantly affect the cell wall of *S. aureus* which invariably may lead to the collapse of the cell wall affecting the entire metabolisms of the organism. Different concentrations of the ethanolic extract of *O. basilicum* used in the present study (20, 40,60,80 and 100 mg/ml), showed positive proportional inhibitory effect to *S. aureus* growth with diameter of zone of inhibition (12.2 ± 0.3 , 15.0 ± 0.1 , 16.0 ± 0.2 , 18.5 ± 0.11 and 20.0 ± 0.20 mm) respectively (increased concentration causes increased inhibition) with a maximal inhibitory effect of 85%. The antimicrobial activity of *O. basilicum* extract may be attributed to the presence of one or more of the active ingredients exhausted from the plant by ethanol (phenols, flavonoid and tannins). More or less similar findings were reported in works of other authors who tested the antimicrobial activity of *O. basilicum* extract against *S. aureus* (28), other common bacteria like *P. aeruginosa* (29), *E.colli*, *Salmonella paratyphi* and *Shigella dysenteriae* (30) and other pathogenic microorganisms causing diarrhea (31), Even though, there are different species of the *Ocimum* plant which have almost the same antibacterial effect particularly against *S. aureus* and *E.colli* species with comparatively some qualitative and quantitative differences in activity which could be attributed to relative differences in the essential chemical constituent of the certain plant species related to several cultivation (irrigation, fertilization, soil..etc), environmental (temperature and humidity) technical (time of collection, process of extraction part of the plant used...etc.) or even physiological (growth period...etc) factors (32,33,34).

Table (1) Means of diameters of inhibition zone (mm) of *S. aureus* growth by using different concentrations of *O. basilicum*, leaves extract

Conc. (mg/ml) <i>O. basilicum</i>	Diameter of Inhibition zone (mm) (M ± SE)
20	12.2 ± 0.30
40	15.0 ± 0.10
60	16.0 ± 0.20
80	18.5 ± 0.11
100	20.0 ± 0.20

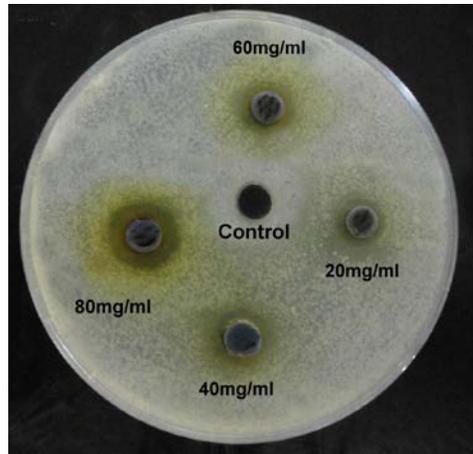


Fig. (1) Inhibition zone of *S. aureus* growth by using different concentrations of *O. basilicum*. leaves ethanolic extract

Table (2) Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of *O. basilicum* ethanolic extract against *Staphylococcus aureus*

Conc. (mg/ml) <i>O. basilicum</i>	MIC	MBC	+ = growth - = No growth
10.960	-	-	
5.480	-	-	
2.740	-	-	
1.370	-	-	
0.680	-	-	
0.340	-	-	
0.170	+	+	
0.085	+	+	
0.043	+	+	

Table (3) Means of diameters of inhibition zone (mm) of *S. aureus* growth inhibited by using different concentrations of Gentamicin

Conc. (ug/ml) Gentamicin	Diameter of Inhibition zone (mm)(M ± SE)
10	9.6 ± 0.10
20	10.0 ± 0.20
40	12.0 ± 0.22
60	14.5 ± 0.10
80	16.2 ± 0.10
100	18.4 ± 0.20



Fig. (2) Inhibition zone of *S. aureus* growth by using different concentrations of Gentamicin (mg/ml)

- **Part II: *In vivo*: Wounds (Open wound).**
- **Clinical signs:** Rabbits of all groups of the present experiment (*in vivo*) with skin incision (30 mm) on the flank area contaminated with (1) ml of *S. aureus* suspension (1.4×10^7) were observed 24 hrs. following contamination. They showed inflammation signs summarized as swelling, redness and warmth of the incised region with the apparent presence of pus (Fig.3). Treatment with formulated plant extract (80 mg/gm) (24 hr). Provoked gradual and remarkable disappearance of these signs (Fig. 4). Treatment with Gentamicin preparation, however, was less effective in this respect and signs of inflammation were still mildly existing at the end of the treatment period 24hr. (Fig.5). Moreover, the animals appeared anorexic and excited. Rabbits treated with the plant extract for (5) days showed complete healing with regrowth of hair without appearance of scar tissue (Fig. 6) in contrast to those treated with Gentamicin and control (untreated) animals (Fig.7). These findings fall in agreement with those of previous studies (35) and suggested that *O.basillicum* increased the formation of granulation tissue, density and activation of fibroblast, keratinization on the surface of the wound, thickness of epidermis and collagen fibers decreasing infection, inflammation, edema and dehiscence (36). Rabbits treated with the plant extract did not show excitement or anorexia; perhaps because active ingredients that cause antimicrobial activity do not have any adverse effect on the central nervous system and their mechanism of action vary depending on the certain phytochemicals involved and intensity of its present (37). This may include mainly enzyme inhibition by the oxidized compounds and act as a source of stable free radical often leading to inactivation of the protein and loss of function. They have the ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls disrupting microbial membranes (38). Some of the active components of the plant may have the ability to intercalate with DNA, formation of ion channels for the microbial proteins to host polysaccharide receptors (39). These results and suggestions are of a significant value that confirms the therapeutic potency of some plants used in traditional medicine and verify reasonability and feasibility of using natural remedies represented by herbal essences and encourage further phytochemical and pharmacological investigation for assessment of alternative therapy for treatment both of human and animal diseases including skin affections and infections (40). These results suggest that *O. basilicum* essential oil could be a beneficial component in preventing infection and enhance wound healing.



Fig. (3) Skin of rabbit, 24 hours after infection with *S aureus* showed inflammation and presence of pus



Fig. (4) Relative disappearance of inflammation signs of wound inoculated with *S. aureus* and treated with *O. basilicum* extract (80mg/ml) for 24 hr. in rabbits



Fig. (5) Existence of inflammation signs on wound with *S. aureus* treated with Gentamicin cream (0.3%) for 24hr. in rabbits



Fig. (6) Regrowth of hair at wound region after treatment with *O. basilicum* extract (80mg/ml) for (5) days in rabbits



Fig. (7) Relatively weak hair regrowth presence of scar tissue at wound region after treatment with Gentamicin (0.3%) for (5) days in rabbits

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