Saudi plants as a source of potential β-lactamase inhibitors

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Abstract: This study was performed to assess the potential β-lactamase inhibitory properties of nineteen crude Saudi plant extracts belonging to eight families against extended spectrum β-lactamase (ESβL) strains of *Klebsiella pneumoniae* and other medically important pathogens. A total of 276 microbial isolates of pathogenic bacteria were used in this study; only 15 of them showed decreased sensitivity to one or several of ceftazidime, aztreonam, cefotaxime or ceftriaxone, which are deemed to be possible producers of ESβL. Antibacterial activities of plant extracts were carried out against ESβL positive isolates by the disc diffusion method. The potential ESβL suppressing activities of plant extracts and prepared fractions, (chloroform and methanol), with or without antibiotic were studied by disc diffusion method. Results revealed that selected plant extracts showed no antibacterial activity against tested strains; meanwhile, only *Echinops viscosus, Pulicaria arabica, Tephrosia nubica, Chrozophora oblongifolia,* and *Clutia myricoides* showed pronounced ESβL inhibitory activities. The extracts were quantified for phenolic compounds and their antioxidant properties. Bio-guided fractionation of the active extracts revealed that the chloroform fraction of *C. myricoides* possess a promising ESβL inhibitory activity. The separation and the structural elucidation of the active compounds from *C. myricoides* will offer beneficial leads for developing β-lactamase inhibitors.

Keywords: β-lactamase inhibitors, *Clutia myricoides*, medicinal plants, anti-bacterial.

INTRODUCTION

Contagious diseases are the second significant reason for death around the world, and the capacity to oppose numerous classes of anti-microbial agents is the key factor empowering pathogenic living beings for nosocomial survival.

Management of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* is greatly troublesome as a result of the resistance to antimicrobials with various resistance mechanisms which are connected with severe infections (Lee *et al.*, 2001). *Klebsiella pneumoniae* is a serious microbe related with numerous hospitals acquired infections. The level of beta-lactamase producing *Escherichia coli* began to wind up progressively harder to be managed, especially those containing *bla* CTX-M, *bla*SHV, and *bla*TEM genes (Kaur and Aggarwal, 2013). The β-lactamases are the principal mechanism of resistance to β-lactam antimicrobials (Hujer *et al.*, 2002). On the other hand, Gram-negative microorganisms are actually more unaffected by antibiotics than Gram-positive ones, and this is attributable to transmembrane efflux (Wright, 2005).

Resistance of these bacteria to antibiotics containing β-lactam ring is solved by combination of these antibiotics with β-lactamase inhibitors. Many well-known examples are marketed like combination of clavulanic acid or sulbactam as β-lactamase inhibitors with antibiotics (Chen *et al.*, 2013).

There is a critical requirement for a possible source of new, potential and safe antimicrobial medications without cross-resistance to others. Unfortunately, the available β-lactamase inhibitors are not efficient against β-lactamase B, C and D, which necessitate discovering either a broad spectrum β-lactamase inhibitors or new resistant β-lactam antibiotics to bacterial enzymes.

Natural products have prompted to disclosure of new compounds and medication leads (Fenical and Jensen, 2006). Many previous works have been achieved on ability of some plants to inhibit β-lactamase enzyme and augment the effect of antibiotics (Aqil *et al.*, 2005; (Gangoué-Piéboji *et al.*, 2007). Some publications reported activity of medicinal plants as β-lactamase inhibitors (Gangoué-Piéboji *et al.*, 2007). In a screening of a group of anti-bacterial Indian plants, *Punica granatum* and *Delonix regia* showed high activity against β-lactamase (Aqil *et al.*, 2005). Moreover, previous work reported antibacterial activity of *Garcinia kola* against multidrug resistance ESβL positive *Escherichia coli*. Moreover, volatile oil constituents from some *Artemisia* species showed antimicrobial activity against ESβL producing *E. coli* and augment the action of antimicrobials (Asili *et al.*, 2015). Myricetin is a flavonol that can inhibit the production of ESβL from *K. pneumoniae* isolates and upon mixing with β-lactam antibiotic and β- lactamase a potentiation of its effect was observed (Lin *et al.*, 2005).

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In this work; some selected Saudi plants were screened for their activity against ESβL strains of *K. pneumoniae* and other medically important pathogens. The most effective plant was subjected to fractionation to identify the bioactive fraction. The effect of bioactive extract was studied against ESβL-producing strains.

MATERIALS AND METHODS

Plant selection

Nineteen plants belonging to eight families were collected from Saudi desert localities, such as al Taif and Abha governorates. Plants were identified by Dr. Emad Al-Sharif, Associate Professor of Plant Ecology, Dept. of Biology, Faculty of Science & Arts, Khulais, King Abdulaziz University, Saudi Arabia. A specimen was reserved in the herbarium of faculty of pharmacy, King Abdulaziz University.

Extraction of Plant material

Dried aerial parts of each plant was separately grinded and extracted with MeOH using ultraturrex homogenizer on cold till exhaustion followed by evaporation of solvent under vacuum followed by freeze drying. The total alcoholic extract was dissolved in DMSO in a concentration of 10mg/ml and kept in the refrigerator as a stock solution for the bioassay preliminary screening. Bioactive alcoholic extracts were suspended in least amount of water and fractionated with chloroform leaving aqueous mother liquor. Prepared fractions were evaporated separately under reduced pressure and kept in the refrigerator for further study.

Quantification of phenolic compounds

Quantification of phenolic constituents in the bioactive extracts was achieved by using the Folin Ciocalteu method. In addition, the radicle scavenging activity was assessed using 2,2 diphenyl 1 picrylhydrazyl radical scavenging assay (Abdallah *et al.*, 2014).

Bacterial strains

A total of 276 microbial isolate of *P. aeruginosa* (n=107), *A. baumannii* (n=82), *K. pneumoniae* (n= 43), *S. maltophilia* (n=24) and *E. coli* (n=20) species were used in this study and were obtained from King Abdulaziz University hospital, Jeddah, Kingdom of Saudi Arabia.

Antibiotic susceptibility testing

The screening for the isolates' antibacterial sensitivity testing was conducted by applying the Kirby Bauer disc diffusion method. This was in agreement with the definition of the Clinical and Laboratory Standards Institute (Wikler, 2009) using the following antibiotics containing discs: Ceftizoxime (30µg), Chloramphinicol (30µg), Colistin (50µg), Gentamicin (10µg), Levofloxacin (5µg), Minocyclin (30µg), Netilmicin (30µg), Piperacillin (100µg), Piperacillin/Tazobactam (75/10 µg), Tobramycin (10µg), Trimethoprim/ Sulfamethoxazole (1.25/ 23.75µg) (Bauer *et al.*, 1966). In short, 20 ml of Mueller- Hinton agar (Difco, USA) was prepared and subsequently put into sterile Petri dishes. The agar medium was left on a flat bench at room temperature to become solid. After this, 1ml of an 18h culture of each bacterium that had been already been adapted to turbidity standard of 0.5 using the McFarland Scale was distributed using a sterile swab across the surface of the solidified agar. Antibiotic discs (Oxoid, UK) were softly and firmly applied to the agar plates, and then placed at room temperature for one hour to enable the antibiotics to diffuse into the agar. Subsequently, the plates were incubated at 35-37°C for 24h (Bauer *et al.*, 1966). Areas of growth inhibition to the nearest millimeter were measured and results were recorded. Isolates were either categorized as resistant or intermediately sensitive or sensitive, according to the standard intermediate chart updated (which was adapted from the standard of the Clinical and Laboratory Standard Institute (Wikler, 2009). An isolate was deemed to be resistant to multiple drugs if it was able to resist a minimum of three antibiotics. Findings were interpreted using zone sizes (Pfaller *et al.*, 2001; Gupta *et al.*, 2004).

Testing for ESβL-production in collected isolates

Isolates that showed diminished susceptibility ceftazidime, aztreonam, cefotaxime or ceftriaxone were deemed to be possible producers of ESβL. Phenotypic confirmation was conducted using double-disc synergy "DDS" test and interpretations were documented according to the criteria provided by the Clinical Laboratory standard Institute (Wikler, 2009). In short, a ceftazidime (30µg) and a ceftazidime + clavulanic acid (30µg/10µg) discs were put 25mm apart on a Mueller-Hinton Agar plate inoculated with a bacterial suspension of 0.5 McFarland turbidity standards.This was then incubated at 35-37°C for 24 =h. An increase of ≥ 5mm in the diameter of the inhibition zone for the combination disc in comparison to the ceftazidime disc ultimately confirm ESβL production (Coudron *et al.*, 1997). *K. pneumonia* (ATCC 700603) and *E. coli* (ATCC 25922) were used as positive and negative controls respectively.

Testing for antibacterial activity

Antibacterial activities of plant extracts were carried out against ESβL positive isolates by applying the disc diffusion method (Murray *et al.*, 1995). Ten µl of 100mg/ml total extracts dissolved in 10% v/v DMSO were applied to sterile paper discs (6 mm in diameter). These were created from Whatman® and spread on the inoculated agar. Negative control and positive controls were used by applying 10% v/v DMSO and suitable antibiotics, respectively. Inoculated plates were incubated at temperature of 37°C for 24 hr. The antibacterial activity was then evaluated by establishing the diameter of inhibition zone compared to the test microorganisms. The assay was conducted three times.

Table 1: Antimicrobial susceptibility pattern among *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*, *S. maltophilia* and *E. coli* isolates

Antimicrobial agents	*P. aeruginosa* (107 isolates)			*A. baumannii* (82 isolates)			*K. pneumoniae* (43 isolates)			*S. maltophilia* (24 isolates)			*E. coli* (20 isolates)		
	Sensitive	Intermediately sensitive	Resistant	Sensitive	Intermediately sensitive	Resistant	Sensitive	Intermediately sensitive	Resistant	Sensitive	Intermediately sensitive	Resistant	Sensitive	Intermediately sensitive	Resistant
Ceftizoxime	15	0	92	27	10	45	-	-	-	-	-	-	-	-	-
Gentamicin	68	15	24	-	-	-	-	-	-	-	-	-	-	-	-
Netlimicin	92	1	14	-	-	-	-	-	-	-	-	-	-	-	-
Piperacillin	65	13	29	-	-	-	17	12	14	-	-	-	2	6	12
Tobramycin	81	3	23	-	-	-	-	-	-	-	-	-	-	-	-
Chloramphenicol	-	-	-	-	-	-	38	0	5	-	-	-	10	7	3
Levofloxacin	-	-	-	14	5	63	37	4	2	17	3	4	9	5	6
Trimethoprim/ Sulfamethoxazole	-	-	-	35	0	47	32	0	11	6	11	7	5	5	10
Colistin	-	-	-	22	0	60	-	-	-	-	-	-	-	-	-
Piperacilin-Tazobactam	-	-	-	27	10	45	-	-	-	-	-	-	-	-	-
Minocycline	-	-	-	-	-	-	-	-	-	24	0	0	-	-	-

Table 2: ESβL producing isolates confirmed by double-disc synergy test

Isolate	Number of isolates	ESβL positive isolates
P. aeruginosa	107	1 (0.93%)
A.baumannii	82	3 (3.6%)
K. pneumoniae	43	7 (16%)
S. maltophilia	24	4 (16.7%)
E. coli	20	2 (10%)
Total	276	17

Table 3: Antibacterial and anti ESβL activity of crude methanol extracts

Family	Plant name	Specimen Number	Antibacterial/ anti ESβL activity*
Asteraceae	*Centaurea pseudosinaica* Mouterde	CP	-/-
	Echinops viscosus Dc.	EV	-/+
	Echinops sheilae Kit Tan	ES	-/-
	Euryops arabicus Steud.	EA1066	-/-
	Onopordum ambiguum Fresen.	OA	-/-
	Osteospermum vaillantii (Decne.) Norl.	OV	-/-
	Psiadia punctulata Vatke	PP1065	-/-
	Pulicaria arabica Cass.	PA	-/+
Hypericaceae	*Hypericum revolutum* R. Keller	HR	-/-
Leguminoseae	*Astragalus abyssinicus* Steud.	AA1012	-/-
	Tephrosia nubica Baker	TN	-/+
Asclepediaceae	*Caralluma tuberculata N.E.Br.*	CT1027	-/-
	Leptadenia pyrotechnica (Forssk.) Decne.	LP	-/-
Euphorbiaceae	*Chrozophora oblongifolia* (Delile) A.Juss. ex Spreng	CO	-/+
	Clutia myricoides Jaub. & Spach	CM	-/++
Clusiaceae	*Garcinia mangostana* L.	GM	-/-
Malvaceae	*Abutilon fruticosum* Guill. & Perr.		-/-
	Triumfetta flavescens Hochst. ex A. Rich.		-/-
Caryophyllaceae	*Cometes abyssinica R.Br.*		-/-

* -, no antibacterial nor anti ESβL activity, +, a ℜ 5 mm increase in the diameter of inhibition zone for the combination disc, ++, a ≥ 5 mm increase in the diameter of inhibition zone for the combination disc, concentration of extract was 100 mg/ml

Table 4: Anti ESβL activity of chloroform and methanolic fractions of positive crude plant extracts

Plant name	Fraction	Anti ESβL activity
Echinops viscosus Dc.	Chloroform	-
	Methanol	-
Tephrosia nubica Baker	Chloroform	-
	Methanol	-
Chrozophora oblongifolia (Delile) A.Juss. ex Spreng	Chloroform	-
	Methanol	-
Pulicaria arabica Cass.	Chloroform	-
	Methanol	-
Clutia myricoides Jaub. & Spach	Chloroform	+
	Methanol	-

Concentration of extract was 10 mg/ml

Characterization of possible ESβL inhibitory activities

The disc diffusion method was used to explore the potentiation between antibiotic discs (methicillin, 100 µg, cephalothin, 30 µg, cephalexin, 30µg, cefuroxime Na, 30 µg, ceftazidime, 30µg, cefixime, 5µg, ceftizoxime, 30 µg and cefepime, 30µg) with crude plant extracts, chloroform and aqueous fractions. In order to establish the anti-ESβL activity of plant extract with antibiotics, the control antibiotic disc and the disc of antibiotic which contained 10µl of each plant extract were put 25mm apart on a Mueller-Hinton agar plate which was inoculated with ESβL pathogens of 0.5 McFarland turbidity standards. These were incubated at a temperature of 37°C for 24hr. A positive interaction is suggested by the enlargement of the size of inhibition zones (Chaudhary, 1996)**.**

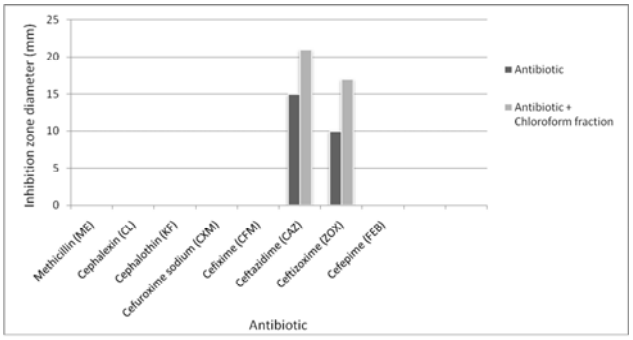

Fig. 1: Antimicrobial susceptibility pattern among ESβL positive *E. coli* isolates with and without addition of chloroform fraction of *Clutia myricoides*

RESULTS

Quantification of phenolic compounds

The results revealed that *C. oblongifolia* displayed the highest phenolic contents (12.45 mg GAE/g dried extract), followed by *P. arabica* (9.76mg GAE/g dried extract), *E. viscosus* (8.27mg GAE/g dried extract), and *C. myricoides* (7.22mg GAE/g dried extract). Furthermore, the bioactive extracts showed distinct anti-oxidant activity in DPPH assay. *P. Arabica* was the most potent as scavenger for DPPH (IC_{50} 22µg/ml) followed by *C. myricoides* (IC_{50} 25µg/ml) and *C. oblongifolia* (IC_{50} 30µg/ml).

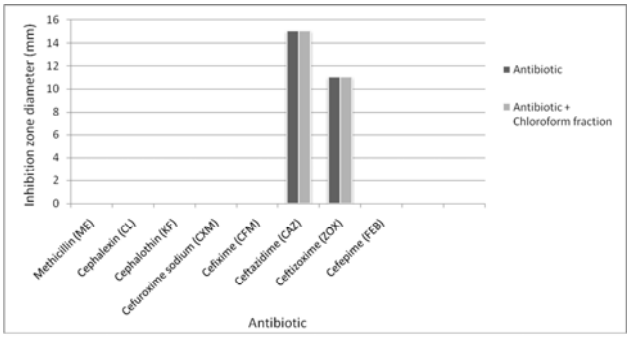

Fig. 2: Antimicrobial susceptibility pattern among ESβL positive *K. pneumoniae* isolates with and without addition of chloroform fraction of *Clutia myricoides*

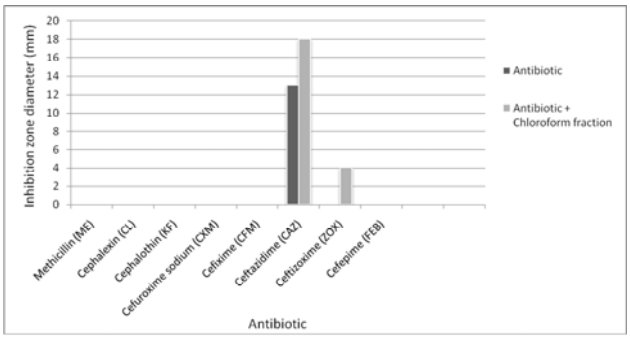

Fig. 3: Antimicrobial susceptibility pattern among ESβL positive *S. maltophilia* isolates with and without addition of chloroform fraction of *Clutia myricoides*

Antibiotic sensitivity testing

The antimicrobial sensitivity testing (table 1) of *P. aeruginosa* isolates revealed that 86% of the isolates were sensitive to netlimicin followed by tobramycin (75.7%), gentamicin (63.5%), pipracillin (60.7%) and ceftizoxime (14%). Regarding *A. baumannii* isolates the results revealed that 42.7% were sensitive to colistin followed by gentamicin, piperacillin/tazobactam and trimethoprim/ sulfamethoxazole (33%, 26.8% and 17%), respectively,

while testing of *Klebsiella* isolates revealed that 88.4% of the isolates were sensitive to chloramphenicol followed by levofloxacin (86%), trimethoprim/sulfamethoxazole (74.4%), followed by piperacillin (39.5%). The antimicrobial susceptibility testing of *S. maltophilia* isolates revealed that all isolates (100%) were sensitive to minocycline, 70.8% levofloxacin and 25% trimethoprim/ sulfamethoxazole. Fifty percent of *E. coli* isolates were found to be sensitive to chloramphenicol, 45% to levofloxacin, 25% to trimethoprim/sulfamethoxazole and 10% were sensitive to piperacillin.

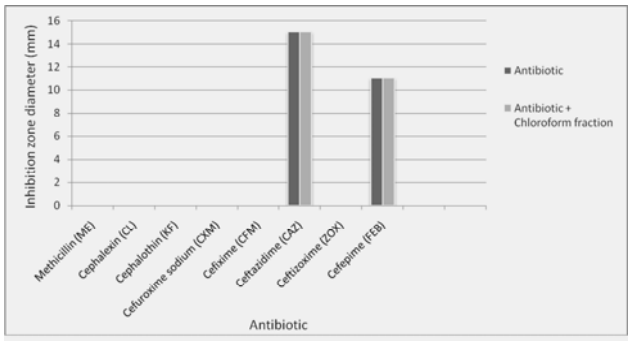

Fig. 4: Antimicrobial susceptibility pattern among ESβL positive *A. baumannii* isolates with and without addition of chloroform fraction of *Clutia myricoides*

Testing for ESβL-production in collected isolates

It was found that *S. maltophilia* isolates were the highest among gram negative ones that showed ESβL activity (16.7%), followed by *K. pneumonia* (16%), *E. coli* (10%), *A. baumannii* (3.6%) and then *P. aeruginosa* (1%) (table 2).

Testing for antibacterial activity

It was found that no antibacterial activities were detected for the tested plant extracts, however, *Echinops viscosus*, *Pulicaria arabica*, *Tephrosia nubica* and *Chrozophora oblongifolia* extracts have shown an enlargement of the diameter of inhibition zone when combined with antibiotic discs less than 5mm, while *Clutia myricoides* extract has shown an enlargement of the diameter of inhibition zone of combination discs more than 5 mm as illustrated in table 3.

Characterization of possible ESβL inhibitory activities

Only chloroform fraction of *C. myricoides* has shown an obvious activity against *E. coli, S. maltophilia* and *P. aeruginosa* with a marked enlargement of inhibition zones (>5mm) in combination with third generation cephalosporin antibiotics (table 4, fig. 1-5).

DISCUSSION

In this study a total of 276Gram negative microbial isolates were collected from King Abdulaziz University hospital, Jeddah, KSA. Isolates were *P. aeruginosa, A. baumannii, K. pneumoniae, S. maltophilia* and *E. coli* with the following percentages respectively (38.7%, 29.8%, 15.5%, 8.8% and 7.2%) .

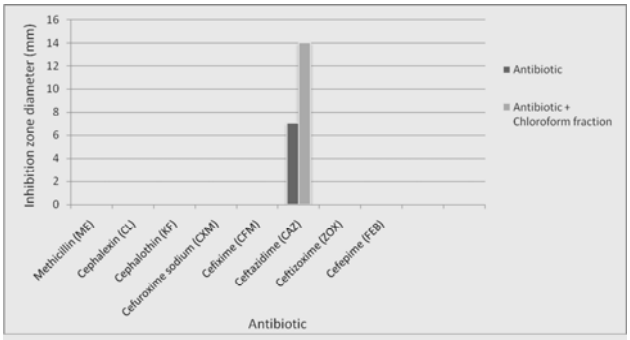

Fig. 5: Antimicrobial susceptibility pattern among ESβL positive *P. aeruginosa* isolates with and without addition of chloroform fraction of *Clutia myricoides*

The results obtained in this work regarding resistance to anti-biotic is in agreement with previous results obtained for developing countries (Kumari *et al.*, 2007; Lekhak and Sherchand, 2007). The resistance of *P. aeruginosa* isolates against aminoglycosides was high (20%) in comparing with a previous hospital study where the resistance rate among *P. aeruginosa* was only 5–9% (Gerding *et al.*, 1991; Bouza *et al.*, 1999). The susceptibility testing of *A. baumannii* isolates revealed highest sensitivity to colistin, that was in agreement with recent research on clinical isolates in the Western Pacific region indicated only 3.3% resistance of *A. baumannii* to colistin (Yau *et al.*, 2009). On the other hand a Korean study revealed higher resistance of this isolates to colistin (30.6%) and polymixin (18.1%) (Ko *et al.*, 2007). Regarding *K. pneumoniae* isolates, the findings were in accordance with reports acquired from Japan (Watanabe *et al.*, 1995), USA (Doern *et al.*, 1988; Jorgensen *et al.*, 1990; Sahm *et al.*, 2008), Turkey (Gonlugur *et al.*, 2004) and Pakistan (Jonaidi *et al.*, 2009). The resistance pattern of *S. maltophilia* isolates against trimethoprim–sulfamethoxazole was 47%, which differ significantly from previous findings, where trimethoprim–sulfamethoxazole has been the drug of choice for treatment of *S. maltophilia* infections (Gales *et al.*, 2001; Cantón *et al.*, 2003).

Isolates were then screened for ESβL activities by double-disc synergy test where some of the collected strains displayed ESβL activity. In this work, it was observed that *E. coli* and *K. pneumoniae* isolates had shown high ESβL activities which is in accordance with previous reports in Asia, where there rates were 41% and 36% respectively in Pakistan (Jabeen *et al.*, 2005), 43.2% and 39.5% respectively in Bangladesh (Rahman *et al.*, 2004), respectively. Moreover, results of research done by the Songklanagarind Hospital revealed that 32% of *K. pneumoniae* and 19% of *E. coli* isolates produced ESβL (Ingviya *et al.*, 2003). The percentage of ESβL producers

detected by the phenotypic confirmatory method was different form a previous study in Turkey, where ESβL rates were 48%, 40% and 14% for *K. pneumoniae*, *K. oxytoca* and *E. coli* isolates respectively (Bülüç *et al.*, 2003; Rodrigues *et al.*, 2004). One the other hand the existence of positive isolates for extended spectrum beta lactamases in the United States of America, Europe, Latin America, the Middle East and Asia/Pacific was 3, 5, 10, 20 and 13% for *E. coli* and 17, 7, 11, 14 and 18% for *Klebsiella* spp. respectively (Paterson *et al.*, 2005). In India, a prevalence of 28% for ESβL production in *Acinetobacter* spp. was identified (Sinha *et al.*, 2007).

In this work, antibacterial and anti ESβL activity of crude methanol extracts of 19 plants belonging to eight families were performed, where no antibacterial activities were detected for the tested extracts. Meanwhile, five plant extracts showed anti ESβL activity (*E. viscosus*, *P. arabica*, *T. nubica*, *C. oblongifolia* and *C. myricoides*), but upon fractionation of these extracts, only chloroform fraction of *C. myricoides* retains anti ESβL activity.

C. myricoides (Soa'bor); which showed promising activity; is used in folkloric medicine as wound healer (Alsufyani *et al.*, 2007). Phytochemical screening revealed the existence of anthraquinone, cardiac glycosides, saponins, flavonoides, coumarins, condensed tannins, triterpenoids, steroids and alkaloids and absence of essential oil and hydrolysable tannins (Alsufyani *et al.*, 2007). Only polar fraction of the plant showed potent antimicrobial activity against *P. aeruginosa.* Furthermore, the daily topical application of the entire ethanolic extract made wounds heal much quicker in rats used in excision models (Alsufyani *et al.*, 2007). In this study only the non-polar fraction demonstrated pronounced activity as β-lactamase inhibitor. The activity of this fraction may be referred to its anthraquinone contents which are known for their activity as β-lactamase inhibitors (Monaghan *et al.*, 1982).

These results support the mixing of this herbal extract with anti-biotic to increase its efficiency. Nevertheless, the interaction between active compounds in the extract and antibiotics should be taken in consideration. Many workers reported potentiation of phyto-compounds and antibiotic effect by plant extracts (Nascimento *et al.*, 2000; Zhao *et al.*, 2001; Aqil *et al.*, 2005). Nonetheless, in order to evaluate how safe these extracts are, more *in vitro* and *in vivo* toxicity assays must be carried out. In addition, further research on the isolation and structural elucidation of the active compounds in *C. myricoides* will offers a beneficial lead for developing *β*-lactamase inhibitors. Moreover, *in vivo* stability and efficacy, is vital for the management of infectious diseases that result from multi drug resistant bacteria.

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