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Isolation of Resistant Gram-Negative Bacterial Strains From Neonatal Intensive Care Units in Tabuk City and its Sensitivity to Some Medicinal Plant Extracts.

KEYWORDS: Multi Drug Resistant Gm –ve bacteria, Cassia angustifolia leaves, Inhibition zone, Neonatal Intensive Care Unit.

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ABSTRACT *The methanolic extract of Cassia angustifolia(sana makkahi) leaves traditionally used for the treatment of infectious disease, were tested for their activity against multi drug resistant (MDR)Gram negative bacteria (Gm –ve b) isolated from Neonatal Intensive Care Unit (NICU) from King Khalid Hospital using the well diffusion method. The Cassia angustifolia extract showed activity against some pathogenic isolates giving inhibition zone diameter of 18 mm at concentration 2.5 mg/ml in case no. 10 of E.coli, 18 mm diameterof inhibition zone at concentration 1.25 mg/ml in case no 12of Pseudomonas aeruginosa and 16 mm diameter of inhibition zone at concentration 5.00 mg/ml in case no. 16 of Klebseilla pneumonia. The antibacterial activity of the Cassia angustifolia leaf extract isreferred to the phytochemistry constituents which prove that the plant leaves contains alkaloids, saponins and tannins whom responsible for the antibacterial activity.*

Introduction:

Infectious diseases were considered curable illnesses, and if one antibiotic in the arsenal of drugs did not clear the infection, another would. Microbes resistant to not one but multiple therapies are common, as a global problem. Ironically, hospitals are large reservoirs for breeding resistant bacterial strains. In fact, approximately 50% of hospital-acquired infections are resistant to different types of antibiotics and become difficult to treat.

The worldwide awareness of health risks associated with non-natural additives, added to the current pathogen concerns, may lead to the investigation of natural products such as; antimicrobials obtained from plant or microbial sources. Several studies have confirmed the antimicrobial activity of phytochemicals from plants that have been used in traditional medicine used as an antiseptic, digestive agent and antihypertensive, however, limited information regarding its antimicrobial activity against pathogens is available so that, our work will try to determine the antimicrobial activity of some medicinal plant extracts against the emerging pathogens.

Infectious diseases were once considered curable illnesses, and if one antibiotic in the arsenal of drugs did not clear the infection, another would. However, that is no longer the case. Microbes resistant to not one but multiple therapies are common and represent a global problem (1). Ironically, hospitals are large reservoirs for breeding resistant bacterial strains. In fact, approximately 50% of hospital-acquired infections are methicillin resistant to different types of antibiotics (2) For example M. tuberculosis (TB), responsible for TB infections, has numerous multi-drug resistant (MDR) strains for which six to seven drugs are needed for effective treatment (3). Also, Acinetobacter spp. has increased over the past decade in the United States (4).

Up to 10%, infants have infections in the first month of life, the matter which results in 30 - 50% of total neonatal deaths in developing countries. These neonatal deaths are attributed principally to infection, birth asphyxia, consequences of premature birth and low birth weight (5).

Natural products have been the greatest source of successful antibiotic compounds throughout history. The penicillins, cephalosporins, macrolides, tetracyclines are all antibacterial classes that were derived from natural product origins. Over the years, 80 % of the bioactive, natural products isolated have been antibiotic compounds and this trend is continuing (6).The worldwide awareness of health risks associated with non-natural additives, added to the current pathogen concerns, may lead to the investigation of natural products such as antimicrobials obtained from plant or microbial sources. Several studies have confirmed the antimicrobial activity of phytochemicals from plants that have been used in traditional medicine used as an antiseptic, digestive agent and antihypertensive, however, limited information regarding its antimicrobial activity against pathogens is available so that our work will try to determine the antibacterial activity of some medicinal plant extracts against the emerging pathogens in intensive care units (ICUs). This will not, only help to reduce the risk in ICU but also be a tool for next generation for better understanding with these microbes and realize an immense potential in production of natural products from medicinal plants to serve as alternate source of combating infections in human beings which may also be of lower cost and lesser toxicity (7).

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Materials and Methods:
Materials:
Microorganisms and media:
Bacterial strains:
Three pathogenic multi drug resistant nosocomial Gm -ve bacteria isolatedfrom neonatal intensive care unit (NICU). These Gm -ve bacteria are; *E-coli,Pseudumonas aeruginosa* and *Klebseillapneumonia*were identified by different means including API 20 Kits and be Confirmed by using VITEK-2 and Micro-scan Apparatus by king Khalid hospital.

Bacteria media:
The bacterial media used in this study for isolation and maintenance of the studied bacteria are: MacConkey Agar, Brain heart infusion broth, MüllerHinton agar, Nutrient Agar and Nutrient broth were purchased from DIFFCO Company.

Plant Collection:
The Plant *Cassiaangustifolia* (Senna or Sana makkahi) leaves were purchased from local herbal shops in Tabuk region, KSA.

Methods:
Antibacterial activity test:
The methanol extract was examined for its antibacterial activity against the multi drug resistant (MDR) nosocomial Gm -ve bacteria from neonatal intensive care unit (NICU) by well diffusion method (8). The experiment was performed using a culture at 37 °C for 24 h. on 10 ml of nutrient Broth for bacteria (20 ml) was poured into sterile petridishes and allowed for solidification. Wells were made in agar plates using sterile cork pore of 4 mm diameter. The cultures were adjusted to approximately 10^6 CFU/ml with sterile saline solution. One hundred and fifty micro liters of the suspensions were spread over the agar plates using a sterile glass spreader. Preliminary antibacterial screening of the isolated studied samples was performed with dry weight concentrations (12.5, 25, 50, 100 and 200 mg sample). Each tested sample was dissolved in 1 ml of dist. water and sterilized by filtration through a 0.22 µm membrane filter (by using Milliopore membrane filter apparatus). 150 µl of each sample were added separately to the appropriate wells in the Petri dishes (9&10).

The antibacterial activity was recorded by measuring the diameter of the clear inhibition zone. In these tests, Impenam (8 ug/disc) for *E.coli* and *Klebsiellapneumonia* or Amikacin (32 ug/disc) for *Pseudomonas aeruginosa* were used as experimental positive controls for bacteria strains and sterile dist. water was used as a negative control for which no inhibitory effect could be observed. Experiments were carried out in triplicate evaluated. The reported results are the average value with standard deviation.

Preparation of the plant extract:
Fresh leaves of *Cassia angustifolia* (sana makkahi) were collected from local herbal shops in tabuk region, washed and cut the large pieces into small pieces and dried at 40 °c. Once dry, plant parts were coarsely powdered using a mortar and pestle and finely mild to form powder using an electric blender and stored in opaque screw tight jars until use and stored in a dark place. (11&12).Each of the powdered plant material was extracted with methanol. For this purpose, 100g of each powdered sample was mixed with 300ml of absolute methanol in a conical flask, plugged, shaken at 100rpm for 30min and extracted by warming at 60 °C in a shaker water bath at 50 rpm for 24h. Then was allowed to cool and filtered rapidly through cotton and filter paper no 3. The debris was extracted one more time with 100 ml of solvent at 60°C in the shaker water bath at 50 rpm for (24h) and filtered again. The two filtrates were then pooled and a more refined filtration was carried out using cotton and Whatman no.3 filter paper. The resulting filtrates were then concentrated in a rotary evaporator under reduced pressure (13). Finally the extracts were collected at small bottle and stored at 40 ℃ until used for determination of antimicrobial activity of extract.

Analytical methods:
Chemical analysis:
Chemical properties of moisture, ash, crude protein, crude lipids, fibers and total carbohydrates were determined according to AOAC, 2000 (14). While the phytochemical analysis is done according to the standard methods of detection of alkaloids, saponins and falvonoids (15)and tannins (16).

Results and Discussion:
Isolation of the highlymulti drug resistant (MDR)Gm -ve bacteria from NICU:
23 from 44 isolates are chosen as Gm –ve bacteriafor this study according to their resistance to antibacterial antibiotics from neonatal intensive care units of King Khalid Hospital (NICU). Tables No 1, 2 and 3 show these isolates, it contains three types of multi drug resistant (MDR) pathogenic bacteria;*E coli*(6 isolates), *Pseudomonas aeruginosa*(5 isolates) and *Klebsiella pneumonia*(12 isolates), respectively.For *E coli* and *Klebsiella pneumonia* the Imipenem antibiotic with concentration 8 µg/ml was usedas the positive control while for *Pseudomonas aeruginosa* was Amikacin with MIC 32 µg/ml is used. For all isolates we can use sterile D.water as negative control.

Table 1. Isolation of multi drug resistant (MDR)Gm -ve bacteria (*E.coli*) from NICU

Organism		E.coli					
Cases		Case9	Case10	Case19	Case26	Case27	Case35
Antibiotics	MIC (µg/ml)						
Amikacin	32	S	S	R	S	S	R
Gentamycin	8	R	R	S	R	R	R
Imipenem	8	S	S	S	S	S	S
Meropenem	8	S	S	S	S	S	S
Cephalothin	16	R	R	I	R	R	R
Cefuroxime	16	R	S	S	R	R	R
Cefoxitin	16	R	I	R	S	S	R
Ceftazidime	16	R	S	S	R	R	R
Ceftriaxone	32	R	R	I	R	R	R
Ampicillin	16	R	R	S	R	R	R
Piperacillin	16	R	R	R	I	R	R
Tetracycline	32	R	R	R	R	R	R

R : resistant, I : intermediate, S : sensetive

Table 2. Isolation of MDRGm -ve bacteria (*Pseudomonas aeruginosa*)from NICU

Organism	Pseudomonas aeruginosa					
Cases		Case12	Case23	Case37	Case38	Case43
Antibiotics	MIC (µg/ml)					
Amikacin	32	S	S	S	S	S

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Organism	Pseudomonas aeruginosa					
Cases		Case12	Case23	Case37	Case38	Case43
Gentamycin	8	I	S	S	S	S
Imipenem	8	S	R	R	R	R
Meropenem	8	S	S	R	R	S
Cephalothin	16	R	R	R	R	R
Cefuroxime	16	R	R	R	R	S
Cefoxitin	16	R	R	R	R	R
Ceftazidime	16	S	I	R	R	S
Ceftriaxone	32	R	R	R	S	R
Ampicillin	16	S	S	R	R	S
Piperacillin	16	R	R	R	R	S
Tetracycline	32	R	R	R	R	R

R : resistant, I : intermediate, S : sensitive

Table 3. Isolation of MDR Gm -ve bacteria (*Klebseilla pneumonia*) from NICU

Organism:	Klebseilla pneumonia												
Cases:	MIC	case2	case5	case13	case14	case15	case16	case17	case21	case25	case31	case32	case33
Amikacin	32	R	R	R	R	R	S	R	R	R	R	R	R
Gentamycin	8	S	R	S	S	S	S	R	R	S	R	S	R
Imipenem	8	S	S	S	S	S	S	S	S	S	S	S	S
Meropenem	8	S	S	S	S	S	I	S	S	S	S	S	S
Cephalothin	16	R	R	S	R	R	S	S	S	R	R	R	R
Cefuroxime	16	R	S	R	R	R	S	R	R	R	R	R	R
Cefoxitin	16	I	I	R	S	S	S	S	R	S	R	R	R
Ceftazidime	16	R	I	R	S	R	S	R	R	R	R	S	R
Ceftriaxone	32	R	R	S	R	R	S	R	S	R	S	S	S
Ampicillin	16	R	I	R	R	R	S	I	R	R	R	R	I
Piperacillin	16	R	R	R	R	R	S	R	R	R	R	R	R
Tetracycline	32	R	R	R	R	I	R	R	R	R	R	R	R

R : resistant, I : intermediate, S : sensetive

Chemical composition of *Cassia angustifolia* (sana makkahi) leaf extract:

Chemical composition of the *Cassia angustifolia* (sana makkahi) leaf extract is shown in table 4. The extract characterized by high moisture content (45%), ash about 18%, the same content of both fat and fiber (12%) with low content of crude protein (4.1%) and total carbohydrates (7.9%).

Table 4. Chemical composition of *Cassia angustifolia* leaves extract

parameter	Yield	moisture	ash	Crude fiber	Crude fat	Crude protein	Total carbohydrates
Percentage %	22.6	45.0 ± 0.1	18.0 ± 0.2	12.0 ± 0.1	12.0 ± 0.1	4.1 ± 0.3	7.9 ± 0.1

Table 5 show that the Phytochemical analysis of the *Cassia angustifolia* (sana makkahi) leaves extract proves that it contains alkaloids, saponins, flavonoids and tanins and this compounds are the responsible for its antibacterial power.

Table 5. phytochemical constituents of *Cassia angustifolia* leaves extract

Sample (g)	Constituents %
Alkaloids	10.7 ± 0.2
Saponins	8.3 ± 0.1
Tanins	5.0 ± 0.1
Flavonoids	19.5 ± 0.3

Antibacterial activity of the *Cassia angustifolia* leaves extract:

After selection to the most resistant bacterial isolates, applying the *Cassia angustifolia* leaves extract on them for testing as antibacterial agent and it clearly shown in tables 6,7 and 8 for *E.coli, Pseudomonas aeruginosa and Klebsiella pneumonia* , respectively. The results clearly shown that, not all isolates were respond positively to the studied extract equally but it varied accordingly to many factors, the most important factors are the type,nature, origin, molecular genetics, byproduct production and the place of isolation. Accordingly, the positive control will be varied from isolate to other (Impenem for *E.coli* and *Klebsiella pneumonia* while Amikacin for *Pseudomonas aeruginosa*. So scientists are now aimed to explore new principles from plant sources(17). Hatano et al., 1999(18)showed that methanolic extract of Cassia tore seed exhibited potent antibacterial effects.

The extract exhibited maximum zone of inhibition against *E coli* (18 mm) at 2.5 mg/ml concentration in case 10 while case 19 show inhibition zone 13 mm diameter at the same concentration while in case of *Pseudomonas aeruginosa*inhibition zone is 18 mm at 1.25 mg/ml in case 12 only and it agree with Mst Rikta Banu, et al., 2013(19) on the other hand the studied extract also affect positively on *Klebsiella pneumonia* and gave maximum inhibition zone 16 mm in case 16 at concentration 5 mg/ml of the extract.

Table 6. Antibacterial activity of *Cassia angustifolia* leaves extract on the selected multi drug resistant (MDR) *E.coli*

Test organism	Diameter of inhibition zone (mm)					Positive control +	Negative control ++
	Leaf extract Concentration (mg/ml)						
	1.25	2.5	5.0	10.0	20.0		
Case 9	--	--	--	--	--	19	--
Case 10	13	18	18	11	10	18	--
Case 19	--	13	11	8	8	20	--
Case 26	--	--	--	--	--	22	--
Case 27	--	--	--	--	--	23	--
Case 35	--	--	--	--	--	23	--

+ positive control : Impenem (MIC : 8 µg/ml), ++ Negative control : sterile D.water

Table 7. Antibacterial activity of *Cassia angustifolia* leaves extract on the selectedMDR*Pseudomonas aeruginosa*

Test organism	Diameter of inhibition zone (mm)	Positive control +	Negative control ++
	Leaf extract Concentration (mg/ml)		

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Test organism	Diameter of inhibition zone (mm)					**Positive control** +	**Negative control** ++
	1.25	2.5	5.0	10.0	20.0		
Case 12	18	14	11	12	12	18	--
Case 27	--	--	--	--	--	18	--
Case 37	--	--	--	--	--	18	--
Case 38	--	--	--	--	--	17	--
Case 43	--	--	--	--	--	17	--

Positive control + : Amikacin (32 µg/ml)., Negative control ++ : sterile D.water

Table 8. Antibacterial activity of *Cassia angustifolia* leaves extract on the selected MDR *Klebsiella pneumonia*

Case No.	Diameter of inhibition zone (mm) Leaf extract Concentration (mg/ml)						
	1.25	2.5	5.0	10.0	20.0	**Positive control** +	**Negative control** ++
Case2	--	--	--	--	--	**20**	--
Case5	--	--	--	--	--	**21**	--
Case13	--	--	--	--	**13**	**21**	--
Case14	--	**13**	**11**	11	**12**	**19**	--
Case15	--	--	--	--	--	**1\9**	--
Case16	10	**14**	**16**	15	**15**	**18**	--
Case17	--	--	--	--	--	**19**	--
case21	9	**10**	**10**	13	**12**	**20**	--
case25	--	--	--	--	--	**21**	--
Case31	--	--	--	--	--	**21**	--
Case32	--	--	--	--	--	**22**	--
Case33	--	--	--	--	--	**19**	--
Case 39	--	--	--	--	--	**19**	--
Case 41	--	--	--	--	--	**19**	--

+ positive control : Impenem MIC + 8 µg/ml, ++ Negative control : sterile D.water

From the above results tables, the antibacterial effect of the studied*Cassia angustifolia* leaves extract is due to the phytochemical constituents which contain Alkaloids, saponins and tannins which have been reported to inhibit the pathogenic bacterial growth, this results agree with Doughari and Okafor, 2008(20). Based on the results of inhibition zones, the phytochemical constituents of the extract showed high significant activity against the tested Gm –ve bacterial strains, in regard with the inhibitory effect of control. This activity may be related to the ability of the tested extract to affect permeability of the Gram –ve bacterial cell wall.Our results agree with Wang and Quinn, 2010(21) whom stated that the Gm–ve bacteria contain an outer membrane composed of phospholipids and lipopolysaccharides leads to increase the negative charge of the cell membrane and helps to stabilize the overall membrane structure.

Conclusion:

The demonstration of the antibacterial activity of the studied *Cassia angustifolia* leaves extract against some multi drug resistant (MDR)Gm -ve bacteria l isolates which isolated from NICU (*E-coli, Pseudumonas aeruginosa* and *Klebseilla*) has provided that plant can be used as medicinal plant in the treatment of the previous pathogens due to itscapability to broke the antibiotic resistance ability of the studiedmulti drug resistant (MDR)pathogens. So we can use this extract as cheap, valuable and effective drug to treat the illness resulted from these pathogens.

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