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Research Article

Evaluation of *Acinetobacter baumanii* isolated from ICUs and herbal disinfectant using *Terminalia chebula*

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Abstract

Acinetobacter baumanii is an opportunistic nosocomial pathogen which is a major contaminant of the ICUs and causes a variety of severe infections like endocarditis, meningitis etc. in the immune compromised patients. It is a gram negative coccobacillus producing white to faint pink colonies on MacConkey agar. The ability of the organism to produce biofilm is one of the reason for its success as a nosocomial pathogen. The organism produces better biofilms on plasic as compared with glass, thus medical devices made of plastic have higher chances of disseminating the pathogen. The organism has developed resistance to most of the commercially used drugs. Cephalosporin is completely resisted by the all the strains whereas imipenem is resisted only by some strains. Gentamicin, amikacin, Ticarcillin and Ciprofloxacin are resisted by most of the strains. *Terminalia chebula*, commonly called as Kadukkai has been used as a traditional medicine from ancient times. Just 150 mg/ml of the ethanolic extract of the fruit of Kadukkai was found to completely inhibit the pathogen. Phytochmical analysis of this fruit showed the presence of a large number of chemicals like tannins, phenols etc. To control the spread of *A. baumanii*, proper disinfection plays a major role and so a novel herbal disinfectant was formulated using the ethanolic extract of *T. Chebula* along with ethanol , isopropanol and SDS, the three of which were proved not capable of completely inhibiting the pathogen. The efficiency of the formulation by MIC testing gave positive results as the pathogen was inhibited by 1 ml of the disinfectant. In comparison with the MIC of ethanol, this gave better results.

Keywords: *Acinetobacter baumanii* , immune compromised patients, *Terminalia chebula*, M.IC

Introduction

Acinetobacter sp. are gram negative aerobic coccobacilli and are generally harmless organisms with the ability to persist in the hospital environment for prolonged periods. Although colonization of patients is common, this organism is a major cause of clinical infection, especially in the immunocompromised patients and those in intensive care units. *Acinetobacter baumannii* is the major species responsible for the infections in humans, causing pneumonia, endocarditis, meningitis, wound and urinary tract infection (Ayan *et al.*, 2002). *Acinetobacter* spp. are commonly found as major contaminants of the environment. The recent development of drug resistance among these organisms has rendered the current antibiotics ineffective (Meritxell *et al.*, 2012).

Biofilm formation is an ability possessed by many bacteria and such organisms are generally highly resistant to environmental stresses. *Acinetobacter baumanii* has also been found to produce biofilms and cause infections associated with medical devices, e.g., vascular catheters, cerebrospinal fluid shunts, foley catheters etc. (Dheepa *et al.*, 2011). The drug resistance exhibited by the organism has been shown

to be due to the ability of the organism to produce biofilms (Srinivasa Rao *et al.*, 2008).

The recent development of Drug resistance in bacteria has made many commonly used antibiotics ineffective. Multiple drug resistance is the resistance developed my microorganisms to certain antibiotics and *A.baumanii* possess this ability to a high extend. This is one another reason why this organism is feared in hospitals (Enoch *et al.*, 2008). As such Colistin and Tigercycline remains the only two antibiotics mainly used against this pathogen and resistance is slowly developing against Tigercycline (Dizbay *et al.*, 2008). Current research should focus upon enhancing the activity of the antibacterial agents (Adwan *et al.*, 2008) as development of novel agents cannot compete the rate at which drug resistance is spreading. Natural herbs has been used as antibacterial agents since ancient times. The chemical compounds possessed by the herb determine its medicinal nature. The compounds like saponins, tannins, alkaloids etc determine the medicinal property of the plant (Edeoga *et al.*, 2005).

Terminalia chebula, commonly known as black myrobalan, belongs to the family "Combretaceae",. It is a medium- to large-sized tree distributed throughout tropical and sub-tropical Asia, including China and Tibet (Kannan *et al*, 2009). It has been used in traditional medicine since ancient times and the antibacterial activity against several bacterial strains have been reported (Kim *et al*., 2006). The fruit of *Terminalia chebula* has been used as an antispasmodic and in treating opthalmia, hemorrhoids, dental caries, bleeding gums, ulcered oral cavity. Its paste has anti-inflammatory, analgesic and wound healing capacity (Anwesa Bag *et al*, 2013).

Materials and Methods

Sample collection

The samples were collected from KMCH, Coimbatore. Swabs taken from ICU patients in various departments were used to isolate the bacteria. Twenty *Acinetobacter baumanii* samples were isolated.

Culture

The samples were cultured on Brain Heart Infusion agar (Dror Marchaim *et al*, 2007) and MacConkey agar plates and incubated for 24 hours at 37^{O}C.

Phenotypic characterization

The phenotypic characterisation was done by Gram's staining and biochemicals testing. IMViC, Catalase, Oxidase, Glucose fermentation and Lactose fermentation was done and incubated for 24 hours at 37^{O}C (Prashanth *et al*, 2000).

Antibiogram

Twenty four hour culture of *Acinetobacter baumanii* was swabbed on Muller Hinton agar plates and antibiotic discs were placed on the plates and incubated for 24 hours at 37 ^{O}C. The discs used were Ticarcillin, Ceftazidime, Imipenem, Gentamicin, Amikacin and Ciprofloxacin (Imane M'hamedi *et al*, 2014).

Biofilm Detection

Biofilm formation is the ability of certain bacteria to produce an extracellular polysaccharide, thus facilitating attachment and matrix formation (Dheepa *et al*., 2001).

I) Congo Red assay

Congo red agar plates prepared using 50gm/l sucrose, 37gm/l Brain Heart Infusion agar and 20gm/l agar. These were sterilised and 0.8gm/l sterilized Congo red stain was added to the media after it has cooled down to 55^{O}C (Yasmeen Taj, 2012) and the samples isolated on MacConkey or Brain Heart Infusion agar were inoculated onto the media and incubated.

II) Tube Assay

Acinetobacter baumanii culture was inoculated into sterile Brain Heart Infusion broth tubes and incubated for 48 hours at 37^{O}C. The tubes were then drained and washed with Phosphate Buffer Saline. Further the tubes were kept for drying at room temperature and 4% Crystal Violet solution was added through the sides of the tube and decanted. The tubes were dried and biofilm formation studied (Mathur *et al*, 2006).

Comparing the biofilm production on different materials

A. Baumanii was inoculated into test tubes made of glass and tubes made of plastic containing 5 ml of

sterilized Brain Heart Infusion broth. The tubes and incubated at $37^{O}C$ for 48 hours. After incubation, the tubes were decanted, washed with Phosphate Buffer Saline and air dried. Staining was done using 4% Crystal Violet solution and the biofilm formation was observed in the tubes (Nadia Kazemi Pour *et al*, 2011).

Plant collection and processing:

The fruits of *Terminalia chebula* were collected from its tree. It was dried in shade for a few weeks to remove all the moisture. The fruits were ground into fine powder using an electronic mixer. By this method about 25gm of the powder was collected.

Ethanolic extraction:

About 20gm of the powder was taken and extracted using ethanol at $70^{O}C$ in a Soxhlet apparatus for two days. The solvent containing the extract was poured into petri dishes and left open overnight to evaporate the solvent and to obtain the extract powder.

Phytochemical analysis:

The powder obtained was then subjected to various Phytochemical analysis**.**

Test for Glycosides: (Sarla Saklani, 2012)

Keller-Killani test (for deoxy sugar):-1ml of glacial acetic acid containing traces of Ferric chloride and 1ml of concentrate sulphuric acid were added to the extract carefully. A reddish brown colour formed at the junction of two layers and upper layer turned Bluish green indicated the presences of glycosides.

Legal test (for cardinolides):-concentrated ethanolic extract was made alkaline with few drops of 10% sodium hydroxide and then freshly prepared sodium nitroprusside Solution was added to the solution. Presence of blue coloration indicated the presence of Glycosides in the extract.

Test for Alkaloids: (Abdul Wadood *et al.*, 2013)

About 0.2g of the plant sample was taken in a test tube and 3 ml of hexane was added, mixed well, shaken and filtered. 5ml of of 2% HCl was added into the tube and heated. The contents were filtered and a few drops of picric acid was added into it. Yellow colour indicates the presence of alkaloids.

Test for flavonoids: (Abdul Wadood *et al.*, 2013)

In a test tube, 0.5 g of the plant extract was taken and to this 10 ml of distilled water was added. To a portion of this, 5 ml of dilute ammonia solution was added followed by 1 mlconcentrated H_2S0_4. Appearance of yellow colour shows the presence of flavanoids.

Test for terpenoids: (Yadav *et al.*, 2011)

Crude extract of the plant was dissolved in 2ml of chloroform and evaporated to dryness. To this, 2ml of concentrated H_2SO_4 was added and heated for about 2 minutes. A grayish colour indicates the presence of terpenoids.

Test for proteins: (Yadav *et al.*, 2011)

Millon's test

Crude extract when mixed with 2ml of Millon's reagent, white precipitate appeared which turned red upon gentle heating that confirmed the presence of protein.

Test for saponins: (Yadav *et al.*, 2011)

In a test tube, crude extract of the plant was mixed with 5ml of distilled water and was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

Test for reducing Sugar: (Abdul Wadood *et al.*, 2013)

An amount of 0.50 g of selected plant sample was added in 5 ml of distilled water. In another test tube, 1 ml of ethanol and 1 ml each of Fehling solution A and Fehling solution B was added. The mixture was heated to boiling and then poured into the aqueous ethanol plant extract. Appearence of colour showed a positive result.

Test for steroids: (Yadav *et al.*, 2011)

Crude extract was mixed with 2ml chloroform and concentrated H_2SO_4 was added sidewise. A red colour produced in the lower chloroform layer indicated the presence of steroids.

Test for Carbohydrates: (Prashanth Tiwari *et al.*, 2011)

Benedict 's test:

The extract was treated with 2 ml Benedict's reagent and heated gently. Orange -
Red precipitate indicates the presence of carbohydrates.

Test for Phenols: (Solomon Charles Ugochukwu *et al.*, 2013)

Ferric chloride test

A fraction of the extract was treated with aqueous 5% ferric chloride and observed for formation of deep blue or black colour.

Minimum Inhibitory Concentration of *Terminalia chebula*

The minimal inhibitory concentrations (MICs) of the extracts of *Terminalia chebula* against all the test strains were determined by macro broth dilution assay method. Two-fold serial dilutions of all the extracts (25mg to 200mg) were prepared in tubes with Brain Heart Infusion Broth (Hi-media, Mumbai, India) as diluents. Each dilution was seeded with 0.1ml of test micro-organisms to the standard concentration. The tubes were incubated at 37 ^{O}C for 24 h. The least concentration of the extract showing no visible growth was taken as the MIC (Rathinamoorthy *et al.*, 2014)

Formulating a Novel disinfectant:

Since *A. baumanii* is an environmental contaminant, disinfection of the environment plays a major role in controlling it spread. Here a disinfectant has been formulated, which contain an alcohol, a foaming surfactant, an emulsifier and a preservative. Here SDS acts as the foaming surfactant and emulsifier, isopropanol acts as the preservative and ethanol acts as the alcohol.

Ethanol – 5 ml
Isopropanol – 30ml
SDS – 0.3g
Terminalia chebula – 0.5 g
Distilled Water – 65 ml

(The above composition was designed based on Patent EP1487949A2- Composition and process for preparing herbal disinfectant and their use ; US 8,268,367 B2- TOPICAL Herbal Formulation for treatment of acne and skin Disorders; Mimoz, Olivier *et al*., 1996).

Determining the MIC of the disinfectant

The disinfectant was taken in varying concentrations (0.5 ml, 1 ml, 1.5 ml, 2 ml) in different test tubes containing sterile Brain Heart Infusion broth. To the tubes, 0.1 ml of *A.baumanii* culture was added and mixed well. From this, 0.1 ml of inoculums was taken and inoculated on to BHI agar plated by spread plate technique. The plates were incubated overnight at 37^0C (Priscila Gava Mazzola *et al*., 2009).

Determining the MIC of Ethanol and comparison of its activity with that of the Formulated Disinfectant

To check the activity of 99% ethanol against *Acinetobacter baumanii*, varying concentrations of ethanol (0.5 ml, 1 ml, 1.5 ml, 2 ml, 2.5 ml and 3ml) was taken in test tubes, which contained 1 ml of sterile Brain Heart Infusion broth. To this, 0.1 ml of 24 hour old culture of the organism adjusted to 0.1 McFarland's standard was added. About 0.1 ml of this inoculums were then inoculated on to sterile Brain Heart Infusion agar plates by spread plate method. All the plates were incubated at 37^0C for 24 hours (Priscila Gava Mazzola *et al*., 2009).

Testing the activity of Individual components of the disinfectants against *A.baumanii* (Smith *et al.*, 2004; Justin Edwards *et al.*, 2007**)**

Ethanol (0.5 %)

In a test tube, 5 ml of 0.5% ethanol was taken and to this 0.1 ml of *A.baumanii* inoculated. From this, 0.1 ml was taken and spread onto BHI agar plated. The plates were incubated overnight at 37^0C and observed for growth.

Isopropanol (30 %)

In a test tube, 5 ml of 30% isopropanol was taken and to this 0.1 ml of *A.baumanii* inoculated. From this, 0.1 ml was taken and spread onto BHI agar plated. The

plates were incubated overnight at 37^0C and observed for growth.

SDS (0.3 %)

To 5 ml of 0.3 % SDS, 0.1 ml of *A.baumanii* inoculated. From this, 0.1 ml was taken and spread onto BHI agar plated. The plates were incubated overnight at 37^0C and observed for growth.

Results and Discussion

Culture

Well isolated colonies of the organism were obtained as the organism grew well on all the media used. It showed faint pinkish taint on MacConkey agar (Lahiri *et al*, 2004) and creamy colour colonies on Brain Heart Infusion agar.

Phenotypic characterisation

The isolated strains were negative for Indole,VP but positive for Citrate. Except one strain all others gave negative result for MR. They showed negative for oxidase and positive for catalase. The samples utilized glucose and lactose but did not ferment them.

Antibiogram

Most of the strains were resistant to most of the antibiotics used except Imipenem or amikacin.

The organism was found to be Multiple Drug Resistant. Most of the strains showed resistance to all the antibiotics except Imipenem and Amikacin. Imipenem was sensitive to 85% of the strains selected (Helen Giamarellou *et al*., 2008). Only some showed sensitivity to Gentamicin and Ciprofloxacin. Only one strain was sensitive to Ticarcillin. Ceftazidime was resisted by all the strains tested.

Biofilm Detection using Tube assay

All the samples tested produced biofilm which, after staining was visible as a layer on the walls and bottom of the tube (Anagha Kinikar *et al*, 2014) and not as a ring in the middle. Though all the samples produced biofilms, the strength of the biofilm varied between the various samples.

Most of the strains produce very strong biofilms. Only Ab8 and Ab 13 produced weak biofilms. Ab6, Ab7, Ab14 and Ab 20 produced moderate biofilms whereas all the other strains produced strong biofilms. This proves that all *A.baumanii* possess the ability to produce biofilms.

Comparing the biofilm production on different materials

The biofilm formation abilities of all the 20 isolates were determined on glass test tubes and also tubes made of plastic and it was seen that the strains produced biofilms more heavily on plastic than on glass (Nadia Kazemi Pour *et al*, 2011).
The phytochemical tests done showed the presence of Glycosides, alkaloids, flavanoids, phenols, carbohydrates, steroids, saponins, reducing sugars, proteins and terpenoids in the plant extract. These compounds are responsible for the anti bacterial activity of the plant (Chetan Sharma *et al.*, 2012).

Ferric chloride reacted with fruit extract and sulphuric acid forming blue colour precipitate, showing the presence of deoxysugars. Cardenoloids reacted with pyridine and sodium nitroprusside producing red colour. Steroids reacted with chloroform and concentrated sulphuric acid to produce red colour. Saponin reacted with mercuric chloride to produce white precipitate as positive result. The reaction ferric chloride with the *T.chebula* fruit extract to form blackish red colour confirm the presence of flavonoids. Phenolic compounds reacted with neutral FeCl3 to colour change as positive result. Terpenoids were confirmed by the grey coloration obtained as a result of reaction of the fruit with chloroform and sulphuric acid. Red precipitate formation on heating Million's reagent with the fruit extract shows that proteins are present. The appearance of yellow precipitate on addition of HCl and hexane to the fruit extract indicates that alkaloida are present (Tensingh Baliah *et al.*, 2014).

Minimum Inhibitory Concentration of *Terminalia chebula*

Complete inhibition was obtained in plates having 150mg/ml onwards. The growth was found to reduce from the lower to higher concentrations of the plant extract with no inhibition at 25mg/ml to good inhibition from 150mg/ml onwards. This shows that

Phenotypic characterization

STRAIN NO.	INDOLE	METHYL RED	VOGES PROSKAUER	CITRATE	CATALASE	OXIDASE	GLUCOSE UTILISATION	LACTOSE UTILISATION
Ab1	-	-	-	+	+	-	+	+
Ab2	-	-	-	+	+	-	+	+
Ab3	-	-	-	+	+	-	+	+
Ab4	-	-	-	+	+	-	+	+
Ab5	-	-	-	+	+	-	+	+
Ab6	-	-	-	+	+	-	+	+
Ab7	-	-	-	+	+	-	+	+
Ab8	-	-	-	+	+	-	+	+
Ab9	-	-	-	+	+	-	+	+
Ab10	-	-	-	+	+	-	+	+
Ab11	-	-	-	+	+	-	+	+
Ab12	-	-	-	+	+	-	+	+
Ab13	-	-	-	+	+	-	+	+
Ab14	-	-	-	+	+	-	+	+
Ab15	-	-	-	+	+		+	+
Ab16	-	-	-	+	+	-	+	+
Ab17	-	-	-	+	+	-	+	+
Ab18	-	-	-	+	+	-	+	+
Ab19	-	-	-	+	+	-	+	+
Ab20	-	-	-	+	+	-	+	+

Antibiogram

STRAIN NO.	Ticarcillin Zone dia. (in mm)			Ceftazidime Zone dia. (in mm)			Imipenem Zone dia. (in mm)			Gentamicin Zone dia. (in mm)			Amikacin Zone dia. (in mm)			Ciprofloxacin Zone dia. (in mm)		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Ab1									10									
Ab2									9									
Ab3									1									
Ab4			21						9			13			18			23
Ab5									9									
Ab6															15			
Ab7									10						14			
Ab8									12						18			9
Ab9																		
Ab10																		
Ab11									10									
Ab12									8									
Ab13									12						13			
Ab14									10						10			
Ab15									10									
Ab16									10						10			
Ab17									13			14			10			14
Ab18									11			13			15			
Ab19									14			13			13			14
Ab20									11			8			10			

Biofilm Detection using Tube assay

STRAIN NO.	BIOFILM STRENGTH
Ab1	Very High
Ab2	Very High
Ab3	Very High
Ab4	Very High
Ab5	High
Ab6	Moderate
Ab7	Moderate
Ab8	Low
Ab9	Very High
Ab10	Very High
Ab11	High
Ab12	Very High
Ab13	Low
Ab14	Moderate
Ab15	Very High
Ab16	High
Ab17	Very High
Ab18	Very high
Ab19	High
Ab20	Moderate

Comparing the biofilm production on different materials

TEST NO.	TEST FOR	OBSERVATION	RESULT
1	Deoxysugars:	Bluish green colouration	Presence
2	Cardenoloids:	Pink colouration	Presence
3	Alkaloids	Yellow precipitate	Presence
4	Flavanoids	Yellow brown precipitate	Presence
5	Terpenoids	Grey colour.	Presence
6	Proteins	Red precipitate	Presence
7	Saponins	Froth after agitation	Presence
8	Reducing Sugar	Brown precipitate	Presence
9	Steroids	Red colouration	Presence
10	Carbohydrates	Brown precipitate.	Presence
11	Phenols	Blue colour	Presence

Determining the MIC of Ethanol and comparison of its activity with that of the Formulated Disinfectant

Amount of Inoculum (in ml)	Growth inhibition by Formulated Disinfectant	Growth inhibition by Ethanol
0.5 ml	Partial Inhibition	Partial Inhibition
1 ml	Complete Inhibition	Partial Inhibition
1.5 ml	Complete Inhibition	Partial Inhibition
2 ml	Complete Inhibition	Complete Inhibition

Terminalia chebula has high activity towards *Acinetobacter baumanii*. The compounds present in the plant extract has the potential to destroy the pathogen and so can be considered as a promising agent in controlling the spread of the pathogen.

Determining the MIC of the disinfectant

The pathogen was completely eradicated where 1 ml of the disinfectant was used whereas partial inhibition was observed in case where 0.5 ml was used. The disinfectant formulated had the ability to destroy *A. baumanii* (Priscila Gava Mazzola *et al*., 2009).

Determining the MIC of Ethanol and comparison of its activity with that of the Formulated Disinfectant

Complete inhibition of *A. baumanii* was seen in plates inoculated with the ethanol concentrations from 2 ml onwards. Ethanol concentraions from 1 to 2 ml showed only partial inhibition of growth.

This result highlights the effectiveness of the formulated disinfectant against *A. baumanii.* The pathogen was completely inhibited by just 1 ml of the disinfectant whereas the same quantity of ethanol gave no or partial inhibition of the pathogen. In comparison with the most common disinfectant of the health care industry, the formulated disinfectant has been proven to be more effective (Priscila Gava Mazzola *et al*., 2009) (Table 5).

Testing the activity of Individual components of the disinfectants against *A.baumanii* (Smith *et al*., 2004; Justin Edwards *et al.*, 2007**)**

Ethanol (0.5 %)

The used amount of ethanol did not inhibit the pathogen completely. Only partial inhibition was seen. This shows that ethanol concentrations as that used in the disinfectant had only a small role in the activity of the disinfectant.

Isopropanol (30 %)

Isopropanol showed similar results as that of ethanol. Only small inhibition was seen in this case as well.

SDS (0.3 %)

Here again, the used percentage of SDS did not completely destroy the pathogen. Hence it has been proven not to be responsible for the complete activity of the disinfectant.

The above results shows that the individual components of the disinfectant are not responsible for its activity and that the disinfectant is actually effective against the pathogen.

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