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In vitro Antibacterial Potential of *Centaurea behen* L. against *Klebsiella pneumoniae* and *Acinetobacter baumannii*

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Abstract
Background: *In most of the developing countries of the world, plants are the main medicinal sources used in treating infectious diseases. Currently, the Centaurea genus has a great of interest due to its beneficial properties and worldwide distribution. The new generation of disease causing pathogens and mutations of existing microorganisms leading to antibiotic resistance are responsible for human morbidity and mortality. Centaurea behen (C. behen) is known to be rich source of bioactive substances that may serve as a natural remedy against antibiotic resistant pathogens.*
Objective of the study*: The study was conducted with an objective to find out antimicrobial potential of Centaurea behen L. against multidrug resistant (MDR) strains of Klebsiella pneumoniae (K. pneumoniae) and Acinetobacter baumannii (A. baumannii).*
Material and methods: *Chloroform, methanol, hexane and aqueous extracts of C. behen dried roots were tested for antimicrobial potential against MDR K. pneumoniae and A. baumannii by cup plate method and MIC (µg/ ml) of each extract was determined.*
Results*: All extracts of C. behen were found to have inhibitory effect against K. pneumoniae. Maximum inhibition was shown by methanol extract followed by aqueous, hexane and least effect was observed with chloroform extract. In case of A. baumannii, chloroform extract showed maximum inhibition followed by aqueous, methanol and hexane extracts.*
Conclusion*: C. behen extracts have potential antibacterial action against multi-drug resistant and challenging superbugs like K. pneumoniae and A. baumannii. This study might open the possibilities of finding new clinically effective herbal remedy against multi-drug resistant bacterial pathogens.*
Keywords*: Centaurea behen L., Multi-drug resistant bacteria, Plant extracts, K. pneumoniae, A. baumannii*

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I. INTRODUCTION

Since several decades, many medicinal plants have been used as a source of natural medicines. About 80 % of developing countries depend on medicinal plant based in their human primary health care.[1] Plants contain many bioactive compounds and majority of these bioactive compounds are secondary metabolites belong to groups of steroids, alkaloids and phenol compounds, resins, fatty acids, tannins flavonoids, etc.[2] Various studies have established that medicinal plants exhibit antioxidant and antimicrobial properties which can safeguard the human body against both pathogens and cellular oxidation reactions. Thus, it is very important to characterize the different types of compounds of medicinal plants for their antimicrobial activity as plant based products have many advantages than synthetic chemicals compounds such as greater activity, less cost, easy availability and decreased side effects.[3] Due to indiscriminate use of antibiotics in last few years, infections caused by multidrug-resistant (MDR) organisms have become difficult to treat. Moreover, these type of infections are associated with increased morbidity and mortality as compared to those caused by susceptible bacteria. Antimicrobial resistance genes may be carried on the bacterial chromosome, plasmid, or transposons. There are several mechanisms of drug resistance in bacteria which include drug inactivation/alteration, modification of drug binding sites/targets, changes in cell permeability resulting in reduced intracellular drug accumulation and biofilm formation.[4,5]

K.pneumoniae causes urinary tract infections, pneumonia, endophthalmitis, meningitis, brain abscess, septic pulmonary embolic, lung abscess, splenic abscess, osteomyelitis etc.The important aspects of *Klebsiella* associated infection is the emergence of multi-drug resistance.[6] Members of Genus *Acinetobacter* have emerged as organisms of questionable pathogenicity and pan resistant nosocomial pathogens worldwide in past two or three decades; especially since 2005-2006. Critically ill patients acquire an infection during their stay in an Intensive care unit (ICU) and the frequency of these infections varies considerably in different populations in clinical settings. Most multidrug resistant *A. baumannii* outbreaks occur in critical care settings and involve resistance to multiple classes of antimicrobial agents. [7]

II. OBJECTIVE OF THE STUDY

The study was conducted with an objective to find out antimicrobial potential of *Centaurea behen* L. against multidrug resistant (MDR) strains of *Klebsiella pneumoniae (K. pneumoniae) and Acinetobacter baumannii (A. baumannii).*

III. MATERIAL AND METHODS

The study was conducted in Centre for Interdisciplinary Biomedical Research and in Bacteriology Laboratory in the Department of Microbiology, Adesh Institute of Medical Sciences & Research (AIMSR), Adesh University Bathinda. The study was approved by Institutional Research Committee and Ethics Committee of Adesh University.

Plant Material

Dried roots of *Centaurea behen* L. were procured from a certified and authorized Herb Store and identity of plant was confirmed through NISCAIR, New Delhi.(Letter No:. NISCAIR/RHMD/CONSULT/2018/3236-37-3)

Solvent Extraction of plant material

Hexane, Chloroform and Methanol were employed for Soxhlet extraction of active ingredients using Soxhlet apparatus and finally the drug was boiled with distilled water to obtain water extract.[8]

Recovery of solvents and Drying of residual mass

Solvents from extracts were recovered under reduced pressure using rotary vacuum evaporator and the dried extracts were preserved in a vacuum desiccator containing anhydrous silica gel. Extracts were filtered, concentrated using rotary vacuum evaporator, and dried in an oven at 40-50 ºC. The dried extracts were preserved in a vacuum desiccator over fused calcium chloride. All the extracts were screened for different classes of phytoconstituents using specific standard reagents.[9]

Bacterial Strains: The bacterial isolates were obtained from Department of Microbiology, AIMSR which were identified through colony characteristics, gram staining morphology, conventional biochemical tests and confirmed with Automated Identification Biomerieux Vitek 2 System using GN cards. *K. pneumoniae* was isolated from urine, pus, endotracheal secretions and *A. baumannii* was isolated from endotracheal secretions and blood samples as shown in Table1.

Table 1: Source of MDR *K. pneumoniae* and *A. baumannii* isolates.

Bacterial strain	Source / Clinical Sample
K. pneumoniae (n=3)	Urine (n=1), Pus (n=1), Endotracheal secretions(n=1)
A. baumannii (n=3)	Endotracheal secretions(n=2), Blood (n=1)

Antimicrobial Susceptibility Testing: Antibiogram of isolates was assessed using the Vitek 2 Compact system (Biomereiux®). Identity of *K. pneumoniae and A. baumannii* was confirmed by using N280 and N281 cards supplied by Biomereiux**..** The organisms which were resistant three or more families of antibiotics tested were considered as Multidrug resistant (MDR).

Stock solutions: Stock solutions of extracts were prepared by dissolving the extracts in DMSO. These solutions were then used to prepare test solutions of desired range of concentrations.

Test solutions

Test solutions of extracts were prepared in DMSO to produce solutions of various concentrations ranging from 50 to 1000µg/ml. Following concentrations were prepared; 50, 100, 200, 500, 1000 µg/ml.

Preparation of inoculum:

To prepare inoculum, a loopful of isolated colony of MDR test strains was taken and inoculated into nutrient broth and incubated at 37^0 C for 6-4 hrs. This cell suspension was used to prepare inoculum (Conc. = 10^8 CFU/ml). The cell suspension was standardized to obtain CFU= 10^8 per ml using Densitometer

(Biomerieux®).This concentration is equivalent to 0.5 Mc Farland conc.; ideal to be used for assaying antimicrobial activity of plant extracts.[10]

Antimicrobial assay:

Cup-plate or cylindrical plate method

The Mueller Hinton agar medium was poured into sterile petri-plates and allowed to solidify. Inoculum of test microorganism was then spread on the surface of agar plate by using sterile cotton swab. Holes of 6 mm in diameter were cut in the medium with sterile cork borer. The volume of solutions added to each cavity or cylinder was kept uniform to fill the holes. 50 microliters of solutions of each concentrations of extracts prepared in DMSO were added in the cavities or cylinder prepared in a solid medium using micropipette under strict aseptic conditions in the laminar flow bench .The plates were left for 1 to 2 hours at room temperature so as to provide a sufficient pre-incubation diffusion period which in turn minimize the effects of variations in time between the applications of different solutions. All the plates were then incubated for about 18 to 24 hours at 37º C. The zones of inhibition obtained after incubation were considered the basis of measurement of antimicrobial activity. Diameter of any resultant zone of inhibition including well size was measured in millimeters. DMSO and Distilled water were used as vehicle control and negative control respectively. The minimum concentration of the extract/s showing a clear zone of inhibition was considered to be MIC of that particular extract on a particular bacterial strain. [11,12]

IV. RESULTS

Phytochemical screening

Hexane extract of *C. behen* showed the presence of phytosterols, fixed oils & fats and terpenoids & triterpenoids. Chloroform extract showed presence of alkaloids, terpenoids & triterpenoids. Glycosides, phenolic compounds & tannins, flavonoids were present in methanolic extract. Aqueous extract showed presence of carbohydrates, terpenoids & triterpenoids. Proteins and amino acids were not found in any of the extracts of *C. behen*. The results of phytochemical evaluation are shown in Table 2.

Table 2: Phytochemical screening of various extractives of *C. behen* L.

S. No.	Name of tests and Test Reagent used	Hexane Extract	Chloroform Extract	Methanol Extract	Aqueous Extract
1.	**Alkaloids**				
	Hager's regent	-	+	-	-
	Wagner's regent	-	+	-	-
	Mayer's regent	-	+	-	-
2.	**Carbohydrates**				
	Molisch's regent	-	-	-	+
	Fehling regent	-	-	-	+
3.	**Proteins and amino acids**				
	Ninhydrin reagent	-	-	-	-
	Biuret test	-	-	-	
	Xanthoproteic test	-	-	-	-
4.	**Phytosterols**				
	Salkowski test	+	-	-	-
	Liebermann Burchard's	+	-	-	-
	Lieberman test	+	-	-	-
5.	**Phenolic compounds and tannins**				
	Lead acetate test	-	-	+	-
	Acetic acid test	-	-	-	-
	Potassium dichromate test	-	-	-	-
	Nitric acid test	-	-	+	-
6.	**Saponins**				
	Foam test	-	-	-	+
	Haemolytic test	-	-	-	+
7.	**Flavonoids**				
	Shinoda test	-	-	+	-
	Sodium hydroxide test	-	-	+	-
8.	**Fixed oils and fats**				
	Staining test	-	-	-	-
	Saponification test	-	-	-	-
9.	**Terpenoids/Triterpenoids**				
	Salkowski test	+	+	-	+
10.	**Glycosides**				
	Borntrager's test	-	-	+	-

Antimicrobial activity

All extracts of *C. behen* were found to have inhibitory effect against *K. pneumoniae.* Maximum inhibition was shown by methanol extract followed by aqueous, hexane and least effect was observed with chloroform extract. But in case of *A. baumannii*, all the extracts showed more activity as compared to *K. pneumoniae* with MIC as 50µg/ml and 100µg/ml respectively**.** Chloroform extract showed maximum inhibition followed by aqueous, methanol and hexane extracts. DMSO (Vehicle control) and Distilled water(negative control) did not show any antimicrobial activity against both the organisms.The results of antimicrobial activity are shown in Table 3. Mueller Hinton Agar plate showing antibacterial activity of *C. behen* extracts on *K. pneumoniae* and *A.baumannii* is illustrated in Figure 1&2 respectively.

Table 3: Antimicrobial activity of extracts of *C. behen* L.

Type of extract	Name of bacterial isolate	Average zone of inhibition(mm)	MIC(µg/ml)
Hexane	*K. pneumoniae*	11.6	100
	A.baumannii	10.3	50
Chloroform	*K. pneumoniae*	10.3	100
	A.baumannii	14.3	50
Methanol	*K. pneumoniae*	14.6	100
	A. baumannii	11.6	50
Aqueous	*K. pneumoniae*	12.6	100
	A. baumannii	12.3	50

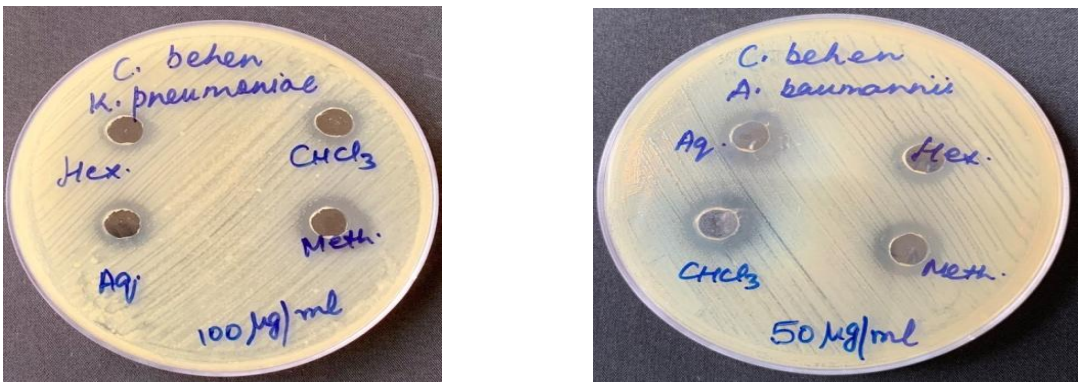

Figure 1 and 2 : Mueller Hinton Agar plate showing antibacterial activity of *C. behen* extracts on *K. pneumoniae* and *A.baumannii* at conc. 100µg/ml and 50µg/ml

V. DISCUSSION

In pre antibiotic era, microbial infections were the major cause of untimely death in humans. Soon after the discovery of antibiotics, death rate of microbial infection has significantly decreased, even though, drug resistant microorganisms remain a major thereat for human beings. Therefore, newer antimicrobial compounds with low/no side effects are desirable for pharmaceutical applications. Higher trees synthesize a variety of phytochemicals compounds as secondary metabolites to protect themselves from the microbial infections and environmental stress conditions. These phytochemicals are the key compounds with many medicinal properties. According to present study**,** hexane extract of *C. behen* showed the presence of phytosterols, fixed oils & fats and terpenoids & triterpenoids. Chloroform extract showed presence of alkaloids, terpenoids & triterpenoids. Glycosides, phenolic compounds & tannins, flavonoids were present in methanolic extract. Aqueous extract showed presence of carbohydrates, terpenoids & triterpenoids. Proteins and amino acids were not found in any of the extracts of *C. behen*. Chougule et al (2012) reported that preliminary photochemical screening of the powdered roots of *C. behen* showed the presence of alkaloids and glycosides.[13] In a study published by Esmaeili et al (2013) reported that phytochemical investigations on *Centaurea* species have shown the presence of flavonoids; sesquiterpene lactones, especially guaianolides; germacranolide type sesquiterpene lactones.[14] Centaurea is also a source of some phytochemical studies for its potentially active substance especially flavonoids sesquiterpene lactones. (Fatih et al 2019).[15] In another study, Escher et al (2018) identified chlorogenic, caffeic, ferulic, and p-coumaric acids, iso quercitrin, and coumarin as major compounds of Centaurea genus.[16]

In the present study, chloroform extract of *C. behen* showed more inhibitory effect against *A. baumannii* as compared to *K. pneumoniae* whereas, methanol extract showed more inhibitory effect against *K. pneumoniae* in comparison with *A. baumannii*. The results is contrary to the results of Cansaran et al (2013) who had reported methanol extract to be inhibitory in case of *K. pneumoniae*.[17] The results of present study finds concordance with Moghannem et al (2016) who had worked particularly using multidrug resistant

bacteria. The study showed that isolates of *K. pneumoniae* and *A. baumannii* were susceptible to the extracts of *Centaurea* spp. [18]

VI. CONCLUSION

The indiscriminate use of antibiotics resulted in the emergence of a number of resistant bacterial strains, and the antimicrobial compounds from plants may inhibit bacteria by a different mechanism than the currently used antibiotics and may have clinical value in the treatment of resistant microbial strains. Based on these results, it is possible to conclude that *C.behen* L. exhibited a broad range of antimicrobial activity particularly methanol and chloroform extracts of the roots showed significant antibacterial activities and could be used as antimicrobial agents in new drugs for therapy. This study might open the possibilities of finding new clinically effective herbal therapy against multi-drug resistant bacterial pathogens.

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