Phytochemical Screening And In-Vitro Studies Of Antimicrobial Activity Of Extracts Of Catharanthus Roseus Against ESBL And MRSA Isolates

S. Parvathy, G. Prabakaran

Abstract : Our aim was to determine the antimicrobial activity of selected plant (Catharanthus roseus) against Extended Spectrum Beta Lactamase (ESBL) producing bacteria and MRSA isolates, which were determined by double disc diffusion method and PCR respectively. The plants were extracted with methanol, acetone, petroleum ether, chloroform and aqueous. The Minimum Inhibitory Concentration (MIC) was determined using broth micro dilution. The MICs ranged between 0.5mg to 2.5 mg/ml. The majority of these microorganisms were inhibited by 4mg/ml concentration of crude extracts and highest inhibitory activity was observed on methanol extract. Furthermore, GCMS analysis of methanolic extract revealed the occurrence of number of bioactive compounds corresponding to major peak. This study was proved to be potentially effective of Catharanthus roseus extract against ESBL and MRSA isolates.

Keywords : C. roseus, ESBL, MRSA, Silane, Cyclopentasiloxane

1 INTRODUCTION

The betalactam has been the antibiotics most frequently prescribed for infectious disease therapy. The mechanism of resistance to these medicines is due to the production of β-lactamases by the appropriate gene in the bacterial chromosome or in the transmission of plasmids. This enzyme producing bacteria were resistant to penicillin, cephalosporins, and monobactams group of antibiotics [1]. The development of resistance to extended-spectrum cephalosporins was triggered by ESBL producing Enterobacteriaceae has been stated to be threatening. These ESBLs was mostly found in Klebsiella species and E. coli as well as in other Enterobacteriaceae family, such as Proteus species, Enterobacter species, Citrobacter species, Providencia species, Serratia species and Salmonella species [2]. Being plasmid DNA mediated, ESBLs are easily transmitted among members of Enterobacteriaceae. The spread of this resistance does not only apply to beta-lactams, but also frequently used antibiotics such as fluoroquinolones, aminoglycosides, and sulphonamides [3]. Consequently, many patients need the 'last stamping ground' antibiotic treatment such as carbapenems. Again the use of carbapenems has led to the rapid selection of carbapenem-resistant Enterobacteriaceae [4]. Only a few antibiotics (e.g. carbapenems, colistin, tigecycline) are available to treat infection caused by ESBL-producing bacterial isolates, even though the in vivo potency and toxicity of these drugs are not well known [5],[2]. Additionally, more and more MDR S.aureus were reported in food poisoning outbreaks and isolated from food product in previous report [6]. ESBL producing Enterobacteriaceae, mainly Escherichia coli and Klebsiella pneumonia have increased adequately during the past 10 years.

- *S. Parvathy, G. Prabakaran*
- *PG& Research Department of Botany, Government Arts College, Dharmapuri- 636705.Tamilnadu, India.*
- *Corresponding author- G.Prabakaran, Email- gpbiotek@gmail.com.*

This rise was a major cause of serious infections worldwide with high mortality and morbidity rates [7]. Against this backdrop, the development of alternative drug classes to cure such infectious diseases is urgently required. Plants have an incredible ability to produce a wide variety of biological activities including antimicrobial, anticancer and antioxidant properties, thereby becoming a great aid to explore for the invention of beneficial and novel antimicrobial products [8]. According to World health organization (WHO) more than 80% of the world population relay on traditional medicine for their primary health care needs [9]. In this present study was designed to investigate the antimicrobial potential of Catharanthus roseus against ESBL producing and MRSA of food isolates.

2 MATERIAL AND METHODS

2.1 Bacterial isolates:

A detail study design was employed whereby a convenience sampling of retail meat outlets from Namakkal was carried out. The homogenate meat samples were streaked on the selective media and incubated aerobically at 37°C for 24 hours. The isolated bacteria were identified based on colony morphology, Gram staining reaction, and biochemical characteristics using established standardized methods according to Bergey's Manual of determinative bacteriology.

2.2 Characterization of meat isolates:

2.2a Isolation of ESBL producing isolates:

Isolated bacterial pathogens were subjected to ESBL characterization. The confirmation of the ESBL producing isolates was done by the phenotypic confirmatory test according to CLSI recommendation. In this test, the first generation of betalactam i.e. Amoxcillin disc (30µg) alone and in combination with clavulanic acid (10 µg) was used. After overnight incubation at 37^{o}C, diameter of zone of inhibition was measured. A 5 mm or more increases in the diameter of zone of inhibition for Amoxcillin tested in combination with clavulanic acid versus its zone when

Amoxcillin tested to alone confirms a ESBLs producing organism

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2.2b Isolation of methicillin resistance S.aureus (MRSA):

The methicillin resistance of S.aureus was observed by PCR amplification procedure, according to Abu Hujier, 2008 [10] method. Following PCR, 20 µl of the reaction mixtures were analyzed by electrophoresis on a 1% agarose gel, containing ethidium bromide (0.2 mg/ml), in the presence of an appropriate DNA molecular weight marker.

2.2c Collection of plant material and extraction:

In the present study, flower of Catharanthus roseus has been used in the present study and they were collected from the Salem area, Tamilnadu, India. The flower sample was cleaned and air-dried and then powdered with grinder. The powder was extracted in a soxhlet extractor successively with 200 ml of Methanol, Acetone, petroleum ether, water and Chloroform until colourless extract was obtained at the top of the extractor. Each of the solvent extract was concentrated separately under reduced pressure. After complete solvent evaporation, each of these solvent extracts was weighed and subjected to further. Extracts were maintained at a temperature between 2 - 8°C for further studies [11].

2.2dPhytochemical Screening and GCMS

Chemical tests were carried out on the solvent extracts using standard procedures to identify the constituents as described by Sofowara (1993). Furthermore phytochemicals were determinate by GCMS method. Gas chromatography (GC) analysis was carried out at TUV analytical laboratory, Tiruppur, Tamilnadu, India.

2.2e Antibacterial activity of Catharanthus roseus

Agar well diffusion method was employed to investigate the antibacterial activity of flower extracts against 4 bacteria. The 24 h old Nutrient broth cultures of test bacteria were swab inoculated on sterile Mueller- Hinton Agar plates and wells of 8 mm were created in the plates with the help of sterile cork-borer. The wells were labeled and filled with different concentration of bark extract, reference antibiotic (Chloramphenicol, 1 mg/ml of sterile distilled water) and relevant extract solvent. The plates were incubated in upright position at 37 °C for 24 h and the zones of inhibition were measured [12].

3 RESULTS AND DISCUSSION

Presently 23 bacterial isolates of 6 genera were observed from meat samples, except S.aureus, all isolates were subjected to ESBL analysis with the double disc diffusion method. Among the 6 genera 8 (34.8%) were ESBL producers, the highest ESBL production were seen in E. coli (50.0%) and E.fecalis (50%) whereas Proteus mirabilis (0%) showed negative for ESBL production (Table 1). Double-disc tests showed synergy for co-amoxiclav with cefotaxime against meat isolates. In recent years, ESBL producing clinical bacterial isolates were creating the major therapeutic problem. These isolates showed resistance to most of commonly used antibiotics. Among the clinical isolates, species of K.pneumoniae were highly producing the ESBL character [13].

Table 1 *Prevalence of ESBL producing isolates*

S. No	Name of isolates	Total no of isolates (n=21)	No. of Positive (n=8)
1.	*E.coli*	4	50%
2.	*E.faecalis*	4	50%
3.	*P.aeruginosa*	6	33.3%
4.	*K.pneumoniae*	5	40
5.	*P.mirabilis*	2	0

In a study made by few researchers, they observed the ESBL producing E.coli and K.pneumoniae from clinical samples [14]. In recently, few scientists observed the 130 isolates of ESBL producing E.coli, K.pneumoniae, and E. aerogenes from clinical samples [15]. The significant procreation of ESBL bacteria globally is causing an increase in the percentage of infected patients in the community and hospitals, as well as raises the mortality rates. In additionally, MRSA isolates were observed by amplification of MecA gene. Among the 2 isolates, single isolate had MecA gene. In 2008, few researches amplified the MecA gene from meat isolates of S.aureus [16]. One recent study was observed the presence of MRSA isolates from various meat samples in Assam, India [17]. Over the past several years different studies conducted all around the world have alarmed the presence of MRSA isolates of bacteria from different sources. These isolates were resistant to another group of antibiotics, which pose the greatest threat to human health by WHO in 2017 [18]. Consequently, herbal assessment is one step closer to accomplishing the natural antibiotic for controlling and treating infection without the look of resistant pressure. In the present study, five different extracts of flower of C.roseus were prepared and subjected to the antimicrobial activity against ESBL producing bacterial isolates. The water, chloroform, petroleum ether, methanol and acetone of flower extracts of Catharanthus roseus exhibited varying degree of inhibitory effect against all tests pathogenic isolates. Among them, methanol extract showed strong antibacterial activity against all bacteria, especially P.aeruginosa and S.aureus. The better inhibition was observed while using 4mg concentration of the extract. The poor inhibitory activity was observed against to E.faecalis and followed by E.coli. This methanol extract exhibited zone of inhibition ranged from 9.6±0.74 mm to 23±0.62 mm (Table 2). These results support earlier results by [19]; they were found antibacterial activity of flower against to S.aureus. Latterly, other hand also found the antibacterial activity of flower extracts against Pseudomonas aeruginosa [20]. Same time few authors were reported that flower extracts were ineffective against Pseudomonas aeruginosa [21].In this study, second most inhibitory activity was observed while using petroleum ether extract, which exhibited zone of inhibitions were ranged from 10.3±0.94 mm to 17±0.81mm (Table 2). Among the five extracts, third most inhibitory activity was shown by acetone extract, which exhibiting zone of inhibition was ranged from 11±0.81mm to 18.8±0.84. The highest inhibitory activity of acetone extract was highly active against to S.aureus (Table 3). Presently no one isolates were suppressed with while using control agents of antibiotic and relevant solvents. In this current investigation, organic extracts were

found to be inhibitory than aqueous extract, same time chloroform extract also showed lowest inhibitory activity (Table 4). Similar results were observed by recent studies conducted by few researchers they were also found that aqueous extract of the flower was not exhibited antimicrobial activity [22]. Furthermore, MIC was carryout with broth dilution method. The MIC was ranged from 0.5mg to 2.5mg of concentration of extracts.

Table 2

Antibacterial activity of methanol and P.ether extract of C.roseus

Isolates	Methanol extract (mg) Zone of inhibition in mm				P.ether extract (mg) Zone of inhibition in mm				Solvent	Antibiotic
	1	2	3	4	1	2	3	4		
E.coli 2	-	10±0.81	13.6±0.62	16±0.81	-	-	-	11±0.81	-	-
E.coli 4	11±0.81	14.5±0.70	16.3±0.47	18.3±0.84	-	-	-	12.5±0.16	-	-
E.faecalis3	-	-	9.66±0.47	12.1±0.84	-	11±0.81	15.5±0.40	17±0.81	-	-
P.aeruginosa 5	15.1±0.62	17.5±0.40	21±0.81	23±0.62	-	-	10.83±0.84	15.3±1.24	-	-
K.pneumoniae1	10±0.81	11.83±0.62	15±0.40	17±0.81	-	10.33±0.94	13.5±0.40	15.83±0.62	-	-
K.pneumoniae2	-	13.1±0.62	15.33±0.47	19.5±0.40	-	-	11.16±0.84	14.33±0.94	-	-
S.aureus 1	10.33±0.942	12.33±0.62	15±0.81	19.33±1.24	-	-	12.5±0.40	17±0.81	-	-

Table 3

Antibacterial activity of acetone and Aqueous extract of C.roseus

Isolates	Acetone extract (mg) Zone of inhibition in mm				Aqueous extract (mg) Zone of inhibition in mm				Solvent	Antibiotic
	1	2	3	4	1	2	3	4		
E.coli 2	-	-	-	11.6±0.84	-	-	10±0.81	12.5±0.08	-	-
E.coli 4	-	-		11±0.81	-	-	14.5±0.40	16.8±0.84	-	-
E.faecalis3	-	-		11.6±0.47	-	-	-	-	-	-
P.aeruginosa 5	-	-	11.5±0.40	16±0.81	-	-	10.5±0.40	12.8±0.62	-	-
K.pneumoniae1	-	-	11.6±0.62	14±0.81	-	-	-	-	-	-
K.pneumoniae2	-	-	11.8±0.62	15±0.81	-	-	-	11±0.81	-	-
S.aureus 1	-	12.5±0.40	16±0.81	18.8±0.84	-	-	10.8±0.23	15.3±0.47	-	-

Table 4

Antibacterial activity of chloroform extract of C.roseus

Isolates	Chloroform extract (mg) Zone of inhibition in mm				Solvent	Antibiotic
	1	2	3	4		
E.coli 2	-	-	12.5±0.40	15±0.81	-	-
E.coli 4	-	-	11.1±0.62	15±0.81	-	-
E.faecalis3	-	-	-	12.6±0.47	-	-
P.aeruginosa 5	-	-	-	13.3±0.47	-	-
K.pneumoniae1	-	-	11.5±0.40	15.1±0.62	-	-
K.pneumoniae2	-	-	12.6±0.47	15±0.81	-	-
S.aureus 1	-	-	-	15.33±0.47	-	-

The polarity of antibacterial compounds makes them more readily extracted by organic solvents, and using organic solvents does not negatively affect their bioactivity against bacterial species. In this study, no appreciable data observed regarding the inter-comparison of antimicrobial activity of non-polar and polar extracts of C.roseus. Because non polar solvent extract of P.ether showed good inhibitory activity, simultaneously chloroform extract showed the low efficiency of susceptibility activity. The significant difference in extractive yield among different solvents might be contributed to the varied polarity of the solvents. The choice of solvent has an awesome affect on the extraction yield, however it does not suggest that the solvent which had maximum yield will show maximum interest under investigation [23][24]. Presently gram positive isolates were found more susceptible as compared to Gram negative isolates. Staphylococcus aureus was found most susceptible, as it was inhibited by almost all the organic extracts. This is phenomenal due to the differences in chemical composition and structure of cell wall of both types of isolates. Similar results were observed by earlier studies [20]. Furthermore, preliminary phytochemicals were analysis of all extracts, among them methanol extract showed the highest number of phytochemicals and followed by the Petroleum ether extract. The Phenol was observed from all solvent and aqueous extract, Alkaloids, and Carbohydrate were observed from four extracts (Table 4). In recent days, the analysis and production of organic compounds from plants have increased. The combination of a best separation technique (GC) with the best identification technique (MS) made GC-MS an ideal technique for qualitative analysis for bioactive compounds. The most abundant components found in the methanolic extract of flowers were Octadecamethyl, Heptasiloxane, Cyclopentasiloxane, Octasiloxane, Hepta methyl, 1H-Indole-2-carboxylic acid, Silane and octadecatrienoic acid Hexadecanoic acid, Hexadecanoic acid and Octadecadienoic acid also observed (Fig 1 to Fig 5). All compounds identified in the flower of C.roseus were reported to have antimicrobial activities. It was reported that Cyclopentasiloxane and Octadecamethyl derivatives has a wide range of pharmaceutical, biological, and medicinal applications [25][26].

Table 4

Preliminary Phytochemicals analysis of Catharanthus roseus

S. No	Phytochemicals test	Water	Chloroform	Acetone	P.ether	Methanol
1.	Alkaloids	+	-	+	+	+
2.	Carbohydrate	-	+	+	+	+
3.	Flavoniods	-	+	-	-	+
4.	Phenols	+	+	+	+	+
5.	Saponins	+	-	+	+	-
6.	Tannin	+	-	-	-	+
7.	Terpenoids	-	-	-	+	-
8.	Quinon	-	-	+	-	+
9.	Sterols	+	+	-	-	+
10.	Proteins	-	+	-	+	-

Fig 1
Mass spectrum analysis of Cholestane

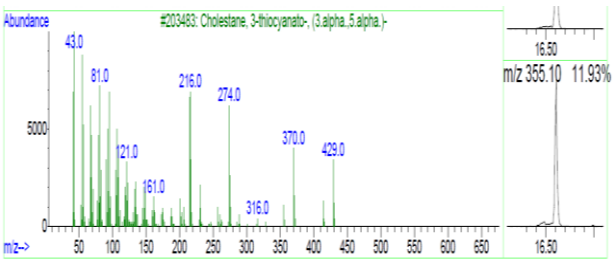


Fig 2
Mass spectrum analysis of Heptasiloxane

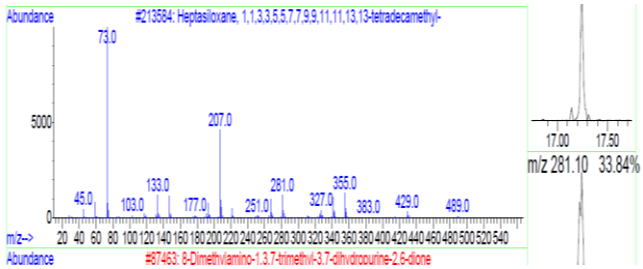


Fig 3
Mass spectrum analysis of Cyclopentasiloxane

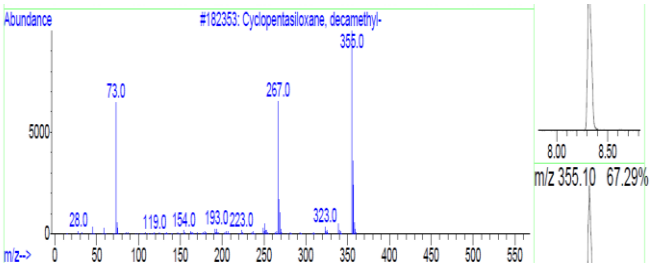


Fig 4
Mass spectrum analysis of Octasiloxane

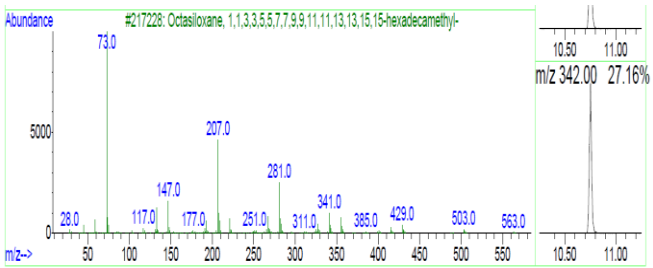
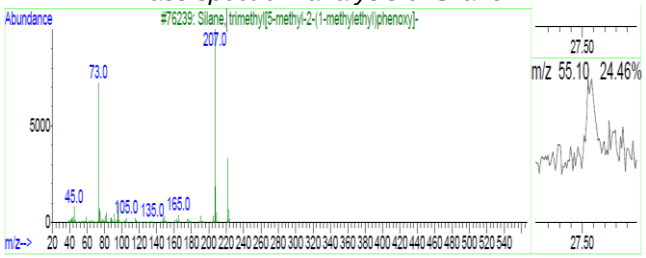


Fig 5
Mass spectrum analysis of Silane



The antimicrobial activity of C. roseus has been shown in many studies, but antibacterial effects of these plants against ESBL-producing isolates have not been yet studied. From the above result, it can be concluded that the methanolic extract of C. roseus (flower) was showed potential antimicrobial activity against ESBL producing and MRSA isolates. Furthermore, flower extract contains a number of phytochemicals constituents, which may act as beneficial substance, therefore, further studies is needed to be determined the specific compounds to utilize as a drug as the main content in the traditional medicine.

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