

# Antimicrobial effects of *Ferula Gummosa* Boiss gum against extended-spectrum $\beta$ -lactamase producing *Acinetobacter* clinical isolates

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## ABSTRACT

**Background and Objectives:** *Acinetobacter* spp. are important causes of nosocomial infections. They possess various antibiotic resistance mechanisms including extended spectrum beta lactamases (ESBLs). The aim of this study was to determine antibiotic resistance profile of *Acinetobacter* clinical isolates especially among ESBL-producing strains and to investigate the antimicrobial effects of oleo-gum-resin extract and essential oil of *Ferula gummosa* Boiss.

**Materials and Methods:** 120 *Acinetobacter* strains were isolated from various clinical samples of hospitalized patients in Baqiyatallah hospital, Tehran during 2011-2012. Antibiotic susceptibility test was performed on the isolates using disk diffusion method. To detect and confirm the ESBL-positive isolates, phenotypic and genotypic tests were performed. Three types of *F. gummosa* oleo-gum-resin extracts and essential oils were prepared and the bioactive components of *F. gummosa* Boiss extracts were determined by GC-Mass chromatography. *F. gummosa* antimicrobial activity was evaluated against standard strain of *Acinetobacter baumannii* (ATCC19606) as well as *Acinetobacter* clinical isolates using well and disk diffusion methods. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by broth microdilution method.

**Results:** 46 isolates were resistant to all tested antibiotics. All clinical isolates were resistant to cefotaxime. 12.94% of the isolates were phenotypically ESBL-producing among which 94.2% carried ESBL genes (*bla*<sub>PER-1</sub>, *bla*<sub>OXA-4</sub> and *bla*<sub>CTX-M</sub>) detected by PCR. Oleo-gum-resin of *F. gummosa* had significant antibacterial activity and alcoholic essential oil had higher inhibitory effect on *Acinetobacter* strains (MIC of 18.75 mg/ml).

**Conclusion:** *Ferula gummosa* extract contained components with well-known antimicrobial effects.

**Keywords:** *Acinetobacter* spp., Antibiotic resistance, Antimicrobial activity, ESBLs, *Ferula gummosa* boiss

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## INTRODUCTION

*Acinetobacter* spp. are Gram-negative, non-motile, obligate aerobic coccobacilli and important causes of nosocomial infections. In recent studies, *Acinetobacter* spp. are introduced as important opportunistic bacteria which are responsible for a wide range of nosocomial infections including ventilator-associated pneumonia, bacteremia, and surgical site infections (1-4). Patients are at higher risk of *Acinetobacter* infections in intensive care units (ICUs) due to the critical conditions, long-term hospitalization, use of various medical equipment and rapid and aggressive treatments such as urinary and intravenous catheters (5-9). Most of the *Acinetobacter* species are resistant to multiple or all antibiotics and are called multidrug resistant (MDR) and pandrug resistant (PDR), respectively (1, 3, 9-11). Various *Acinetobacter* species use different mechanisms for being resistant to antibiotics. Examples of such mechanisms are enhancing efflux pumps, alteration of membrane porins to reduce antibiotic permeability or uptake and secretion of degrading enzymes like  $\beta$ -lactamase (which degrades  $\beta$ -lactam antibiotics) (10, 12). Extended spectrum  $\beta$ -lactamase (ESBL) was first reported in 1983 (13) and quickly became a healthcare problem worldwide. Also, it may increase the prevalence of infections due to the long-term hospitalization and consequently may increase mortality rate.

*F. gummosa* boiss belongs to Umbelliferae family, a wild native plant of Iran (14). This plant has been used in traditional medicine and has been shown to contain various medicinal and therapeutic properties such as antispasmodic, analgesic, expectorant, anti-parasitic and antibacterial effects (15, 16). *F. gummosa* contains 6% volatile oil, 67% resin and 19% gum (17). There are different compounds in the plant extract including terpenoids, alkaloids, alpha-pinene and cardenolids (16). Phytochemical investigations have revealed that *F. gummosa* is a source of biologically active compounds making it a potent antibacterial candidate. So far, few studies have been conducted on the antimicrobial effects of *F. gummosa* extracts showing significant inhibitory effects against Gram-positive, Gram-negative bacteria and fungi (18-20). Based on previous studies on antibacterial properties of *F. gummosa* essential oil, its minimum inhibitory concentration (MIC) was 8, 12.5, 50, 6.25 and 1.56  $\mu$ l/ml for clinical *Staphylococcus*

*aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *S. enteritidis* and *Listeria monocytogenes*, respectively (20). In addition 8, 25, 50, 12.5 and 12.5  $\mu$ l/ml were reported as MBC for the above strains, respectively. In a study by Mahbuobi et al. a lower MIC of essential oils was reported (21). The MIC for *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* was 2, 4 and 4  $\mu$ l/ml, respectively. The MBC for the relevant species was 4, 8 and 8  $\mu$ l/ml, respectively. They attributed the antibacterial activities to the quantity of  $\beta$ -pinene and higher concentrations of  $\beta$ -pinene (21). Moreover, Gram positive bacteria are more sensitive to the essential oils of *F. gummosa* compared with Gram negative strains. In addition to essential oils, different kinds of *F. gummosa* extracts derived from root (particularly ethanolic extract) had a potent antibacterial effect. 12.5 mg/ml of ethanolic and 6.25 mg/ml of methanolic root extracts had inhibitory effects on *Pseudomonas aeruginosa* (22). In contrast to *Proteus mirabilis*, *Escherichia coli* and *Klebsiella pneumoniae*, sensitivity towards the ethanolic extract was observed in *Serratia marcescens*, *Shigella dysenteriae* and *Yersinia enterocolitica* (18). The aim of this study was to determine the prevalence and antibiotic resistance profile of *Acinetobacter* clinical isolates especially among ESBL-producing strains and evaluate antibacterial effects of oleo-gum (Galbanum) extract and essential oil of *F. gummosa* against clinical isolates of *Acinetobacter*.

## MATERIALS AND METHODS

**Biochemical characterization of *Acinetobacter* isolates.** Several clinical isolates were collected from different clinical samples such as blood, wound, sputum, stool, urine, bronchoalveolar lavage (BAL), pleural fluid and cerebrospinal fluid. After performing differential biochemical tests including oxidase, catalase, TSI, SIM and MR/VP tests, 120 *Acinetobacter* strains were characterized and stored at -80 °C until used.

**Determination of antibiotic resistance profile in *Acinetobacter* isolates.** Antibiotic susceptibility pattern of 120 *Acinetobacter* isolates were determined against 15 conventional antibiotics including ceftriaxone (30  $\mu$ g), ceftazidime (30  $\mu$ g), cefotaxime (30  $\mu$ g), cefepime (30  $\mu$ g), piperacillin-tazobactam

(100/10 µg), ampicillin-sulbactam (10/10 µg), imipenem (10 µg), meropenem (10 µg), gentamicin (30 µg), amikacin (30 µg), tetracycline (30 µg), ciprofloxacin (5 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), norfloxacin (10 µg) and ampicillin-clavulanic acid (20/10 µg). Bacterial suspension was prepared and normalized to 0.5 McFarland turbidity standard ( $1.5 \times 10^8$  CFU/ml). Antibigram was performed on Muller Hinton agar by disk diffusion agar method according to CLSI guidelines (23, 24). *Acinetobacter baumannii* ATCC 19606 was used as the control strain.

**Phenotypic determination of ESBL producing isolates of *Acinetobacter*.** All *Acinetobacter* isolates were phenotypically analyzed for ESBL production. Detection test was performed to confirm the ESBL-positive isolates by combined disk method (23, 24). Bacterial suspensions were prepared and spread evenly on Muller Hinton agar plates (suspension concentrations equal to 0.5 McFarland). Ceftazidime and cefotaxime disks (30 µg each) were applied on the plate 20 mm apart (center-to-center) from ceftazidime/clavulanic acid (30/10 µg) and cefotaxime/clavulanic acid (30/10 µg) disks, respectively. According to CLSI protocols, in cases where the inhibition zone around clavulanic acid disks was at least 5 mm greater than the one around the disks without clavulanic acid, the relevant isolate was considered as ESBL-producing strain. In all cases, ESBL-positive *Klebsiella pneumoniae* reference strain ATCC 700603 was used as a positive control strain.

**Genotypic detection of ESBLs.** PCR was conducted in order to detect *Acinetobacter* isolates carrying ESBL genes, *bla*<sub>PER-1</sub>, *bla*<sub>OXA-4</sub> and *bla*<sub>CTX-M</sub>. Genomic DNA was extracted using genomic DNA Extraction kit (Bioneer, South Korea) based on the manufacturer's instructions. Briefly, 25 µl mixture reaction consisting 10× reaction buffer, 0.1 mM deoxynucleoside triphosphates (dNTPs), 2 mM MgCl<sub>2</sub>, 10 pM specific designed primers and 1 U Taq DNA Polymerase (CinnaGen, Iran) was prepared and 1 µl of each DNA sample was amplified in the mixture. *P. aeruginosa* KOAS, *K. pneumoniae* 7881, *K. pneumoniae* CHU de BICETRE were used as positive controls harboring *bla*<sub>PER-1</sub>, *bla*<sub>OXA-4</sub> and *bla*<sub>CTX-M</sub> genes, respectively. Specific primers and the size of the PCR products were as follow: *bla*<sub>CTX-M</sub> (gene ID: 6383814; 550 bp), F: 5'-cgctttgcgatgtgcag-3' and

R: 5'-accgcgatatcgttggt-3' (25); *bla*<sub>OXA-4</sub> (gene ID: 6762749; 216 bp), F: 5'-tcttcaacggatatctctactgt-3' and R: 5'-tgctttatcccatttgaatatgt-3' (developed in the present study; 927 bp); *bla*<sub>PER-1</sub> (gene ID: 12104212), F: 5'-atgaatgtcattataaaagc-3' and R: 5'-aattgggcttag-ggcagaa-3' (25).

***F. gummosa* extract preparation.** The gum of *F. gummosa* was provided from Semnan Province located in the central Iran. Extraction was performed using maceration method (26). Briefly, collected oleo-gum-resin was dried and crushed into a soft powder by mortar and was completely dissolved in 30 g dry weight per 100 ml solvent (distilled water, methanol or distilled water/methanol mix) and stored at room temperature for 48 h in a dark container. Then, the extract was purified by filter paper and solvent was removed by rotary evaporator (Heidolph, Germany) at 40 °C under reduced pressure. The dry residue was dissolved in dimethyl sulfoxide (DMSO) (Merck, Germany) (1:1) and stored at 4 °C in a dark jar until used.

**Preparation of *F. gummosa* essential oil.** Essential oils were prepared by hydro-distillation method using Clevenger apparatus (Shahab Chemistry, Iran) (27). Dried oleo-gum-resin was crushed into a soft powder and was completely dissolved in solvent (30% w/v) in the same way as the extract was prepared and poured into a distilling flask. Distillation was continued until no additional volume was added to the oil. Three types of essential oils including aqueous, methanolic and aqueous-methanolic (50:50) were produce to compare the potency of the them. Finally, solvents were removed as previously mentioned and the dry materials were dissolved in DMSO (1:1) and stored at 4 °C in a dark jar until used.

**GC-Mass analysis.** To determine the components of *Ferula gummosa* Boiss extracts, GC-Mass chromatography was performed using Mass Hunter system equipped with HP-5MS 5% Phenyl Methyl Silox column (30 m × 0.25 mm). Oven temperature was ramped up from 50 to 290 °C at a rate of 5 °C. The temperature of transfer line was 300 °C. Helium was utilized as a carrier gas with an average velocity of 36.445 cm/sec and split ratio of 5:1. Determination of compounds was carried out according to the retention time relative to toluene. Finally, the findings were matched with the spectra presented in Wiley

275L library to confirm the presence of the components.

**Evaluating antibacterial effects of *F. gummosa* extracts.** A solution of 300 mg/ml of dried extract in DMSO (30% w/v) was prepared and serially diluted (150, 75, 37.5 and 18.75 mg/ml). These dilutions were used for all antibacterial tests through disk, well diffusion agar and broth microdilution methods. *Acinetobacter baumannii* ATCC 19606 reference strain was used for evaluation of *F. gummosa* antibacterial effects by standard well and disk diffusion agar methods.

**Disk diffusion method.** 20 µl of each dilution was loaded onto 6.4 mm disks. A disk containing DMSO (20 µl per disk) also was prepared as negative control. All procedures were performed under sterile conditions. Bacterial suspensions were prepared after 18-24 h culture on blood agar at a concentration equal to 0.5 McFarland ( $1.5 \times 10^8$  CFU/ml) and were spread evenly on Muller Hinton agar plates. Afterwards, disks containing different concentrations of *F. gummosa* extract were applied on the agar surface with a distance of 24 mm from each other (center-to-center) and 15 mm away from the edge of plate. After incubation at 37 °C for 18-24 h, the diameter of inhibition zones around the disks was measured.

**MIC determination; broth microdilution method.** Minimal inhibitory concentrations (MIC) of *F. gummosa* extract were determined against the reference strain and all 120 clinical isolates of *Acinetobacter*. For this purpose, 5 ml bacterial suspension with a concentration equal to 0.5 McFarland turbidity standard ( $1.5 \times 10^8$  CFU/ml) was prepared from an overnight grown culture of each test strain. Then, 100 µl of a 10% dilution of the suspension in sterile normal saline was transferred into a 96 well microplate. Each well contained 100 µl Muller Hinton broth and 100 µl different concentrations of *F. gummosa* extract or essential oil. The final concentration of the bacterial suspension was  $5 \times 10^5$  in each well. Afterwards, 8 serial dilutions were prepared from the initial concentration across the row. A well containing 100 µl Muller Hinton broth and 100 µl *F. gummosa* extract or essential oil was used as negative control. To confirm the results, 10 µl contents of each well (containing different treatments), were spotted on a Muller Hinton agar plate. Finally, the last concentra-

tion of the plant extract in which bacterial growth was inhibited (in microplate) or decreased (on agar) was considered as MIC. The concentration where no growth (no colony) was observed was considered as MBC. The plate was read in a microplate reader at 600 nm. The results were analyzed by Chi-square test and Fisher's exact test using SPSS (v. 17) software.

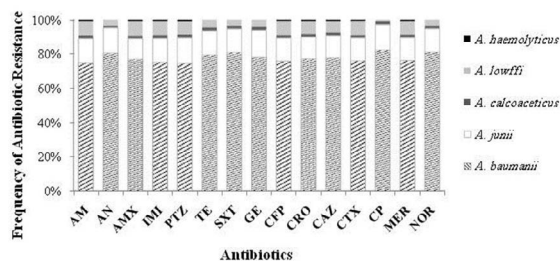
## RESULTS

**Frequency of *Acinetobacter* isolates in different clinical samples.** In one year period, 85 *Acinetobacter* clinical isolates were obtained from various clinical samples in a teaching hospital in Iran. The studied clinical samples were collected from patients in the range of 17 to 90 years of age. Among 85 *Acinetobacter* isolates, frequency of identified strains was 67.05% for *A. baumannii*, 18.82% (*A. junii*), 11.76% (*A. lwoffii*) and 2.35 % (*A. calcoaceticus*).

**Relationship between *Acinetobacter* isolates and origin of clinical samples.** Wound samples were the most frequent samples and the pleural effusion, ascites and abscess had minimum frequency among collected samples. 73.3% of all *A. baumannii* isolates were frequently found in wounds and bronchial lavages (*P* value= 0.00).

**Disk diffusion method and antibiotic resistance profile of *Acinetobacter* isolates.** 48 strains (56.47%) were completely resistant to all tested antibiotics. Maximum resistance to cefotaxime (100%) was observed and only 57 strains (67%) showed resistance towards amikacin. Frequency of antibiotic resistance among isolated *Acinetobacter* species was estimated. Correlation between isolated species and resistance against tetracycline, trimethoprim, gentamicin, sulfamethoxazole, ceftazidime, ciprofloxacin and norfloxacin were significant ( $P < 0.05$ ) but no significant association was observed with other tested antibiotics. According to the Fig. 1, maximum resistance against all tested antibiotic belonged to *A. baumannii* isolates.

Interestingly resistance pattern of *Acinetobacter* isolates varied in various clinical samples. For example, in wound samples highest resistance was observed against amikacin (27.05%). In bronchial samples resistance was mostly against gentamicin



**Fig. 1.** Antibiotic resistance among *Acinetobacter* isolates. AM: Ampicillin; AN: Amikacin; AMX: Amoxicillin; IMI: Imidazole; PTZ: Piperacillin-Tazobactam; TE: Tetracycline; SXT: Sulfamethoxazole-Trimethoprim; GE: Gentamicin; CFP: Cefoperazone; CRO: Ceftriaxone; CAZ: Ceftazidime; CTX: Cefotaxime; CP: Ciprofloxacin; MER: Meropenem; NOR: Norfloxacin

(31.7%) and in pleural effusion samples was against gentamicin, ciprofloxacin and piperacillin-tazobactam. A significant association was found between antibiotic resistance and type of sample in cases of gentamicin, ciprofloxacin and piperacillin-tazobactam ( $P<0.05$ ).

**Phenotypic detection of ESBL producing *Acinetobacter* clinical isolates.** Phenotypic evaluation of ESBLs production in *Acinetobacter* clinical isolates was performed by combined disk method. In this technique ceftazidime and cefotaxime disks (30 µg each) in proximity of ceftazidime/clavulanic acid and cefotaxime/clavulanic acid (30/10 µg) disks, indicated that 11 (12.94%) isolates were positive for ESBL production. Among 11 ESBL-positive strains, 9 (81.81%) isolates had positive phenotype for CTX and CAZ and 2 others (18.18%) were CAZ-positive phenotypes. According to CLSI protocols, an extension of inhibition zone greater than 5 mm around clavulanic acid disks towards the one without clavulanic acid was considered as ESBLs producing strain.

Only *A. baumannii* isolates were found to produce ESBLs. Other *Acinetobacter* species have shown no sign of ESBLs production. According to these results, the pattern of ESBLs production varies among *Acinetobacter* clinical isolates depending on the infection site. Samples collected from trachea contained highest rate of ESBL production (24.7%), blood and wound samples came second and third on the ranking (21.17% and 3.52% ESBL-producing strains, respectively). Other samples including mucus, urine, abscess, pleural effusion and ascites were

negative for ESBL production. To determine the relationship between sample type and ESBL production, Fisher's exact test was performed which was significant ( $P=0.03$ ). 45.8% of all the ESBL producing bacteria were isolated from ICU. Following ICU, women surgery wards, pulmonary division (17.6% of each), ear nose and throat (ENT), orthopedic and renal transplantation wards had the highest rate of ESBL isolation (9.4% of each section).

Among ESBL producing *Acinetobacter* isolates, the maximum resistance rate was found against cefotaxime (100%) and the lowest rate was observed against amikacin (72.9%). On the other hand, among ESBL-negative isolates highest rate of resistance was against cefotaxime, cefepime, ceftriaxone and ceftazidime (100%) and the lowest resistance rate was against amikacin (17.6%).

**GC-Mass findings.** Table 1 outlined chemical compounds and their quantities identified in *Ferula gummosa* Boiss extracts. Totally, 33 compounds were determined. Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-, [2R-(2α,4α,8αβ)] and Guaiol were the most frequent compounds.

**Agar disk diffusion method.** In order to confirm *F. gummosa* antibacterial effects, *A. baumannii* reference strain ATCC 19606 was used. For this purpose, serial dilutions were prepared (1/2, 1/4, 1/8 and 1/16) from stock solution (300 mg/ml) of each aqueous, methanolic and aqueous-methanolic extracts and tests were accomplished in triplicate. Aqueous-methanolic extract had the highest inhibition zone ( $16 \pm 2$  mm) at 150 mg/ml. Methanolic extract exerted an inhibition zone of 14 mm at the same concentration. In overall, the aqueous extract had the lowest inhibitory effect among all the extracts (highest inhibition zone was  $10 \pm 2$  mm at 300 mg/ml). Testing *F. gummosa* extracts by disk diffusion method indicated that all three types of extracts had antibacterial effects on *A. baumannii* reference strain and aqueous-methanolic had the highest antibacterial effect at 150 mg/ml.

**MIC and MBC of *F. gummosa* gum.** MIC and MBC of *F. gummosa* were determined against *A. baumannii* reference strain ATCC 19606. The aqueous-methanolic extracts had the highest antibacterial effects containing MIC and MBC of 37.5 mg/ml.

Table 1. Chemical compounds identified in the *Ferula gummosa* Boiss extracts

Compounds	Percentage (%)	Retention time (min)
Ethyl benzene	1.24	6.91
p-Xylene	7.43	7.11
β-Pinene	1.52	10.10
Isopinocarveol	2.54	15.03
2(10)-Pinen-3-one, (±)-	0.87	15.67
(-)-Myrtenol	2.87	16.70
Fenchyl acetate	1.88	17.20
Isoborneol, allyldimethylsilyl ether	0.58	19.09
3-Cyclohexene-1-methanol, α,α,4-trimethyl-, acetate	1.13	20.71
γ-Elemene	0.40	22.87
Cedran-diol, 8S,14-	4.91	23.46
Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-, [2R-(2 α,4α,8aβ)]-	19.70	24.28
Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1 α,4α,7α)]-	0.31	24.48
Isolongifolan-8-ol	3.72	24.63
Epiglobulol	1.22	25.07
Isoaromadendrene epoxide	1.27	25.73
10-Heptadecen-8-ynoic acid, methyl ester, (E)-	0.44	26.65
Guaiol	13.66	26.87
Cubenol	3.12	27.27
Agarospinol	7.27	27.76
Aristolene	6.77	27.97
2,2,6,7-Tetramethyl-10-oxatricyclo[4.3.1.0(1,6)]decan-5-ol	2.05	29.26
2,5-Octadecadiynoic acid, methyl ester	0.76	30.25
Aromadendrene oxide-(1)	1.55	30.70
Isoaromadendrene epoxide	0.96	30.93
Cholestan-3-ol, 2-methylene-, (3β,5α)-	5.27	31.56
1-Heptatriacotanol	1.35	32.22
2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	0.52	33.76
trans-Z-α-Bisabolene epoxide	1.00	36.68
Aromadendrene oxide-(2)	0.70	36.80
Methyl 7,10,13-hexadecatrienoate	0.40	37.24
β-Guaiene	0.84	38.53
Octadecanal, 2-bromo	0.38	41.17

Aqueous-methanolic and methanolic essential oil had the lowest MIC and MBC of 18.75 mg/ml. Results obtained from disk diffusion, well diffusion and broth microdilution tests indicated that the broth microdilution method is much more suitable for evaluating the antimicrobial effects of *F. gummosa* extracts and essential oils compared with the other two methods. This would be due to the high density of *F. gummosa* extracts and essential oils which

makes it difficult to diffuse in agar. Hence, we used broth microdilution method to evaluate antimicrobial effects of *F. gummosa* against *A. baumannii* clinical isolates. Table 2 provided a summary of MIC and MBC of *F. gummosa* extract and essential oil against ESBL-positive and ESBL-negative *A. baumannii* clinical isolates. In overall, it showed that among ESBL-negative *Acinetobacter* isolates the lowest inhibitory concentration of methanolic extract was 37.5

mg/ml and that of ESBL positive strains was 75 mg/ml. The MIC of 36.4% ESBL-negative strains was 75 mg/ml compared to detected MIC of 150 and 300 mg/ml in 44.7% ESBL-positive strains. The MBC of 45.9% of ESBL-negative isolates was also 75 mg/ml. In ESBL-positive strains the lowest concentration of methanolic extract with a bactericidal effect was 150 mg/ml but the MBC of 81.1% of these strains was 300 mg/ml. The MIC and MBC data of other types of extracts are summarized in Table 3. The same approach was taken to determine MIC and MBC of *F. gummosa* essential oil at various concentrations on ESBL-positive and ESBL-negative *Acinetobacter* clinical isolates. Data indicated that the lowest inhibitory concentration of *F. gummosa* methanolic essential oils was 18.75 mg/ml amongst ESBL-negative *Acinetobacter* strains, a concentration in which the growth of 45.9% of ESBL-negative *Acinetobacter* isolates was inhibited. Among ESBL-positive *Acine-*

*tobacter* isolates, 75 mg/ml concentration was chosen as the best inhibitory concentration. Considering Table 2, we found that all three types of *F. gummosa* extracts and essential oils had antibacterial activity against both *Acinetobacter* ESBL-positive and ESBL-negative strains. Comparing MIC among these extracts (aqueous, aqueous-methanolic and methanolic) it became clear that the aqueous extract had the highest inhibitory effect ( $92.06 \pm 38.8$  mg/ml) on ESBL-negative isolates whereas maximum inhibition on ESBL-positive strains was exerted by aqueous-methanolic extracts ( $124.47 \pm 98.55$  mg/ml). On the other hand, methanolic *F. gummosa* essential oil had maximum inhibitory effect (MIC) on both ESBL-positive ( $129.54 \pm 35.03$  mg/ml) and ESBL-negative isolates ( $56.29 \pm 50.98$  mg/ml). By comparing MBC among three types *F. gummosa* extracts it has been revealed that the aqueous-methanolic extract had the highest bactericidal effect ( $92.06 \pm 38.8$  mg/

Table 2. MIC and MBC of *F. gummosa* gum against ESBL-negative and ESBL-positive *A. baumannii* clinical isolates

	Extracts (mg/ml)						Essential oils (mg/ml)					
	Aqueous		Aqueous-Methanolic		Methanol		Aqueous		Aqueous-Methanolic		Methanol	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
ESBL (-)	92.1	156.8	138.	146.6	119.3	156.8	265.9	265.9	95.5	143.2	56.2	68.3
	±	±	±	±	±	±	±	±	±	±	±	±
	38.8	52.5	111.9	107.9	97.4	97.5	77.6	77.6	79.2	89.9	50.9	43.6
ESBL (+)	146.5	225.9	124.4	184.1	211.3	272.7	268.6	268.6	146.6	225	129.5	211.3
	±	±	±	±	±	±	±	±	±	±	±	±
	95.2	89.6	98.5	116.6	87.5	60.6	79.1	79.1	63.7	88.7	35.03	87.5

Table 3. Frequency of ESBL-positive and ESBL-negative *Acinetobacter* clinical isolates based on MIC and MBC of *F. gummosa* extracts

	Methanolic extract (mg/ml)									
	300		150		75		37.5		18.75	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
ESBL (-) (%)	17.6	27.1	17.6	27.1	36.4	45.9	27.1	-	-	-
ESBL (+) (%)	45.9	81.1	45.9	17.6	9.4	-	-	-	-	-
Aqueous-Methanolic extract (mg/ml)										
ESBL (-) (%)	27.05	27.05	17.64	27.05	27.05	17.64	17.64	27.05	9.4	-
ESBL (+) (%)	17.64	45.9	27.05	17.64	27.05	17.64	17.64	17.64	9.4	-
Aqueous extract* (mg/ml)										
ESBL (-) (%)	-	9.4	27.1	81.1	36.4	9.4	9.4	-	-	-
ESBL (+) (%)	17.6	45.9	36.4	36.4	36.4	9.4	-	-	-	-

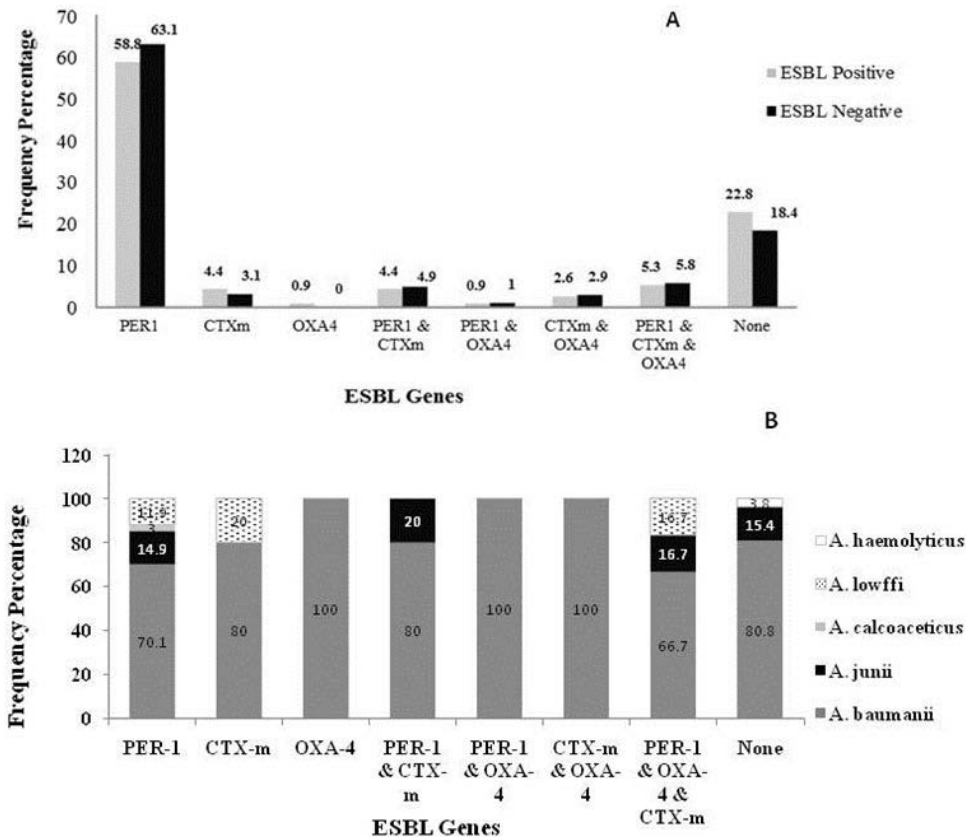
\*In aqueous extract, 9.1% of ESBL-positive and ESBL-negative strains of *Acinetobacter* had a MIC and MBC at concentrations higher than 300 mg/ml.

ml) on ESBL-negative ( $146.65 \pm 107.9$  mg/ml) as well as ESBL-positive ( $184.13 \pm 116.67$  mg/ml) isolates. *F. gummosa* methanolic extract had maximum bactericidal effects (MBC) on ESBL-negative ( $68.31 \pm 43.68$  mg/ml) as well as ESBL-positive ( $184.13 \pm 116.67$  mg/ml) isolates.

**Genotyping of ESBLs.** In addition to the phenotypic methods, detection of ESBL producing *Acinetobacter* strains was conducted with PCR by means of primers related to *bla*<sub>PER-1</sub>, *bla*<sub>OXA-4</sub> and *bla*<sub>CTX-M</sub> genes representing ESBL genes. Among 85 *Acinetobacter* isolates, 12 strains (7.08%) were confirmed as ESBL-positive by phenotypic method (well diffusion agar) containing 10 *caz*-positive and 2 *ctx*-positive strains. However, 80 isolates (94.2%) carried ESBL genes (*bla*<sub>PER-1</sub>, *bla*<sub>OXA-4</sub> and *bla*<sub>CTX-M</sub>) when tested by PCR. As shown in Fig. 2A, *bla*<sub>PER-1</sub> gene had the high-

est frequency among isolated *Acinetobacter* strains which were phenotypically confirmed as either ESBL-positive or –negative strains. Also, 22.3% of the phenotypically ESBL-positive and 17.6% of phenotypically confirmed ESBL-negative isolates did not contain any of these genes and might contain other ESBL representative genes such as *bla*<sub>OXA-1</sub>, *bla*<sub>OXA-10</sub>, *bla*<sub>GES-1</sub> or *bla*<sub>VEB-1</sub>.

All ESBL-positive strains were confirmed as *A. baumannii* when tested phenotypically. 81.1% were *caz*-positive and 17.6% were *ctx*-positive whereas the rest were ESBL-negative phenotypically. On the other hand, 15.2% of *A. junii*, 3.5% of *A. calcoaceticus* and 11.7% of *A. lwoffii* which were tested by PCR, carried *bla*<sub>PER-1</sub> gene as a representative of ESBL genes. The frequency of the other ESBL genes (*bla*<sub>OXA-4</sub> and *bla*<sub>CTX-M</sub>) among *Acinetobacter* species are shown in Fig. 2B.



**Fig. 2.** Genotypic and phenotypic detection of ESBL genes in *Acinetobacter* isolates. A) Frequency of ESBL genes (*bla*<sub>PER-1</sub>, *bla*<sub>OXA-4</sub> and *bla*<sub>CTX-M</sub>) among *Acinetobacter* isolates were phenotypically confirmed as either ESBL-positive or –negative; B) Frequency of ESBL genes (*bla*<sub>PER-1</sub>, *bla*<sub>OXA-4</sub> and *bla*<sub>CTX-M</sub>) among different *Acinetobacter* species tested with PCR.



## DISCUSSION

*Acinetobacter* species are common etiologic agents in nosocomial infections. According to the recent reports the occurrence of multidrug resistant *Acinetobacter* spp. is increasing worldwide (7-9). In this study, 84 *Acinetobacter* isolates were collected from various clinical samples in various units within the hospital. Most frequently *Acinetobacter* isolates were collected from wounds (25%) and bronchial specimens (24.2%). In accordance with our results, other studies (1, 5, 8, 10) have shown that respiratory specimens (bronchial, tracheal, sputum and pleural) contained high frequency of *Acinetobacter* isolates. Therefore it can be concluded that the respiratory tract is the primary site of *Acinetobacter* infections through use of ventilators, tracheal tubes and respiratory catheters. Most of the *Acinetobacter* isolates were *A. baumannii* which corresponded with previous studies (28) and confirms the dominance of *A. baumannii* in *Acinetobacter* associated nosocomial infections. In the present study *Acinetobacter* isolates showed a high resistance rate against routine antibiotics. All isolates were multidrug resistant (MDR) and were resistant to more than two different classes of antibiotics. 43.3% of the isolates were also pandrug resistant (PDR) and were resistant to all tested antibiotics. High resistance to cefotaxime, cefepime, ceftriaxone and ceftazidime showed that 3<sup>rd</sup> generation of cephalosporins is not suitable choice for the treatment of *Acinetobacter* infections. In a study performed in Nashville, USA (1), the highest antibiotic resistance was reported against imipenem, ampicillin, sulbactam and amikacin (58%) that was considerably lower than observed results in this study and other studies from Iran and other Asian countries. This may imply that antibiotic resistance rate in developed countries is lower than developing ones probably because of avoiding indiscriminate use of antibiotics. The group of 70-79 years old showed the highest frequency of infection which can be due to the weakness of the immune system making these subjects susceptible to the opportunistic infections. On the other hand, most of the isolates (48.3%) were collected from intensive care unit (ICU) perhaps because of long hospitalization of patients with special conditions and use of intensive treatments like tracheal tube, urinal and intravascular catheters.

Number of *Acinetobacter* strains which were phenotypically confirmed as ESBL-positive was lower than that of confirmed through PCR. It can be due to

the improper expression of some ESBL genes despite of the presence within bacterial genome.

Considering low incidence of ESBL-producing *Acinetobacter* isolates in the present study, it is obvious that in addition to ESBL enzymes other mechanisms such as efflux pumps and membrane porins may be involved in the development of antibiotic resistance. Hence, performing phenotypic tests alone for detection of ESBL-positive strains is not sufficient and genotype profiling is strongly recommended. However, the rise of antibiotic resistance among ESBL-producing *Acinetobacter* strains illustrates the major role of this enzyme in bacterial resistance to the antibiotics. Genotypic and phenotypic detection of ESBL producing strains play important roles in the treatment of related infections and prevention of developing antibiotic resistant strains.

The results obtained from well diffusion agar show that all types of *F. gummosa* essential oils and extracts had inhibitory effects on *A. baumannii* reference strain ATCC 19606. Methanolic extract and essential oil at concentration of 300 mg/ml had the highest antibacterial effect. To our knowledge, there are few reports on bacterial inhibitory effects of *F. gummosa* essential oil and extract especially on *Acinetobacter* strains as MDR nosocomial agents.

*Ferula gummosa* methanolic extract prepared in this study was analyzed by GC/Mass. Among 33 components, a naphthalene derivative and guaiol oil were accounted for most of the compounds. It has been found that naphthalene and its derivatives have antimicrobial effects when tested against most of pathogenic Gram-negative microorganisms in addition to *Staphylococcus aureus* and the tested yeast species and can utilize as novel antimicrobial compounds (29-31). Guaiol oil, as a bioactive molecule, also has shown activity against several bacterial strains (32). Extraction of natural products directly from *F. gummosa* is time consuming and cost effective compared to the synthesis methods. *F. gummosa* is a rich source of novel bioactive molecules with significant antimicrobial and antifungal activities and oil extracts from other parts of *F. gummosa* contain various components. In a study conducted in 2005, *F. gummosa* Boiss fruit volatile oil was analyzed by GC/Mass and major detected components were  $\beta$ -pinene (43.78%),  $\alpha$ -pinene (27.27%) and myrcene (3.37%) (33). In another study in 2011 (18), antimicrobial effect of *F. gummosa* root methanolic extract was tested against bacteria causing gastroenteritis. The highest MIC (1/1024) was shown

against *Proteus mirabilis*, *Escherichia coli* and *Klebsiella pneumoniae* which was higher than the activity of the aqueous extract (1/4 dilution) of our study. Oleo-gum-resin of *F. gummosa* has considerable antibacterial activity and its alcoholic essential oil showed considerable antibacterial activity (MIC of 1/16). Furthermore, metanolic and etanolic extracts have led to the inhibition of *P. aeruginosa* growth at minimum of 6.25 and 12.5 mg/ml concentrations respectively (22) that were more lower than our findings.

In comparison to a few previous studies carried out on Gram-negative bacteria, MIC and MBC values were considerably lower than our findings. However, none of the previous studies examined *Acinetobacter* strains. In accordance with findings reported by Mahboubi, et al. MIC and MBC values of *F. gummosa* essential oil were 4 and 8 µl/ml for both *Escherichia coli* and *Pseudomonas aeruginosa*, respectively (21). These findings probably are due to the higher quantity of β-pinene (62.7%) in their study compared with ours (10.1%).

# CONCLUSION

This study covered a wide range of hospital units and various clinical samples. We also used three types of extracts and essential oils (aqueous, aqueous -methanolic and methanolic) to investigate the antibacterial effects of different types of extracts and essential oils. Considering the prevalence of antibiotic resistant *Acinetobacter* strains in various clinical samples of hospitalized patients, it is important to equip diagnostic laboratories with phenotypic and molecular detection methods to assess the prevalence of MDR *Acinetobacter* strains and enforce strategies for treatment and control of antibiotic resistance in bacteria.

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