Antimicrobial activity of carnosic acid isolated from *Rosmarinus officinalis* L. leaves

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Abstract
Carnosic acid $C_{20}H_{28}O_4$, the major compound in *Rosmarinus officinalis* L. (Lamiaceae) leaves was isolated and purified through silica gel column chromatography and detected on TLC plates in comparison with standard carnosic acid that served as positive control. Moreover, FTIR spectrometry and HPLC analysis were used to confirm the purity and identity of carnosic acid. The separated acid was investigated for its antimicrobial activity against six selected pathogenic microorganisms: *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and the yeast *Candida albicans*. Carnosic acid at different concentrations (5, 2.5, 1.25 and 0.62 mg/ml) was active against all tested microorganisms. High inhibitory effects were observed against *Bacillus cereus* and *Staphylococcus aureus* (zone of inhibition: 21.7 and 20.3 mm respectively) using the disc diffusion method. The minimal inhibitory concentration (MIC) of carnosic acid was determined using a broth microdilution method in 96-well microtiter plates. MIC values ranged from 15.6 to 125.0 µg/ml, and the highest value was recorded against *Bacillus cereus* and *Staphylococcus aureus* (MIC 15.6 µg/ml), followed by *Escherichia coli*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* respectively. Moreover, carnosic acid observed good antifungal activity against the yeast *Candida albicans* (zone of inhibition range: 11.2-17.4mm; MIC: 125.0 µg/ml).

Introduction
Antimicrobial properties of herbs and spices have been recognized and used since ancient times for food preservation and medicine [1,2]. The use of natural antimicrobial compounds is important not only in the preservation of food but also in the control of human and plant diseases of microbial origin [3].

Rosmarinus officinalis L. (Rosemary), member of the Lamiaceae family is an attractive evergreen shrub with pine needle-like leaves that grows wild in most Mediterranean countries. It's trusses of blue flowers last through spring and summer in a warm, humid environment. It will grow to a height of between 3 and 5 feet. In Iraq the plant grows in the northern provinces. It is used for flavoring food, a beverage drink as well as a fragrant additive in soaps and other cosmetics.

In folk medicine it is believed that the extract of this plant affect the menstrual cycle, relieves menstrual cramps, increase urine flow, reduce kidney pam (for example, from kidney stones), in relieving respiratory disorders, stimulate growth of hair and to support the circulatory and nervous systems [4]. Multiple pharmacological activities such as antimicrobial effects, antiviral activities, antiulcerogenicity, antiturnerogenic and antimutagenesis activities, hyperglycaemic action, diuretic effect, antioxidant effects, hepatoprotective activities and acting as an abortifacient (inducing miscarriage) have been reported for this plant [5]. Moreover, rosemary has tonic stimulant properties, it is used as a pulmonary antiseptic, a choleretic and a colagoguic. It has also stomachic, antidiarrhoic and antirheumatic properties [6].

Rosemary contains a number of potentially biologically active compounds, including antioxidants, such as carnosic acid (the major component in the phenolic diterpenoid fraction from *R. officinalis*) and rosmarinic acid. Other bioactive compounds include camphor (up to 20% in dry rosemary leaves), caffeic acid, ursolic acid, betulinic acid, rosmaridiphenol, rosmanol, saponins, tannins and flavonoids [7].

Extracts of *R. officinalis* have been widely used as a preservative in food industry due to the antioxidant activity and slowing the growth of a number of bacteria that are involved in food spoilage [8]. The present study's goal is to isolate and identify the major compound responsible for the antimicrobial activity using different spectral techniques.

Material and Methods

Chemicals and Reagents
Petroleum ether, ethyl acetate EtOAc, hexane C_6H_{14}, potassium iodide, potassium iodate, starch, vanillin, sulfuric acid H_2SO_4, acetonitrile CH_3CN, acetic acid CH_3COOH, and dimethyl sulfoxide "DMSO" were supplied from BDH Analar (England). Standard carnosic acid (powder) ≥ 91% purity (molecular weight 332.43, molecular formula $C_{20}H_{28}O_4$) and p-iodonitrotetrazolium violet (INT) were obtained from Sigma-Aldrich Chemical Company.

Plant Material
Rosemary leaves were obtained commercially from a local market in Mosul city, Nineveh province, Iraq and identified by an experience botanical taxonomist at college of Agriculture and Forestry, University of Mosul. The leaves were washed first under running tap water, followed by sterilized distilled water and dried at room temperature in dark without applying any heat treatment to minimize the loss of active components, then grinded to powder using an electrical blender (SME GmbH).

Isolation of carnosic acid
A stocking of thin cloth containing 200 g ground rosemary leaves was placed in the extraction container of a Soxhlet extractor. The height of the vegetable mass in the extractor measured 20 cm. The

extractor was placed under an inert atmosphere and the rosemary was extracted with petroleum ether (500 ml: Bp. 40°-60°C.) in a total of 4 filling and siphoning cycles each lasting 75 minutes. The solvent was removed in a rotary evaporator and 20 g of a dark oily extract was collected.

The extract was further dissolved in 150 ml ethyl acetate, separated, and purified through silica gel column chromatography (developing solvent, ethyl acetate : hexane 1:4 v/v). These were recrystallized from hexane to leave pale yellow carnosic acid crystals [9].

Characterization of carnosic acid

Chemical detection

Carnosic acid was detected according to [10]. 5mg of the acid was placed in a test tube, a few drops of 2% potassium iodide solution, 4% potassium iodate solution and 1% of starch solution was added to the test tube resulting a blue positive color.

Thin-layer chromatography (TLC)

The isolated compound was dissolved in appropriate solvent. 5 µl of reference solution of carnosic acid and 5 µl of investigated carnosic acid were applied to silica gel plates, Merck (Germany) 20 × 20 cm, 0.25 mm in thickness. Plates were developed using the solvent system hexane and ethyl acetate (7:3 v/v) and the separated zones were visualized by spraying developed plates with 1% vanillin / H_2SO_4, followed by heating at 100°C for 3 min [11]. Standard carnosic acid served as positive control.

FTIR studies

The IR spectrum of carnosic acid was recorded in the College of Education, Department of Chemistry, University of Mosul, using a computerized Tensor 27 FTIR spectrometer (Bruker Co., Germany) in the range of 400–4000 cm-1 by the KBr pellet technique.

High-performance liquid chromatography (HPLC)

HPLC analysis was performed in College of Science, University of Mosul, using a Shimadzo LC 2010 HPLC system (Kyoto, Japan), equipped with a Shimadzo LC 2010 UV-VIS detector with a thermostatted flow cell and a selectable two wavelengths of 190–370 nm or 371–600 nm. The detector signal was recorded on a Shimadzo LC 2010 integrator. The column used was a C18 block heating-type Shim-pack VP-ODS (4.6 mm interior diameter × 150 mm long) with a particle size of 5 µm. The column was eluted with 2 (v/v)% acetic acid and acetonitrile (45:55 v/v) at a flow rate of 1.0 ml/min, column temperature 25°C. Injection volume was 10 µl and the elute was monitored at 230 nm [9].

Antimicrobial activity

Microbial cultures

Five strains of bacteria and one yeast were used as test microorganisms. The bacterial strains included Gram-positive: *Staphylococcus aureus* and *Bacillus cereus* (due to its medical importance), Gram-negative: *Escherichia coli*, *Salmonella typhimurium* and *Pseudomonas aeruginosa*; and the yeast *Candida albicans*. All microorganisms were clinical isolates, obtained from the Microbiology Laboratory at Department of Basic Science, College of Nursing, University of Mosul, Iraq.

Inoculum preparation

Brain heart infusion broth and Sabouraud dextrose agar (SDA) were used for growing and diluting the microorganism suspensions. Bacterial strains were grown to exponential phase in nutrient broth at 37°C for 6-8 h and adjusted to a final density of 10^8 cfu/ml by diluting fresh cultures and comparison to McFarland density. *C. albicans* was aseptically inoculated on petri dishes containing autoclaved, cooled, and settled SDA medium. The petri dishes were incubated at 31°C for 48 h to give white (creamy) round colonies against a yellowish background. These were aseptically sub-cultured on SDA slants. The yeast colonies from SDA slants were suspended in sterilized 0.9% sodium chloride solution (normal saline), which was compared with McFarland solution. According to the manufacturer's directions, 1 ml of yeast suspension in normal saline was added to 74 ml of sterile medium and kept at 45°C to give a concentration of 2×10^7 cells/ml.

Disc diffusion assay

A modified agar diffusion method was used to determine antimicrobial activity of carnosic acid. Nutrient agar was inoculated with microbial cell suspension (200 µl in 20 ml medium) and poured into sterile petri dishes. Sterile filter paper discs 6 mm in diameter were impregnated with 20 µl of carnosic acid in different concentrations (5, 2.5, 1.25 and 0.62 mg/ml initially prepared by disolving in DMSO and sterilized by filtration through 0.45 µm millipore filters), and placed on the inoculated agar surface. Standard 6 mm discs containing gentamycin 10 µg/disc and amphotericin B 10 µg/disc (Bioanalyse) were used as positive controls. The plates were incubated overnight at 37°C for 18–24 h. In contrast, *C. albicans* was incubated at 31°C for 48 h. and the diameter of any resulting zones of growth inhibition was measured (mm). Each experiment was tested in triplicate [12].

Micro-well dilution assay

Minimal inhibitory concentrations of carnosic acid isolated from *R. officinalis* was determined based on a microdilution method in 96 multi-well microtiter plates as previously described [13]. Briefly, bacterial strains were cultured overnight at 37°C on nutrient broth and adjusted to a final density of 10^8 cfu/ml, and used as an inoculum. Carnosic acid was dissolved in dimethyl sulfoxide and then in nutrient broth to reach a final concentration of 500.0 µg/ml. Serial twofold dilutions were made in a concentration range from 7.8 to 500.0 µg/ml. In each microtiter plate, a column with broad-spectrum antibiotic was used as positive control (gentamycin in serial dilutions 7.8-500.0 µg/ml). As an indicator of bacterial growth, 50 µl of 0.2 mg/ml p-iodonitrotetrazolium chloride (INT)

was added to the wells and incubated at 37°C for 30 min. The lowest concentration of compound showing no growth was taken as its minimal inhibitory concentration MIC. The colorless tetrazolium salt acts as an electron acceptor and is reduced to a red-colored formazan product by biologically active organisms. Where bacterial growth was inhibited, the solution in the well remained clear after incubation with INT [14].

As for *C. albicans*, a simple turbidity test was used to determine the MIC value of carnosic acid. A volume of 0.1 ml from each serial dilution of carnosic acid concentrations (7.8-500.0 µg/ml) was added into tubes containing 9.8 ml of sterile nutrient broth, and then the tubes were inoculated with 0.1 ml of yeast suspension and incubated at 31°C for 48 h. Amphotericin B (7.8-500.0 µg/ml) was used as a positive control. The optical density was determined using a SERIES CECIL 1021, 1000 spectrophotometer at 630 nm. The MIC value was the lowest concentration of compound that showed no growth after 48 h of incubation in comparison with the control tube, which included 9.8 ml of nutrient broth and 0.1 ml of yeast suspension in addition to 0.1 ml of each compound concentration (unincubated) [15].

Results

The present study was conducted to isolate the main bioactive compound from *R. officinalis*. Carnosic acid was isolated from the leaves, and then detected on TLC plates in comparison with standard carnosic acid. An orange zone with a retention factor (R_f) value of 0.8 was identified as carnosic acid in comparison with standard carnosic acid that had the same R_f value. The FTIR spectrum confirmed the material isolated from *R. officinalis* leaves as carnosic acid (Figure 1). Significant peaks were found at: 1728 cm^{-1} that corresponds to carbonyl group of carboxylic group; 1163 cm^{-1} attributes to (C–O) bond of hydroxyl group in conjugation with phenyl ring; 1377 cm^{-1} refers to [–C$(CH_3)_2$] group; 3034 cm^{-1} ascribes to the aromatic system and 2958-2925 cm^{-1} corresponds to the methyl group. Moreover, carnosic acid was characterized using the HPLC system (Figure 2) and identified by comparing its retention time (t_R) and UV spectrum with that of the standard compound. The retention time 30–31 min and UV spectra of the isolated compound on HPLC were completely identical to that of standard carnosic acid.

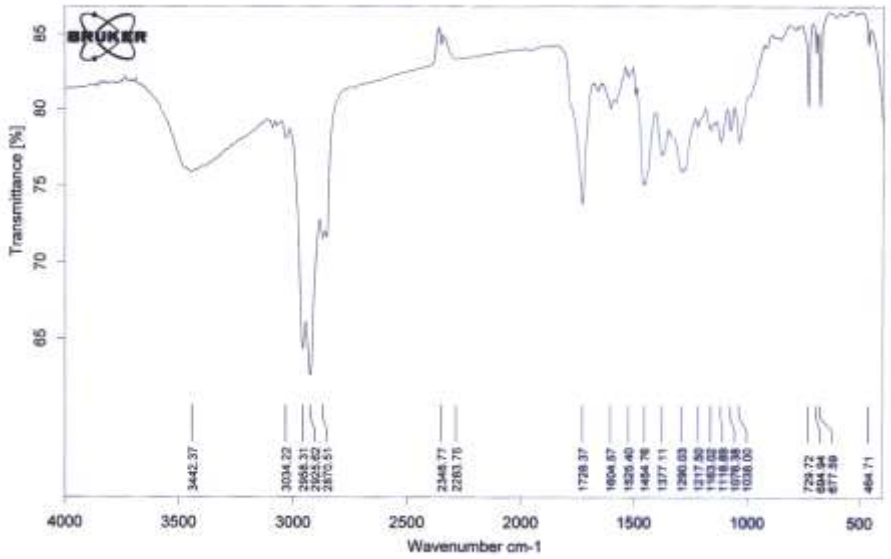

Figure (1): FTIR spectra of carnosic acid isolated from *R. officinalis* leaves.

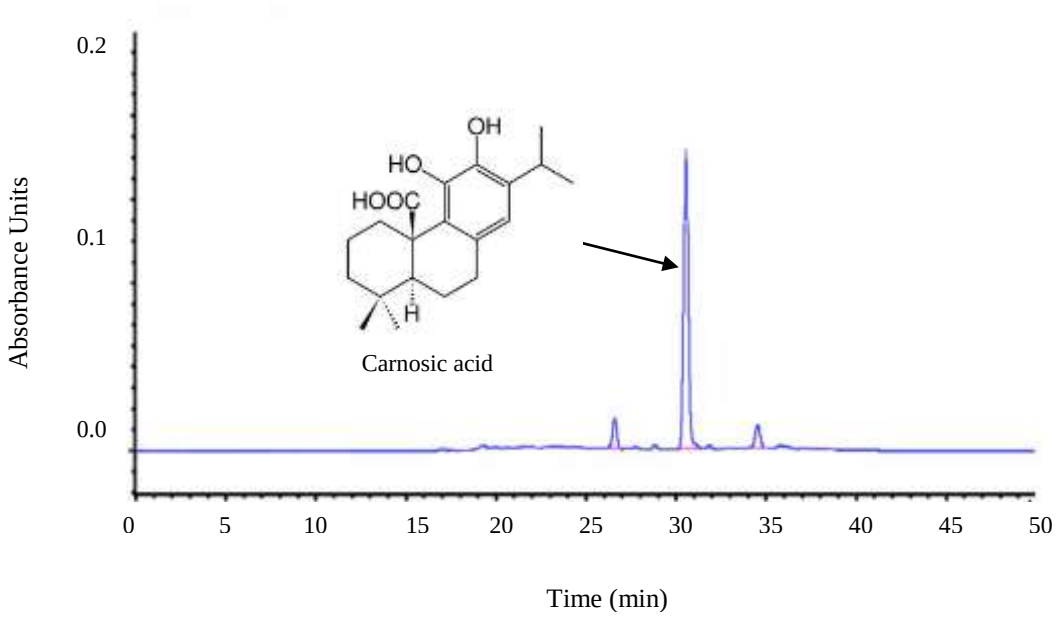

Figure (2): HPLC chromatogram of carnosic acid isolated from *R. officinalis* leaves.

The isolated acid was investigated for its antimicrobial activity against five bacterial species and one yeast. The initial screening of antibacterial activity of carnosic acid was assayed *in vitro* by the agar diffusion method using four concentrations (5, 2.5,1.25 and 0.62 mg/ml). All carnosic acid concentrations were active against all tested bacteria (Table 1). The highest inhibitory effect was observed against *B. cereus* (zone of inhibition: ٢1.7mm) using the concentration 5 mg/ml, while the weakest activity was demonstrated against *P. aeruginosa* (zone of inhibition: ٨.2mm) using the concentration 0.62 mg /ml.

Table (1): Antimicrobial activity of carnosic acid isolated from *R. officinalis* leaves.

Microorganisms	Zone of inhibition (mm)					
	Carnosic acid concentrations (mg/ml)				Control (µg/disc)	
	5	2.5	1.25	0.62	G	A
S. aureus	20.3	18.7	16.5	14.3	20.9	N.T
B. cereus	٢1.7	19.9	17.7	15.3	22.5	N.T
E. coli	17.2	15.7	13.4	11.9	18.3	N.T
S. typhimurium	17.1	15.2	12.6	10.2	19.2	N.T
P. aeruginosa	١٤,٤	12.1	١٠,٥	٨,٢	15.2	N.T
C. albicans	17.4	15.5	13.3	١1.2	N.T	12.4

G: Gentamycin (10 µg/disc), A: Amphotericin B (10 µg/disc), N.T: Not tested

In view of the study results obtained by the disc diffusion method, the minimal inhibitory concentration MIC of carnosic acid was determined by broth microdilution assay (Table 2). The highest MIC value (15.6 µg/ml), was observed against *S. aureus* and *B. cereus*, while *E. coli* ranked next (MIC 31.2 µg/ml) followed by *S. typhimurium* (MIC 62.5 µg/ml) and finally, *P. aeruginosa* with a MIC value of ١٢٥.0 µg/ml. Moreover, carnosic acid observed good antifungal activity against the yeast *C. albicans* (zone of inhibition range: ١1.2–17.4 mm; MIC: 125.0).

Table (2): Minimum inhibitory concentration (MIC) of carnosic acid isolated from *R. officinalis* leaves.

Microorganisms	MIC values (μg/ml)		
	Carnosic acid	Control	
		G	A
S. aureus	15.6	7.8	N.T
B. cereus	15.6	7.8	N.T
E. coli	31.2	7.8	N.T
S. typhimurium	62.5	7.8	N.T
P. aeruginosa	١٢٥.0	15.6	N.T
C. albicans	125.0	N.T	7.8

G: Gentamycin, A: Amphotericin B, N.T: Not tested.

The standard drug gentamycin was active against all reference bacteria (zone of inhibition range: 15.2–22.5 mm; MIC range: 7.8–15.6 μg/ml). In addition, amphotericin B demonstrated good antifungal activity against *C. albicans* (zone of inhibition: 12.4 mm; MIC: 7.8 μg/ml).

Discussion

There has been an increasing interest in the use of plants and natural products, such as tocopherols, flavonoids and rosemary extracts for the preservation of food materials in recent years [16,17]. Plants, including herbs and spices, have many phytochemicals which are potential sources of natural antioxidants, anti-inflammatory, anticancer and antimicrobials [18].

In the present study, different physical methods were employed to characterize carnosic acid. Among them, infrared spectra and HPLC indicated the absolute purity of the isolated material. The retention time and UV spectra of the isolated material on HPLC were completely identical to that of the standard. HPLC is the most widely used qualitative and quantitative determination and separation method. The method is popular because it is non-destructive and may be applied to thermally labile compounds (unlike GC); it is also a very sensitive technique since it incorporates a wide choice of detection methods [19].

Rosemary plants are rich sources of phenolic compounds with high antioxidative and antimicrobial properties, but their antimicrobial activities have not been deeply characterized [20]. On the basis of results obtained with agar diffusion and micro-well dilution methods, it was noticeable that carnosic acid in the present study inhibited the growth of all tested microorganisms. The zones of inhibition ranged from ٨,٢–21.7 mm and ١1.2–17.4 mm in diameter against *C. albicans* using the disc diffusion method. Furthermore, MIC values ranged from 15.6–١٢٥.0 μg/ml against tested bacteria and 125.0 μg/ml against *C. albicans*. Generally, plants possessing strong antimicrobial properties against pathogens contain a high percentage of phenolic compounds [21]. It seems reasonable that their mechanism of action would therefore be similar to other phenolics; this is generally considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force (PMF), electron flow, active transport and coagulation of cell contents [22].

Candida albicans remains the most common medically important fungi; about 45% of clinical fungal infections are caused by *C. albicans* [23]. The present study showed that carnosic acid had potent anti-*C. albicans* activity. The action mechanism of the antifungal phenolic diterpenoids is not known so far but may lie in damage to the membrane and leakage of cellular materials, ultimately leading to cell death [24].

In view of the results obtained using both disc diffusion and micro-well dilution assays, carnosic acid was found active against both Gram-positive and Gram-negative bacteria. Although that Gram-negative organisms were slightly less susceptible to the action of carnosic acid since they possess an outer membrane surrounding the cell wall [25] which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering [26]. Moreover, the weakest activity was observed against *P. aeruginosa* using both antibacterial methods. Several studies have reported that the Gram-negative bacteria, Pseudomonas, and in particular *P. aeruginosa*, appear to be least sensitive to the action of natural products, plant extracts and essential oils [27,28]. Bacterial resistance can result from modification of antibacterial's target or from bypassing of that target, or it can be contingent on impermeability, efflux, or enzymatic deactivation. Some bacteria are inherently resistant. In others, resistance may arise in hitherto susceptible organisms via mutation or horizontal gene transfer by plasmids, transposons and lysogenic bacteriophage [29].

The study can conclude that carnosic acid isolated from *R. officinalis* leaves had strong antimicrobial activities against six different pathogenic microorganisms and that carnosic acid is very much responsible for the antimicrobial activity of rosemary leaves. Additional *in vivo* studies and clinical trials would be needed to justify and further evaluate the potential of this compound as an antimicrobial agent in topical or oral applications. It is also recommended that more safety studies should be carried out before active substances are more widely used or at greater concentrations in food preservation.

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الفعالية ضد الميكروبية لحامض الكارنوسيك المفصول من أوراق نبات إكليل الجبل *Rosmarinus officinalis* L.

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الملخص

تضمنت الدراسة الحالية فصل وتنقية حامض الكارنوسيك $C_{20}H_{28}O_4$ ، المركب الرئيسي الموجود في أوراق نبات إكليل الجبل *Rosmarinus officinalis* L. باستخدام كروماتوكرافيا عمود الفصل، والكشف عنه باستخدام طريقة كروماتوكرافيا الطبقة الرقيقة TLC بالمقارنة مع عينة السيطرة (Carnosic acid). فضلا عن ذلك تم التأكد من نقاوته وماهيته باستخدام مطياف الأشعة تحت الحمراء FTIR و تحليل كروماتوكرافيا السائل عالي الكفاءة HPLC. تم التحري عن الفعالية البايولوجية للحامض المفصول ضد ستة أنواع مختارة من الأحياء المجهرية المرضية: *Staphylococcus aureus*، *Bacillus cereus*، *Escherichia coli* ، *Salmonella typhimurium* ، *Pseudomonas aeruginosa* و الخميرة *Candida albicans*. أظهرت المادة المفصولة فعالية تثبيطية ضد كل الأحياء المجهرية قيد الدراسة عند التراكيز (٥، 2.5، 1.25 و 0.62 ملغم/مل)، ولوحظ أعلى تثبيط ضد بكتريا *Bacillus cereus* و *Staphylococcus aureus* (قطر التثبيط: 21.7 و 20.3 ملم على التوالي) باستخدام طريقة الانتشار بالأقراص. تم التحري عن التركيز المثبط الأدنى (MIC) لحامض الكارنوسيك باستخدام طريقة الـ Microdilution assay وبمعدل تركيز مثبط تراوح بين (15.6 – 125.0 مايكروغرام/مل). لوحظ أن أعلى قيمة تثبيط كانت ضد بكتريا *Bacillus cereus* و *Staphylococcus aureus* عند التركيز (15.6 مايكروغرام/مل)، تلتهما كل من *Escherichia coli*، *Salmonella typhimurium* و *Pseudomonas aeruginosa* على التوالي. إضافة إلى ذلك، اظهر حامض الكارنوسيك تأثيرا تثبيطيا جيدا ضد الخميرة *Candida albicans* وبقطر تثبيط تراوح بين (11.2–17.4 ملم) وبتركيز مثبط أدنى عند (125.0 مايكروغرام/مل).