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Antibacterial activity of Fenugreek essential Oil against *Pseudomonas aeroginosa*: *In vitro* and *in vivo* Studies

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Abstract

Background Multiple drugs resistance has increased due to the random use of available antimicrobial drugs in treatment of infectious diseases.

Objective To investigate probable antibacterial effects of fenugreek essential oil extract against *Pseudomonas aeruginosa*

Methods Twenty eight isolates of *P. aeruginosa* were collected from skin infected patients in Al- Yarmook teaching hospital in Baghdad. Antimicrobial susceptibility tests of 14 antibiotics were performed using Vitek2 compact system. The antibacterial activity of essential oil was evaluated using agar well diffusion method with minor modifications**.** The broth micro dilution method was used to determine minimum inhibitory concentration. Then animal experiment was performed in five groups of mice (n=7, for each) as following: control, induction, treated with fenugreek alone, treated with Gentamycin alone, treated with combination of fenugreek and Gentamycin. Then histopathological examination was done after seven days of the treatment.

Results *P. aeruginosa* isolates are highly resistance to trimethoprime/ sulfamethaxazole, while sensitive to amikacin. Minimum inhibitory concentration of fenugreek essential oil for highly resistance *P. aeruginosa* isolates (n=10) as followed: 6 isolates with minimum inhibitory concentration = 1.2gm/100µl, and 4 isolates with MIC= 0.6gm/100µl**.** Minimum inhibitory concentration of gentamycin was equal to>=16.

Conclusion Fenugreek essential oil has higher antibacterial effect alone and in combination with gentamycin than gentamycin alone.

Keywords Antibacterial activity, Fenugreek, *Pseudomonas aeruginosa.*

List of abbreviation: FICs = Fractional inhibitory concentration values, MDR = multidrug resistance, FLC = Fast Liquid Chromatography, MIC = minimum inhibitory concentration.

Introduction

Multiple drugs resistance has increased due to the random use of available antimicrobial drugs in treatment of infectious diseases. Bacteria are more common on normal skin than other microorganisms [(1,2)]. A number of antimicrobial agents, including a number of Beta–lactams are active against *P. aeruginosa.* Extended-spectrum penicillins often used to treat infections caused by this bacterium. Although most cephalosporins are not active against *P. aeruginosa* [(3)]. Of the carbapenems, meropenem has slightly better activity against *P. aeruginosa* than imipenem-cilastatin. The fluoroquinolones have the advantage of being exists in both oral and intravenous formulations and are thus attractive options for treating *P. aeruginosa* infections in the outpatient setting. Of the fluoroquinolones agents, Ciprofloxacin is the most active against *P. aeruginosa*. In

conclusion, the aminoglycosides have been mainstays in the treatment of these infections [4].

The aim of the study is to investigate probable antibacterial effects of fenugreek essential oil extract against *P. aeruginosa* in sample from skin infections and detection of active component behind these antibacterial effects, and to study the combination effect of fenugreek essential oil with gentamycin against *P. aeruginosa*.

Methods

Isolation and detection of *P. aeruginosa*

All specimens were diagnosis microscopically (Gram stain), morphorgically and biochemically according to standard methods [5,6], and some biochemical tests were achieved bycommercial kits (GN VITEK2 gram negative colorimetric identification kit) for *P. aeruginosa* bacteria (BioMerieux, France).

Antibiotics susceptibility

Antibiotics susceptibility tests by using the Biomérieux VITEK2 compact system (BioMerieux, France) against the following antibiotics: pipracillin, pipracillin- tazobzctum, ticarcillin, ticarcillin/ clavulonic acid, ceftazidim, cefipim, imepenem, meropenem, amikacin, tobramycin, gentamycin, trimethoprim / sulfamethaxazole, and ciprofloxacin.

Extraction of fenugreek essential oil

Fenugreekessential oil is extracted from seeds of plant (supply from local markets in Baghdad). The oil is extracted by steam distillation methods from dried plant and yield 1.2gm for fenugreek for each 100gm of plant materials [7].

Assessment of antibacterial activity of fenugreek essential oil

It was evaluated using agar well diffusion method with minor modifications [8]. The broth micro dilution method was used to determine minimum inhibitory concentration (MIC). All tests were performed in muellerhinton broth Salucea (Netherlands) supplemented with Tween 80 (BDH (England) at a final concentration of 0.5% (v/v) [9]. Fractional inhibitory concentration values (FICs) for antimicrobials combinationswas used to determine the effect of antimicrobials combinations on multidrug resistance (MDR) isolates of bacteria. FIC values used to assess the synergism between gentamycin with fenugreek essential oil for *P. aeruginosa* [10].

Separation active ingredient of fenugreek essential oil

Separation of active ingredients of fenugreek essential oil was done by Fast Liquid Chromatography (FSL) (Shimadzu, North America) equipped with binary delivery pump model 2010, using 3µ particle size column (50 × 4.6 mm H.D) C-18 (Injection 10 µl of essential oil in column), Mobile phase: 0.01M ammonium phosphate buffer (BDH, England) A: acetoitrile B (BDH, England). Eluted by linear gradient from 0-100% B in 10 min. Detection of eluted peak were monitored by UV-Vis spectrophotometer (Cecil, France) set at 254nm, flow rate 1.0 ml/min, temperature 30˚C [11] (Fig. 1).

Animals experiment (*In vivo* method)

Thirty five healthy, domestic male mice, weighing 23-25 gram were used in this study; they were obtained from animal house in High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, in the period between Aug. 2014 and Oct. 2015. These mice were kept in separated cages; the room temperature was maintained at 20 -25^{o}C.

Animals grouping

The mice randomly divided into five groups (n=7, each) according to following:

Group 1 (control): control group infected just by phosphate buffer saline (China).

Group 2 (induction group): infected by bacteria without treatment

Group3: treated with fenugreek essential oil alone for 7 days.

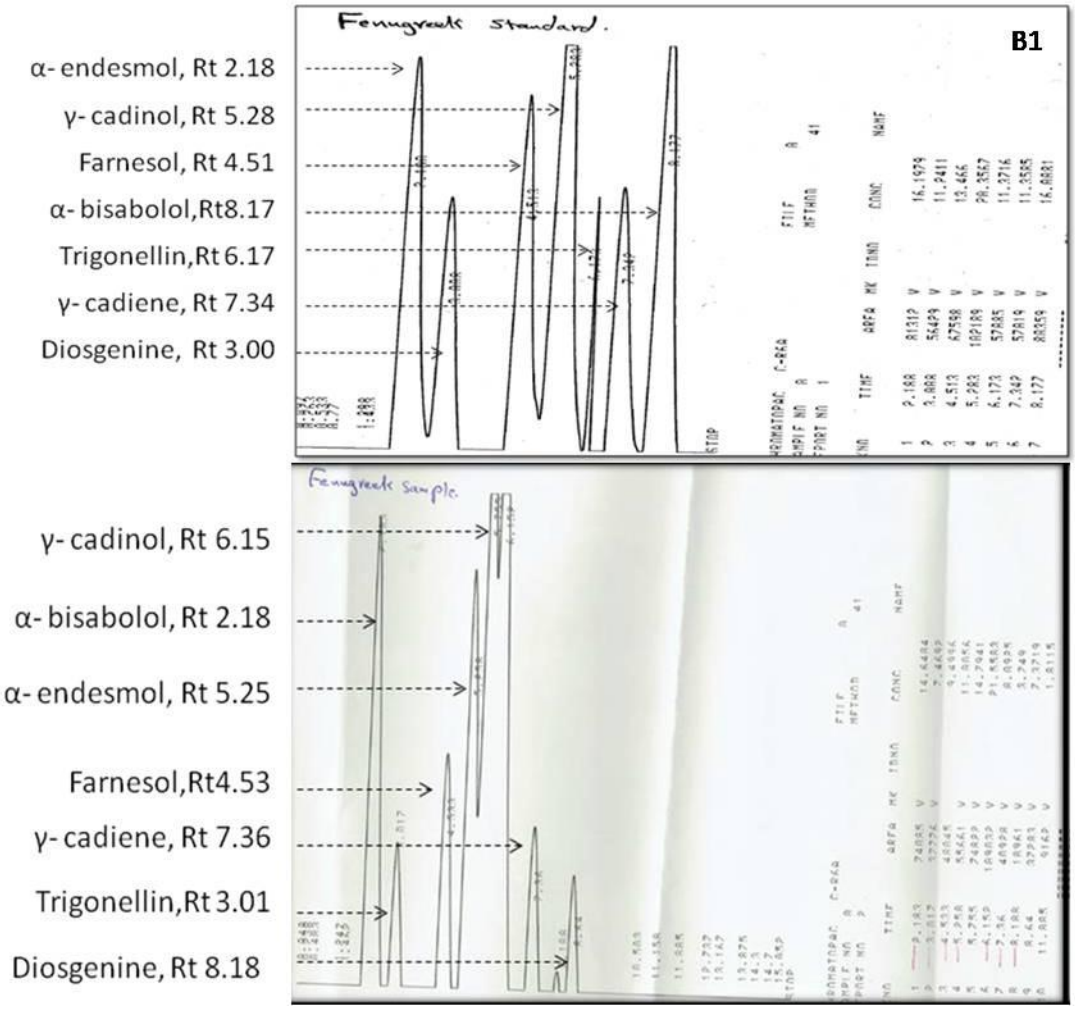

Fig. 1. HPLC chromatography of fenugreek standard and sample, B1: fenugreek standard, B2: fenugreek (Rt = retention time)

Group 4: treated with gentamycin cream (SDI) alone for 7 days.

Group 5: treated with combination of Gentamycin cream and fenugreek essential oil for 7 days.

For preparation of inoculate, the bacteria were sub cultured onto brain heart agar (BHA) (Oxoid, England) and incubated at 37^{o}C overnight. Then one colony was inoculated into brain heart broth (BHB) (Oxoid, England) and incubated overnight at 37^{o}C, the overnight culture was diluted 1:100 in fresh BHB and grown until the mid-exponential phase (approximately 3 hours). The bacteria washed twice and resuspended in sterile phosphate buffered saline (PBS) [12].

Before inoculation the mouse models of bacterial skin infection were sedated with ether. The flanks of the sedated mice were shaved with clippers when necessary and cleansed with an ethanol solution (BDH (England), and then make wound by scalpel cuts. The wounds were subsequently inoculated by 50 µl of the bacterial suspension. Then the mice were returned to their cages and observed. All mice had free access to food and water throughout the duration of the experiments. Animals were observed daily and skin lesion size, swelling, redness, amount of buss were noticed. The treatments with antimicrobial used in this study begin after 4 hrs of bacterial inoculation and continued at the regimens of 7 days [12,13].

Statistical Analysis: Data were analyzed using SPSS version 16 and Microsoft Office Word and Excel 2007. Nominal data were expressed as

number and percent. Independent sample T-test was used for comparison of mean. *P*-value less than 0.05 were considered significant.

Results

Out of 300 specimens obtained from different skin infection, 28 isolates (9.3%) were *P. aeruginosa* which are highly resistance to most of antimicrobial agents while show moderate resistance to ciprofloxacin, imepenem, meropenem and amikacin. The percentage of resistant isolates to each antibiotic is shown in fig. 2.

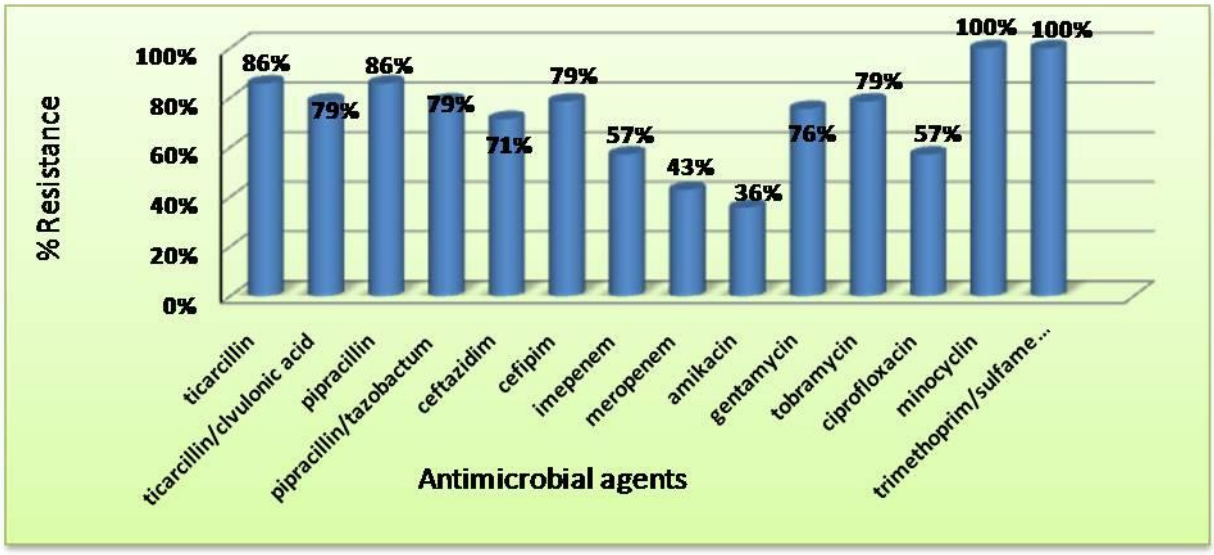

Fig. 2. Resistant *Pseudomonas aeruginosa* isolates to antimicrobial agents (n=28)

MIC of fenugreek essential oil for highly resistance *P. aeruginosa* isolates (n=10) as followed: 6 isolates with MIC= 1.2gm/100µl, and 4 isolates with MIC= 0.6gm/100µl (Table 1). MIC of gentamycin for isolates was determined using VITEK 2 AST method which represent >=16.

The effects of fenugreek essential oil alone and in combination against *P. aeruginosa* are shown in table 1.

Table 1. Effect of fenugreek essential oil alone and in combination with gentamycin on multidrug resistance *P. aeruginosa* isolates

Isolates no.	Fenugreek MICgm/100µl	Fenugreek 1/4 MIC	Gentamycin 1/4 MIC	1/4+1/4 MIC	1/2+1/2 MIC	FIC Values FIC/Interpretation	
1	1.2	+	+	+	-	1	Indifference
2	1.2	-	+	-	-	0.5	synergism
3	0.6	+	-	+	-	1	Indifference
4	1.2	-	+	-	-	0.5	synergism
5	0.6	+	+	-	-	0.5	synergism
6	0.6	+	+	-	-	0.5	synergism
7	1.2	-	+	-	-	0.5	synergism
8	1.2	+	-	-	-	0.5	synergism
9	1.2	+	+	-	-	0.5	synergism
10	0.6	+	+	+	-	1	Indifference
240 mg		240 mg	0.00325 mg	2.4 mg			
0.77		0.77		0.77			

– (no growth), + (growth), fractional inhibitory concentration (FIC) was determine as follow: ≤0.5 = synergism, 0.5-<1 = additive, 1-<4 = indifference, ≥4 = antagonism, *P*-value less than 0.05 were considered significant.

The treatments with antimicrobial used in this study for *in vivo* study begin after 4 hrs of bacterial inoculation and continued at the regimens of 7 days. Then after seven day part of lesion area was tested for histopathological examination.

Group 1: the mice infected just by phosphate buffer saline, no lesion, no redness, no swelling, no death, and histopathological section showed normal skin without inflammatory cell infiltration, also no vascular congestion, no edema and no necrosis as showed in fig. 3.

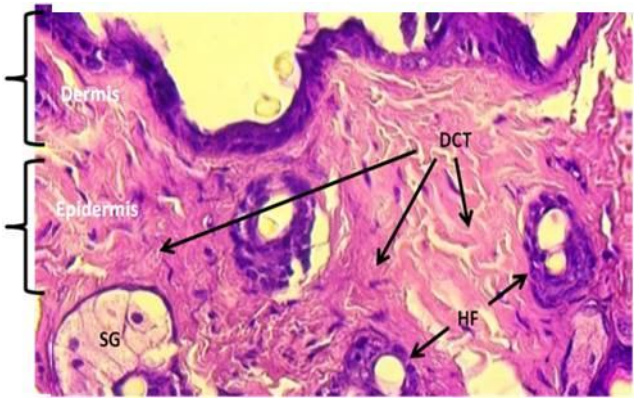

Fig. 3. Section of mouse infected by phosphate buffer saline showing DCT = dermatomal connective tissue, HF = hair follicle, SG = Sebaceous gland (H&E) x40

Group 2: this group infected by *P. aeruginosa* isolate without treatment. The skin swallowed, red, heavy pus, lesion size=0.8 cm, four mice died, and under histopathological examination the section showed market inflammatory cell infiltration, market edema and market vascular congestion of dermis and subcutaneous tissue as shown in fig. 4.

Group 3: Redness, swallowed, lesion (0.6 cm), and pus continued from first day until four day. Few pus and redness were remaining, no death was occurred, the histopathological examination showed moderate edema, moderate inflammatory cell infiltration and moderate vascular congestion as showed in fig. 5.

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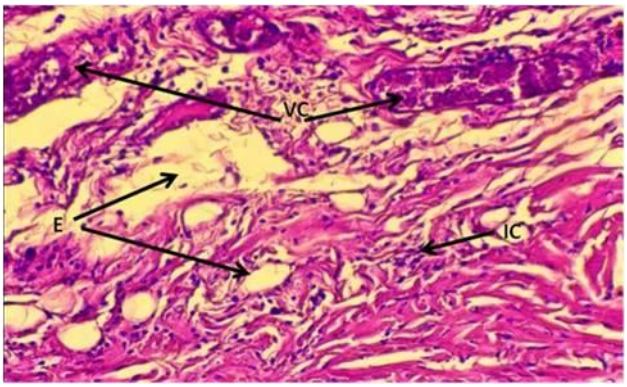

Fig. 4. Section of mouse infected by *P. aeruginosa* without treatment showing IC = inflammatory cells, E = edema, VC = vascular congestion (H&E) x40

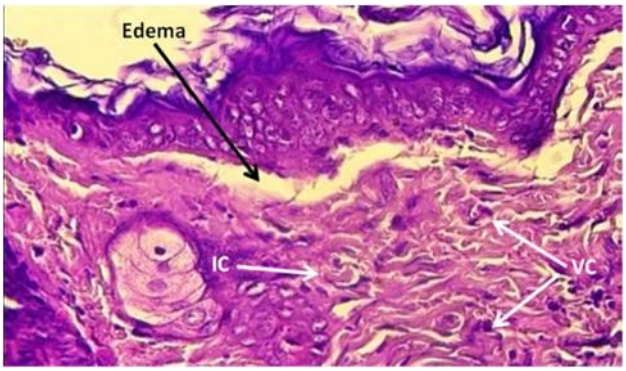

Fig. 5. Section of mouse infected by *P. aeruginosa* treated with fenugreek showing VC = vascular congestion, IC = inflammatory cells (H&E) x40

Group 4: the degree of redness, lesion (1cm), swelling and pus were decreased until third day, healing begun in day six and only one mouse was died in third day. Histopathological examination showed moderate edema, and moderate inflammatory cell infiltration as showed in fig. 6.

Group 5: in this group swallowed, lesion (0.8cm) and pus continue until day five and only one mouse was died in third day.

Histopathological examination showed just mild edema, no vascular congestion and scanty inflammatory cell infiltration as shown in fig. 7.

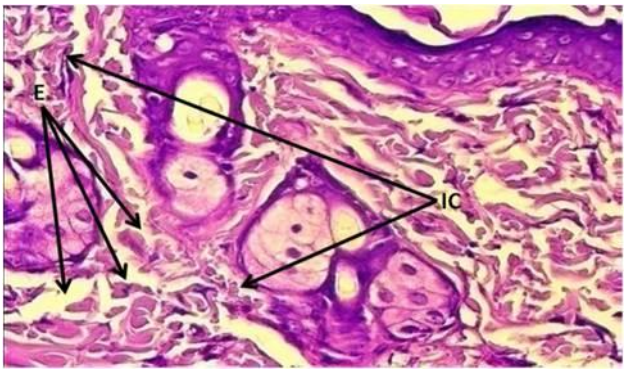

Fig. 6. Section of mouse infected by *P. aeruginosa* treated with gentamycin showing E = edema, IC = inflammatory cells (H&E) x40

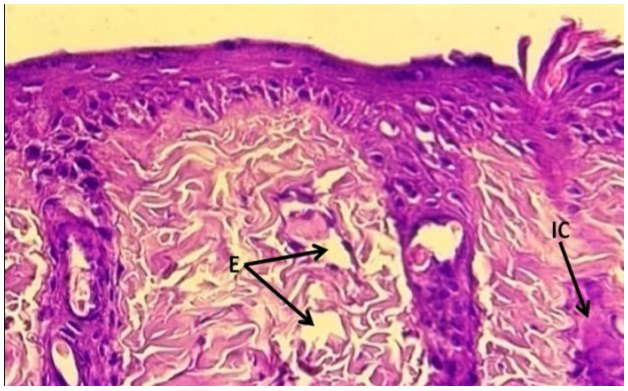

Fig. 7. Section of mouse infected by *P. aeruginosa* treated with combination of fenugreek and gentamycin showing E = edema, IC = inflammatory cells (H&E) x40

Discussion

In the present study, correct identification rate of *P. aeruginosa* was 100 % (28 isolate/28 total isolates). Ines *et al* [14] found that correct identification rates of *P. aeruginosa* were 90.1%.The result of current study demonstrate that *P. aeruginosa* represent 9.3% among patients with skin and soft tissue infection, the results of present study coincides with previous studies in Iraq [15] in which 9.7% of *P. aeruginosa* isolates obtained from burn wound swabs in patients admitted to Al-Karama teaching hospital in Baghdad.

Zone of inhibition of fenugreek essential oil after 48 hrs against *P. aeruginosa* range from 12 to 22 mm of concentrated essential oil (100%), and some essential oil did not have any antibacterial effect against two isolates. Mayssaa [16] revealed that zone of inhibition of fenugreek against *P. aeruginosa* was 16mm and some isolates showed resistant to fenugreek extract. In this study it was found that MIC of fenugreek essential oil range from 0.6-1.2 gm/100ml. Other investigations revealed that MIC values of fenugreek essential oil were ranging from 0.8-6.4 gm/100ml against both gram positive and gram negative bacteria [17].

The result of the present study was in agreement with the finding of Al-Derzi [18] that has found the resistance of *P. aeruginosa* to gentamicin was 79.1%, while in agreement with him that the resistance rate to amikacin was 89.7% and this result is higher than the result of the current study.

The aminoglycosides inhibit protein synthesis in bacterial cell by binding to 30S subunit of the ribosome and the aminoglycoside- resistance in *Pseudomonas sp.* is primarily due to modification in target enzymes and in activation of the antibiotics [19,20].

Aminoglycosides have numerous groups of resistance mechanisms: enzyme modification, low outer membrane permeability, active efflux and, not often, target modification [21,22].

Synergistic effect was seen from combination of gentamycin with fenugreek essential oil against most isolates of *P. aeruginosa* as shown in table 1. The important point of Abascal and Yarnell [23] study is on the combining of herbs with antibiotics to decrease drug resistance acting synergistically with drugs to kill microbes. Generally, their action is the result of the combined effect of together active and inactive compounds [24,25].

The growth of the organism was clearly observed in all inoculated mice. Lesions cultures was confirmed the infections by bacteria. After usage of the plants as topical treatment for one week, the lesions and

wounds were healed dramatically. Control groups were used to prove that healing was not spontaneously.

In recent years, different reports from different countries were indicated that there were antimicrobial activities of medicinal plants, for many years, the effect of herbal medicine on burn wound has been noted. Herbal products seem to possess moderate efficacy and are less expensive as compared with synthetic drugs. Many plants and plants-derived products have been shown to possess potent wound-healing activity [26].

In-vivo-sensitivity of the plants studied on the infected mice proved to be very active. All the infected mice were cured by local application of the plants on the lesions. No spontaneous improvement was detected on the infected control mice. The result of histopathological examination in the present studies show that antibacterial activity of fenugreek essential oil alone and in combination with Gentamycin is greater than antibacterial activity of gentamycin alone, this effect may belong to the active compound in the plants which have bactericidal effect against *P. aeruginosa*. This indicates that thecure of the tested mice was due to the action of theseplants studied. The use of that plant in the form of topical therapy in infected mice was proved the affectivity of fenugreek plants as medicinal purpose [27].

Most of the medicines are mixture of many plants, but none of these traditional ointments were scientifically studied.In our study, fenugreek extract was compared with gentamycin as the standard treatment for burn wounds in mice. The actual mechanism of improved healing is still unclear, the probable mechanism are providing necessary material for healing, increasing blood flow to burn area, decreased inflammatory response, and decreasing rate of infection. A new skin medication can be introduced by usage of herbal medicines with fewer adverse effects and shorten the period of healing thus decrease the rate of hypertrophic scar. The result findings denotes of fenugreek in healing of burn injuries as an inexpensive and available herbal medicine [28].

In conclusion, fenugreek essential oil has antibacterial effect against skin infection with *Pseudomonas aeruginosa* and combination of fenugreek with gentamycin shows synergistic effect and is more effective than gentamycin alone.

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Author contribution

Dr. Abdul Kahaleq conducted the study; Dr. Abu-Raghif organizes the idea, finalizes the protocol, and selects the herbs; and Dr. Kadhim contributes through technical support of the research.

Conflict of interest

The authors declare no conflict of interest.

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