

Study of Phytochemical Composition and Antibacterial Activity of *Emblica officinalis* (Amla) Fruit Extract

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Abstract

In present study we focused our interest on phytochemistry and antibacterial activity of plant against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* isolates. TLC screening of phytochemical analysis showed a content of Emblicanin compounds, Gallic and Ellagic acids, Quercetin, Chebulinic and Chebulagic acids. Results showed antibacterial activity of E.O extract against *Staph aureus*, *Klebsella pneumonia* and *Pseudomonus aurginosa* at different concentrations. The highest inhibition zone was recorded for *S. aureus* bacteria ranged from 16 ± 0.57 to 33.3 ± 0.3 at 10 mg/ml to 100 mg/ml concentrations respectively in comparison with inhibition zones which were recorded for *K. pneumonia* 11.3 ± 0.6 mm. to 28 ± 0.06 mm. and *P. aeruginosa* 10.6 ± 1.2 to 22.6 ± 1.2 mm. at same concentrations.

دراسة التركيب الكيميائي والفعالية البكتيرية لخلصة فاكهة الاملا

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الخلاصة

ركزت هذه الدراسة على فعالية مركبات فاكهة الاملا في تثبيط نمو بعض العزلات البكتيرية وباستخدام تراكيز مختلفة من المستخلص النباتي المحضر من هذه الفاكهة التحليل الكيميائي لفاكهة الاملا بطريقة أستشراب الطبقة الرقيقة أظهرت محتوى عال من مركبات التانينات، القلويدات بالإضافة إلى الفلافونيدات. بينت النتائج امتلاك المستخلص تأثيرا تثبيطيا واضحا على نمو العزلات البكتيرية المستخدمة في الدراسة حيث تم تسجيل أعلى منطقة تثبيط في عزلة *S. aureus* تراوحت بين 16 ± 0.57 ملم إلى 33.3 ± 0.3 ملم وبتركيز 10 ملغم/مل و 100 ملغم/مل على التوالي مقارنة بتلك المسجلة في عزلتي (*K. pneumonia*) 11.3 ± 0.6 ملم إلى 28 ± 0.06 ملم و (*P. aeruginosa*) 10.6 ± 1.2 ملم إلى 22.6 ± 1.2 ملم باستخدام نفس التراكيز.

Introduction

Plants produce wide array of bioactive molecules or phytochemical which probably evolved as chemical defense against predation or infection but, are now found to be useful for treatment of various ailments. Due to the myriad of potential benefits they posses, plants have been widely exploited in traditional medicine and their curative potentials are well documented. The clinical efficacy of many existing antibiotics is being threatened by rapid emergence of multidrug resistant pathogens (1). Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind (2). There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structure and mechanisms of action for new and re-emerging infectious diseases (3). The present study was carried out to identify the antibacterial property of *Emblica officinalis* (E.O) as well as its phytochemical composition against some isolated pathogens, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia*. E.O commonly known as alma belongs to *Ephorbiaceae* widely distributed in the most of tropical and subtropical countries. Early studies have demonstrated potent antibacterial activity against a wide range of bacteria used traditionally in treatment of respiratory diseases (4). Beside

antibacterial activity, E.O. used for its anti-inflammatory, analgesic and antipyretic properties (5), hepatoprotective (6), antioxidant (7) and anti-tumor activities (8) the fruit is rich with Tannins (ambilicanins compounds), Alkaloids (phylantidine and phylantine), Flavanoids (Campherol 3-L α 6 ethyl, Campherol 3-L α 6 methyl, quercetin, chebulinic acid and chebulagic acid) (9).

Material and Methods

Plant Material: Dried fruit of E.O were purchased from a local market in Baghdad and identified in the National Herbarium at Abu-Graib.

Preparation of E.O Extract: The dried fruits of E.O. were infused with 70% ethanol (BDH, England) for six days, then on the 7th day, the hydro-alcoholic infusion of E. O. was heated and stirred with hotplate stirrer for 24 hrs, then the infusion were evaporated with room temperature and the resultant extract were kept tightly till use (10).

Preparation of Micro Organism: The following micro organisms were used: *Staphylococcus aureus*, *Psuedomonas aeruginosa* and *Klebsiela pneumoniae* locally isolated from Preventive and Internal Medicine Department of Veterinary Medicine College of Baghdad University. Bacteria were diagnosed depending upon standard morphological and physiological characteristics (Gram staining, and hemolytic zone on blood agar). Biochemical tests (Catalase, Coagulase, Oxidase , IMViC).

The obtained cultures were brought to the laboratory and subcultured on brain heart infusion media. After 24 hour of incubation at 37C° the cultures were preserved aseptically in refrigerator until further use.

Phytochemical Analysis of E.O Extract: A small portion of dried extract was used for the phytochemical analysis (11, 12). Mayer's reagent, Hager's reagent, Wagner's reagent were used to test Alkaloids, Ferric Chloride for Tannins, Trill-Hill reagent for Eridoids, Benedictridoids, Benedict's solution for testing Saponins (Frothing Test).

Thin Layer Chromatography Analysis (TLC): The preliminary analysis was conducted with TLC according to the method developed by (13). TLC plate used was silica gel 60F254 coated with aluminum. E.O sample was dissolved in 96% ethyl alcohol and the aliquots were applied to the plate with micropipette. Two mobile phases were used, containing different concentrations of toluene, ethyl acetate and formic acid 5: 4: 1, V/V/V and 3.6: 1.2: 1.5, V/V/V respectively (14). The mobile phase used for TLC in the present sample was toluene: ethyl acetate: formic acid 5: 4: 1, V/V/V because it's best resolution. The TLC chamber was saturated with the mobile phase at least 1 hour before analysis. The developed plates were air dried and heated for 10 minutes at 110C° to facilitate the development of spots. The phenolic acids and flavonoids were visualized under long 366 nm. And short 254 nm. Ultra violet lights before and after spraying with reagent 1% W/V methanolic solution of diphenylboric acid aminoethyl ester followed by 5% V/V ethanolic solution of poly ethylene glycol 4000. The position of the spots on TLC plate was expressed as the retention factor R. F. The distance of the components travelled divided by the distance solvent travelled from the base (15).

Preparation of Inoculums: All bacteria were cultured for 8 hours at 37C° in a liquid medium (brain heart infusion) and were used as inoculums. The turbidity of the suspension was adjusted to the Mcfarland 0.5 turbidity standard (16).

Antibacterial Activity of the Extract: Screening of antibacterial activity was preformed by well diffusion technique (17). The Muller Hinton Broth (MHB, Merck) plates were seeded with 0.1 ml of standardized inoculums of each tested organism incubated at 37C° for 24 hour. Final inoculums was adjusted to cfu ml⁻¹ with reference to Mcfarland turbidometry. The inoculums was spread evenly over the plates with loop or sterile glass spreader. A standard cork borer of 8 mm diameter was used to cut

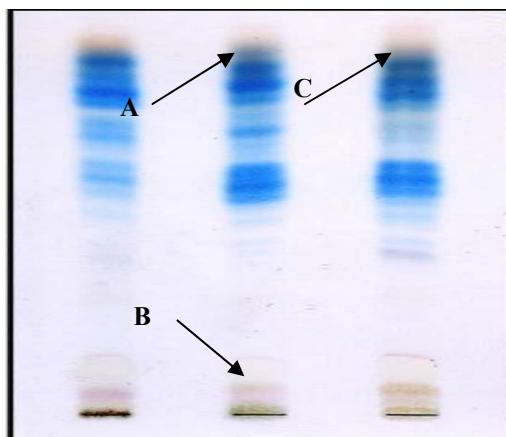
uniform wells on the surface of MHB and 100 ml, 80 ml, 60 ml, 40 ml, 20 ml and 10 ml of the extract was introduced in well and distilled water was taken as a negative control. The plates were incubated at 37°C for 24 hour and examined for zones of complete inhibition to the nearest mm. The experiment was done three times and mean values were calculated.

Antibiotic sensitivity: The plates were prepared as mentioned above. The antibiotic discs of Clindomycin 2 mg, Erythromycin 15 mg, Ampicilin 10 mg, Streptomycin 10mg, Rifampin 5 mg, Imperem 10mg, Cefotaxime 30 mg, Trimthoprim 5mg and Novobiocin 30mg were used. All used antibiotics were produced by Difco. The plates were incubated at 37°C for 24 hour and examined for zones of complete inhibition to the nearest mm. The experiment was done three times and the mean values were calculated.

Statistical Analysis: All data were analyzed by using SPSS and to compare between means Duncan Multiple Test was used (18).

Results

The results showed a highly antibacterial activity of E.O extract against some isolates as shown in Pic. (1, 2, 3). The highest inhibition zone (33.33 ± 0.33 mm, 28.00 ± 0.06 mm, 22.6 ± 1.20 mm) was recorded against *Staph aureus*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* respectively in disc diffusion method from extract at a concentration of 100 mg/ml, while inhibition zone diameters increased proportionally with using each of 20 mg/ml, 40 mg/ml, 60 mg/ml and 80 mg/ml concentrations against same tested isolates and this increase was statistically significant at ($p < 0.01$) except for those diameters that belong to *Pseudomonas aeruginosa* where as showed statistically insignificance between 20 mg/ml and 40 mg/ml concentrations at ($p < 0.01$). The lowest inhibition zone (16.0 ± 0.57 mm, 11.3 ± 0.6 mm, 10.6 ± 0.3 mm) was recorded against the same tested isolates above from extract at a concentration of 10 mg/ml. as shown in Table (1). Ethanolic extract of E.O was effective as well as antibacterial standard imperem, streptomycin and erythromycin on *S. aureus*, *K. pneumonia*, *P. aeruginosa* with significant differences at ($p < 0.01$) all of the tested isolates were resistant to clindomycin, ampicilin and cefotaxime as shown in Table (2). The TLC screening of phytochemical analysis of the extract showed a content of Flavonoids (kaempferol), Alkaloids (quericitin) and Tannins (emblicanin A&B) were indicated by spots of different distances on the TLC plate upon spray by chemical reagent as shown in Fig.(1).



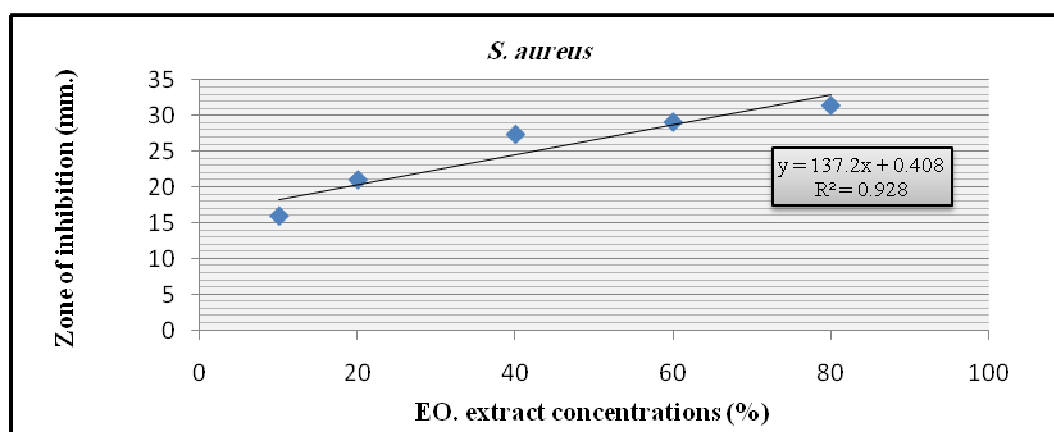
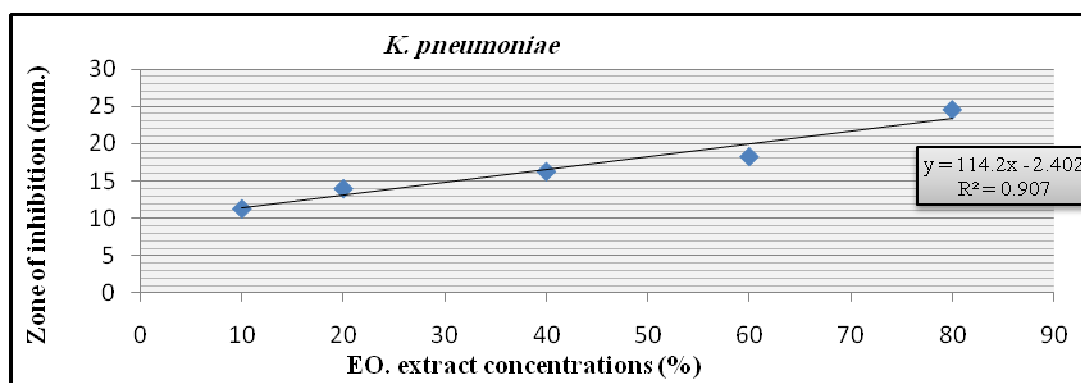
$$R.F = \frac{\text{Distance of the spot from the base}}{\text{Distance of the solvent moved from the base}} = 1.8 \text{ mm.}$$

Fig. (1) TLC pattern of E.O extract: (A) Tannins (emblicanin A & B), (B) Flavonoids (kaempferol), (C) Alkaloids (quericitin)

Table (1) Diameter of mean (triplicate) zone of inhibition (mm)						
Bacterial Spp.	Concentrations of E. O.					
	10%	20%	40%	60%	80%	100%
<i>S. aureus</i>	16±0.57 F a	21±0.57 E a	27.3±0.6 D a	29±0.57 C a	31.3±0.6 B a	33.3±0.3 A a
<i>K. pneumoniae</i>	11.3±0.6 F b	14±0.57 E b	16.3±0.3 D b	18.3±0.3 C b	24.6±0.3 B b	28±0.06 A b
<i>P. aeruginosa</i>	10.6±0.3 E b	12±0.06 D c	12.6±0.3 D c	15.3±0.3 C c	18±0.57 B c	22.6±1.2 A c
Capital letters denoted that significant differences between concentrations at (p<0.01) while small letters denoted that significant differences between isolates at (p<0.01).						

Table (2) Diameter of mean (triplicate) zone of inhibition (mm)

Bacterial Spp.	Antibacterial					
	Imperem µg	Streptomycin µg	Erythromycin µg	Clindamycin µg	Ampicillin µg	Cefatoxime µg
<i>S. aureus</i>	32	15	13	-	-	-
<i>K. pneumoniae</i>	26	8	17	-	-	-
<i>P. aeruginosa</i>	31	8	18	-	-	-

Fig. (2) Relationship between (E.O). concentrations (%) and zone of inhibition (mm) for *S. aureus*Fig. (3) Relationship between (E.O). concentrations (%) and zone of inhibition (mm) for *K. pneumoniae*

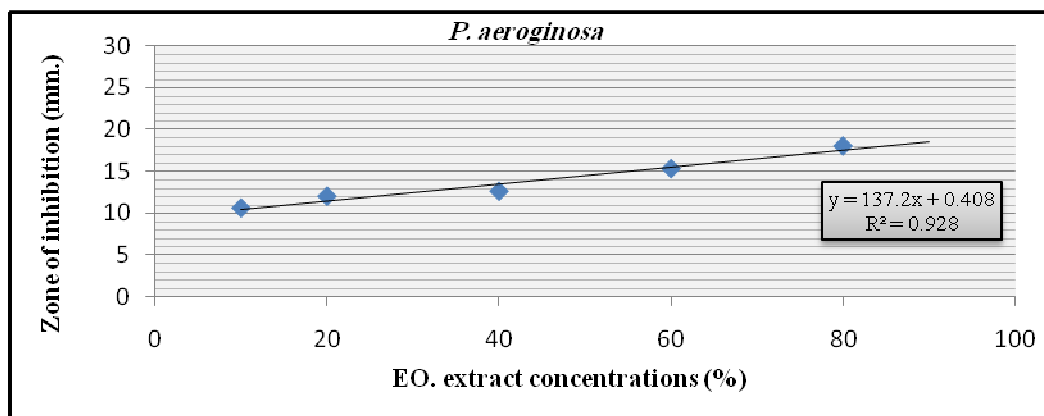


Fig. (4) Relationship between (E.O). concentrations (%) and zone of inhibition (mm) for *P. aeruginosa*



Pic. (1) Inhibition zones resulted from using different concentrations of E.O extract against *S. aureus*



Pic. (2) Inhibition zones resulted from using different concentrations of E.O extract against *P. aeruginosa*



Pic. (3) Inhibition zones resulted from using different concentrations of E.O extract against *K. pneumoniae*

Discussion

Antibacterial activity of Amla (E.O) extract on Gram negative and Gram positive bacteria have been studied earlier (19). The possible reason for this activity can be attributed to the presence of flavonoids in Amla composition, which are phenolic structures their antibacterial activity is probably due to their ability to form a complex with extracellular and soluble proteins or with bacterial cell walls which disrupts bacterial membranes (20). Results of antibacterial study of E.O extract showed that all used isolates were sensitive to the extract at different concentrations. The highest inhibition zone was recorded for Gram positive *S. aureus* bacteria ranged from (16 ± 0.57 to 33.3 ± 0.3) at concentration of 10 to 100 mg/ml and this can be attributed to the flavonoids compounds which have the ability to inhibit RNA synthesis, Authors suggested that B-ring of flavonoids may play a role in intercalation or hydrogen bonding with the stacking of nucleic acid bases and this may explain the inhibitory action on DNA or RNA synthesis (21). Also hydrosable tannins (A&B) as well as alkaloids as active constituents with a significant in vitro antimicrobial properties and this agree with (22, 23). This study showed that E.O extract has a huge impact on Gram positive bacteria rather than Gram negative bacteria and this may be ascribed to the chemical composition of Gram negative bacteria which is more complex than Gram positive bacteria (24). TLC analysis was observed mainly because the presence of most active compounds such as Kaempferol, Quercetin, Emblicanin A & B, Phyllanthidine and Phyllantine which have a highly inhibitory activity against *S. aureus* bacteria (21) and (25).

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