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Research Article

Quorum Sensing Inhibitory Activity of *Calotropis gigantea*: A Tropical Indian Medicinal Plant

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ABSTRACT

Development of multidrug resistant pathogenic bacterial strains has become more prevalent. Novel therapeutic approaches to treat drug resistant bacterial infections are gaining momentum. Disruption of quorum sensing (Quorum Quenching) attenuates the pathogenicity without imposing resistance in bacteria. Preliminary evaluation of QSI activity of certain medicinal plants of Indian origin were tested against an established pathogen of *Pseudomonas sps*. Out of the plant extracts tested *Calotropis* leaf extract had the potential QSI activity. *Calotropis* leaf extract could not affect the growth proving that it does not have any antibactericidal activity but at the same time it could reduce two out of three virulence characters like pyocyanin and protease activity. QSI activity was confirmed by a bio-inidicator organism *C. Violecium* 12472 as violacein inhibition assay. Concentration of AHL molecule were tested in treated cultures of *Pseudomonas* using *CV026* as violacein induction assay. Qualitative tests for phytochemicals of the extract had revealed that it has phenolic compounds, terpenoids, alkaloids and saponins. TLC and HPLC analysis along with fractional separation of phytochemicals proved that it has mixture of saponins, alkaloids, terpenoids and phenolics. *Calotropis* leaf extract can be further exploited as potential QSI agent as these phytochemicals could decrease the drug resistance of certain clinical bacterial isolates like *Enterococcus faecalis* and *Proteus vulgaris.*

INTRODUCTION

Development of multidrug resistant pathogenic bacterial strains has become more prevalent. Novel therapeutic approaches to treat drug resistant bacterial infections are gaining momentum. The discovery of quorum-sensing systems (QS), which coordinate important sequential events during the infection process, has provided a novel target to fight bacterial infection[1]. Compounds which are capable of interfering bacterial signalling processes known as quorum quenchers were discovered in the recent years[2]. Disruption of quorum sensing (Quorum Quenching) attenuates the pathogenicity without imposing resistance in bacteria. Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Several research papers were published indicating the potential of the plant extracts in treating microbial infections through quorum sensing inhibition[3]. Antagonists of QS that have been found are catechin (from *Combretum albiflorum* bark extract), halogenated furanones (from red alga *Delisea pulchra*), raspberry, basil and vanilla extracts [4,5,6,7].

Pseudomonas aeruginosa is increasingly recognized as an emerging opportunistic pathogen of clinical significance. Being Gram-negative bacteria, most *Pseudomonas sps*. are naturally resistant to penicillin and the majority of related beta-lactam antibiotics. According to a survey conducted by Centre for Disease Control and Prevention, *Pseudomonas aeruginosa* is the second most prevalent organism in nosocomial infections, third in causing urinary tract infections, fifth in post surgical infections. *Pseudomonas aeruginosa* produces N-Acetyl Homoserine lactone as quorum sensing signalling molecule which is used for regulation of genes for extracellular virulence factors which catalyze reactions in the host leading to cell death and tissue necrosis. *Pseudomonas* quorum sensing system is operated by two components i.e., LasR/LasI system and RhlL-RhlR system. LasR/LasI is activated by autoinducer N-3-oxo dodecanoyl Homoserine

Lactone system and RhlL-RhlR system is triggered by auoinducer N-butanoyl Homoserine Lactone.

Many researchers have reported phytochemicals as quorum quenchers or QSI agents on this pathogen and published their work on QSI activity of Malaysian plant extracts *Melicope lunu-ankenda* against *Chromobacterium violaceum CV026* and *Pseudomonas*

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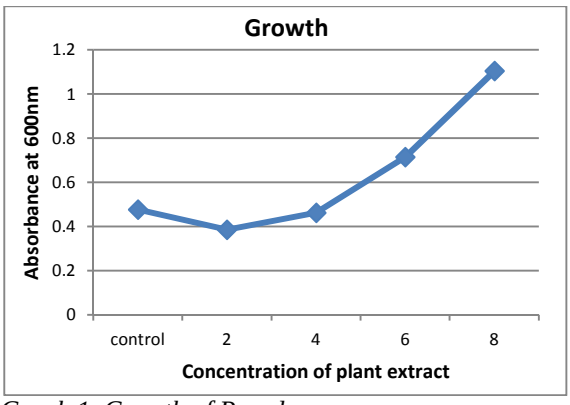
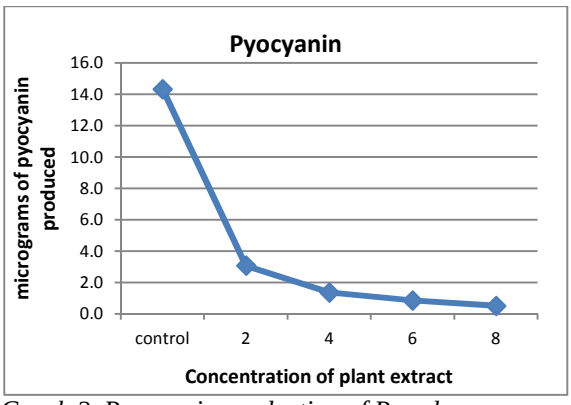
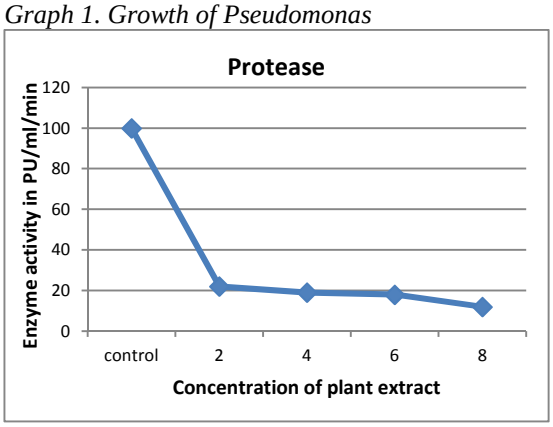


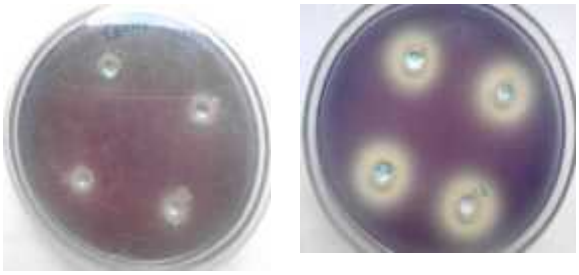
Graph 1. Growth of Pseudomonas



Graph 2. Pyocyanin production of Pseudomonas

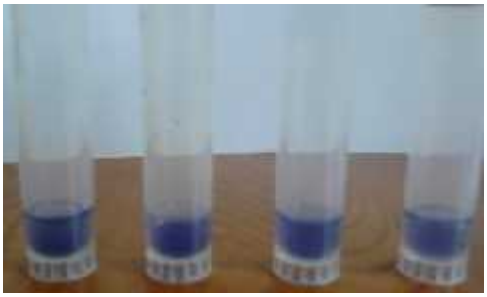


Graph 3. Protease activity of Pseudomonas

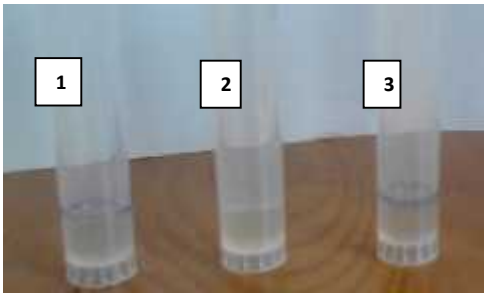


Control *Calotropis*

Fig. 1. Quorum quenching activity of different concentrations of Calotropis leaf extracts



Different concentration of standard C_6 AHL



AHL extraction from treated pseudomonas culture
1 Culture control
2 Treatment at 8mg
3 Treatment at 6mg

Fig. 2. Violacein induction assay with CV026

Table 1: List of Plant extracts used in the study & their significance

Common Name	Scientific name	Medicinal Uses
Tulsi	*Ocimum sanctum*	Important constituent of expectorants, helps to mobilize mucus in bronchitis, asthma, relieves cold, flu and brings down fever.
Mint	*Mentha spicata*	Well known for its properties related to indigestion, cramps, flatulence, nausea and appetite stimulant.
Betel	*Piper betle*	Effective against aphrodisiac, cold, chest congestion, perspiration, diuretic, aids in digestion, inflammation, sore throat and constipation.
Neem	*Azadirachta indica*	Used for the treatment of acne, arthritis, boils/ulcers, chicken Pox, herpes, malaria, dandruff, dental care, diabetes and skin disorders.
Crown flower	*Calotropis gigantea*	Treatment of skin allergies, Eczema, boils, painful swellings, quick healing and black spots on the face.

Fig. 3: *TLC plate of Calotropis leaf extract*s

aeruginosa PAO1[8] and also reported inhibition of quorum sensing controlled virulence factors production in *Pseudomonas aeruginosa* PAO1 by Ayurveda spice clove (*Syzygium Aromati*cum) bud extract[9]. Though the exact nature of bioactive compounds in these extracts were not established, these papers were in support of the view that the phytochemicals in crude extracts could be a better QSI agents. In the present investigation an attempt was made to evaluate medicinal plant extracts of Indian origin for Quroum sensing inhibition (QSI) activity on *Pseudomonas sps.* All these plant extracts were reported with good therapeutical value and often used in Ayurveda and Unani medicine (Table1).

Out of all the plant extracts, *Calotropis gigantea* leaf extract was showing promising QSI activity against pathogenic bacteria *Pseudomonas.* In ancient ayurvedic medicine the plant *Calotropis gigantea* is known as "Sweta Arka". Different parts of the plant have immense potential to cure various diseases and disorders. *Calotropis* is used in various polyherbal preparations to cure variety of human and animals ailments[10,11].

MATERIAL AND METHODS

Microorganisms used in the study**:** All the strains were cultured in Luria Bertani (LB) broth. *Chromobacterium violaceum 12472 and* CV026 was grown at 28°C supplemented with antibiotic when necessary while *Pseudomonas sps* (a lab isolate) was routinely cultured at 37°C. Other organisms used in the study are tabulated (Table 2).

Preparation of crude extracts of medicinal Plants: The crude plant extracts were prepared by making aqueous extracts to get a final concentration of 1g/10ml. The extract were used to test the Quorum sensing inhibitory activity. Plant extracts used in the study were *Calotropis gigantea* (Giant rubber bush or King's Crown), *Ocimum sanctum* (Tulsi), *Mentha spicata* (Mint), *Azadirachta indica* (Neem), *Piper betle* (Betle leaves).

Assay methods: Aqueous extract of each plant was serially diluted to 2, 4, 6, 8 mg/ml concentrations in nutrient broth. Overnight grown culture of *Pseudomonas* were added to these tubes and incubated at 37ºC. Triplicates of each set were made. Control test tubes were set with nutrient broth without the culture. Only culture without any plant extract was taken as culture control. After incubation, growth was monitored by recording absorbance at 600nm.

The Virulence factors of *Pseudomonas* like pyocyanin production, protease activity, swarming nature were assayed in the treated samples at 24hrs. Pyocyanin assay was carried out by using Essar method[12]. Protease activity was estimated by using casein as a substrate[13]. Swarming motility was evaluated by plate based assay method[14] on nutrient agar media and AHL was extracted with ethylacetate[15]. Quorum quenching activity was tested using indicator organism *Chromobacterium violaceum* 12472[16]. Violacein induction assay was performed by employing CV026[17]. Antimicrobial Sensitivity test was carried by Kirby bauer method.

Penicillinase assay was performed by Iodometric Assay[18]. Phytochemical analysis and TLC was performed as per the standard protocols[19,20,21].

Fractionation and HPLC methods were performed as per the protocols mentioned by Harborne JB[22].

RESULTS

Quorum quenching activity of plant extracts against *Pseudomonas:* Effect of crude plant extracts on *Psuedomonas* growth was monitored to know whether they had any antimicrobial activity. Extracellular virulence factors like pyocyanin (pigment) production,

Table 2. Organisms used in the study

Strain	Description	Source
Chromobacterium violaceum 12472	Wild, used in quorum signal inhibition screens, indirect acyl HSL detection.	Organism was obtained from Dr. Hammeda bee, Asst.Professor, Depar tment of Microbiology, Osmania University.
Chromobacterium violaceum CV026	Mini-Tn*5* mutant derived from *C. violaceum* ATCC 31532 HgR, *cvil*::Tn*5xylE*, KanR, plus spontaneous StrR, AHL biosensor producing a purple pigment in respond to C_4 and C_6 AHL.	Purchased from CECT, Spain
Pseudomonas sps	-	A lab isolate
Enterococcus faecalis	-	Obtained from Chromogenic
Staphylococcus epidermidis	-	Clinical isolate obtained from diagnostics
Bacillus sps	-	A lab isolate
Proteus vulgaris	-	Obtained from Chromogenic
Klebsiella pneumonia	-	Obtained from Chromogenic

Table 3: Phytochemical Analysis of *Calotropis* leaf extract

S.No	Test and Methods	Reaction
1.	Phenols	
	Ferric chloride test	+
	Libermann's test	+
2.	Tannins	
	Ferric chloride test	-
	Gelatin test	-
3.	Flavonoids	
	Dilute NaOH	+/-
	Ammonia	+/-
	Lead acetate	+/-
	Shinoda test	-
4.	Quinones	
	Conc. H_2SO_4	-
5.	Saponins	
	Froth test	+
	Foam test	+
	Honey comb froth test	++
6.	Cardiac Glycosides	
	Keller Killiani test	-
	Sodium nitroprusside test	+/-
7.	Glycosides	
	Ferric Chloride test	-
	Modified Borntrager's test	-
8.	Terpenoids	
	Terpenes test	+
	Iridoids(Monoterpenes)	-
	Diterpenes test	-
	*Libermann Burchard's(Triterpenes)	+(upper layer green)
	Salkowski's test(Triterpenes)	+
9.	Steroids/Sterols	
	Fluorescence test	-
	*Libermann Burchard's	- (No brown ring)
10.	Alkaloids	
	Mayer's test	+/-
	Wagner's test	+
	Dragendroff's test	-
	Hager's test	++
11.	Carbohydrates	
	Molisch's test	+
	Benedict's test	+
	Fehling's test	+
	Iodine test	+(Glycogen)
12.	Proteins & Aminoacids	
	Xanthoproteic test	+
	Ninhydrin test	+
13.	Resins	
	Precipitation test	+
	Turbidity test	-
14.	Coumarins	
	Fluorescence test	-
	Alcoholic NaOH test	-
15.	Volatile oils	
	Colour test	-
16.	Phlobatannins	
	Precipitation test	-
17.	Anthraquinones	
	Colour test	-
18.	Peroxides	
	Iodide test	-

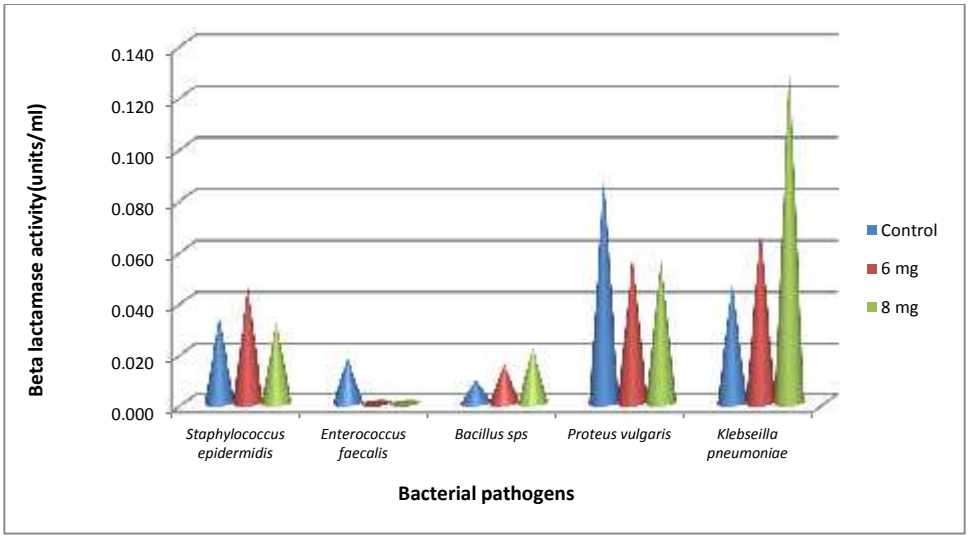
**Libermann Burchard's test: Formation of brown ring at the junction (Sterols)*
Upper layer turns green colour (Triterpenoids)
Upper layer turns red or pink colour (Terpenoids)
++ :- Strong reaction
+ :- Presence
- :- Absence
+/- :- Uncertain

protease activity, swarming nature were assayed in order to know the effect of phytochemicals present in the crude extracts. Experiments were done in triplicates and the data procured was represented in graphs (1, 2, 3).

From the graph 1, it was observed that the extract could not influence the growth of *Pseudomonas* proving that it does not have any antibactericidal activity. Data collected on virulence factors like pyocyanin production and protease activity (graph no 2 & 3) indicated that the extract could drastically reduce these two virulence factors.

Pyocyanin is produced by strains of *Pseudomonas aeruginosa* as a water soluble blue-green pigment, which belongs to the Phenazine family. It stimulates redox cycling in bacteria, liver cells, and human epithelial cell lines. Pyocyanin enhances oxidative metabolism, which increases the formation of intracellular reactive oxygen species (ROS) via reduction of NADPH. This is advantageous for bacterial survival and thus the infection sustains. Chemicals which could reduce the pyocyanin production will definitely control the virulence behaviour of the pathogen. From the present data of pyocyanin production, maximum of 96.5% reduction was recorded after treating with *Calotropis* extract at 8mg concentration. Protease activity was also observed to be effected at 2mg concentration and maximum of 88% reduction was recorded at 8mg concentration. *P. aeruginosa* protease was believed to play a major role in pathogenesis via host tissue degradation. Another important virulent factor like swarming nature was not influenced by the treatment of *Calotropis* extract. As two out of three virulent characters were observed to be effected by this extract, the studies were carried out further by checking QSI activity Violacein inhibition assay by *Chromabacterium violaceum* 12472 and Violacein induction assay by *CV026*.

Crude extracts of *Calotropis* were loaded directly onto Luria Bertani (LB) plates spread



Graph 4. Beta-lactamase (Penicillinase) activity before and after treatment with Calotropis extract

with *C. violaceum* 12472. Plates were incubated overnight at 30°C, and QS inhibition was detected by a ring of colourless,

but viable cells around the well. Loss of purple pigment in *C. violaceum* is indicative of QS inhibition by leaf extract (Fig 1). Strong QS activity was observed at all dilution of leaf extracts against distilled water as negative control.

A second bio-indicator organism *CV026* was employed in order to study the AHL concentration in the treated cultures of *Pseudomonas.* For this purpose AHL extraction was extracted. To the extracted AHL samples, *CV026* culture broth was added in order to look for violacein induction assay. Control tubes with varying concentration of standard AHL was set to record the concentration of AHL at which the violacein induction occurs. There was no violacein induction at higher concentration (8mg) of extract indicating that the AHL concentration is too low for the induction. From these results it was given to understand that *Calotropis* extract could effect and decrease the concentration of AHL molecule in the treated samples. Further studies were carried out to investigate the phytochemical components present in the crude leaf extract. As most of the plant extracts are known to possess a mixture of phytochemicals like phenols, tannins, flavonoids, quinones, saponins, cardiac glycosides, terpenoids, sterols, alkaloids, resins, coumarins, volatile oils, phlobatannins, anthraquinones, peroxides etc., specific qualitative tests were performed for the detection of these phytochemicals[19,20,21].

Based on qualitative reactions performed, the following phytochemicals i.e., phenolic compounds, saponins, terpenoids, alkaloids, carbohydrates, proteins, aminoacids & resins were identified.

Further efforts were put on to separate these phytochemicals in the crude extract. This was achieved by TLC analysis wherein silica gel was stationary phase and Ethylacetate : Methanol : water (3:0.5:0.5) was mobile phase (fig 3). Three spots of saponins, terpenoids and alkaloids were observed when TLC sheet exposed to Iodine fumes. Further identification and separation of these phytochemicals were being carried out by fractionation of crude extract using different solvents[22] and HPLC analysis (fig 5).

Fractionation of crude extract of *Calotropis* leaf was carried out as per the general procedure mentioned for fresh plant tissues into different classes of phytochemicals according to their polarity (fig 4). Samples at various steps of fractionation protocol were subjected to TLC,

HPLC analysis and QSI activity by using bio-indicator organism *C. violaceum* 12472. TLC and HPLC methods helped to separate the different phytochemicals while the bio-indicator organism could confirm the QSI activity of the fractionated samples.

Separation of phytochemicals into phenolic compounds (sample no. 5), terpenoids (sample no. 5) and alkaloids (sample no. 6, 7) was achieved at the end of this method. The QSI activity of these separated components were tested against bio-indicator organism and found to be the same for all classes of phytochemicals. These results indicate that all these phytochemicals were equally responsible for QSI activity. Hence it was observed that these phytochemicals were more effective as a mixture rather than the individual components.

As the QSI agents were reported to attenuate the bacterial pathogens and reduce the antibiotic resistance, effect of these phytochemicals were tested on clinical bacterial isolates like *Staphylococcus epidermidis, Enterococcus faecalis, Proteus vulgaris* and *Bacillus sps.* These isolates were found to be resistant to antibiotic Penicillin which is a beta-lactam antibiotic. Most of the drug resistant bacteria were found to produce beta-lactamase enzyme which help these organism to survive in the presence of penicillin. In the present study experiments were conducted to measure beta-lactamase activity[18] of these clinical isolates before and after treatment with *Calotropis* leaf crude extract. The results obtained in these experiment were represented in graphs 4. From the graph it was observed that *Calotropis* extract could reduce beta-lactamase activity in *Enterococcus faecalis and Proteus*

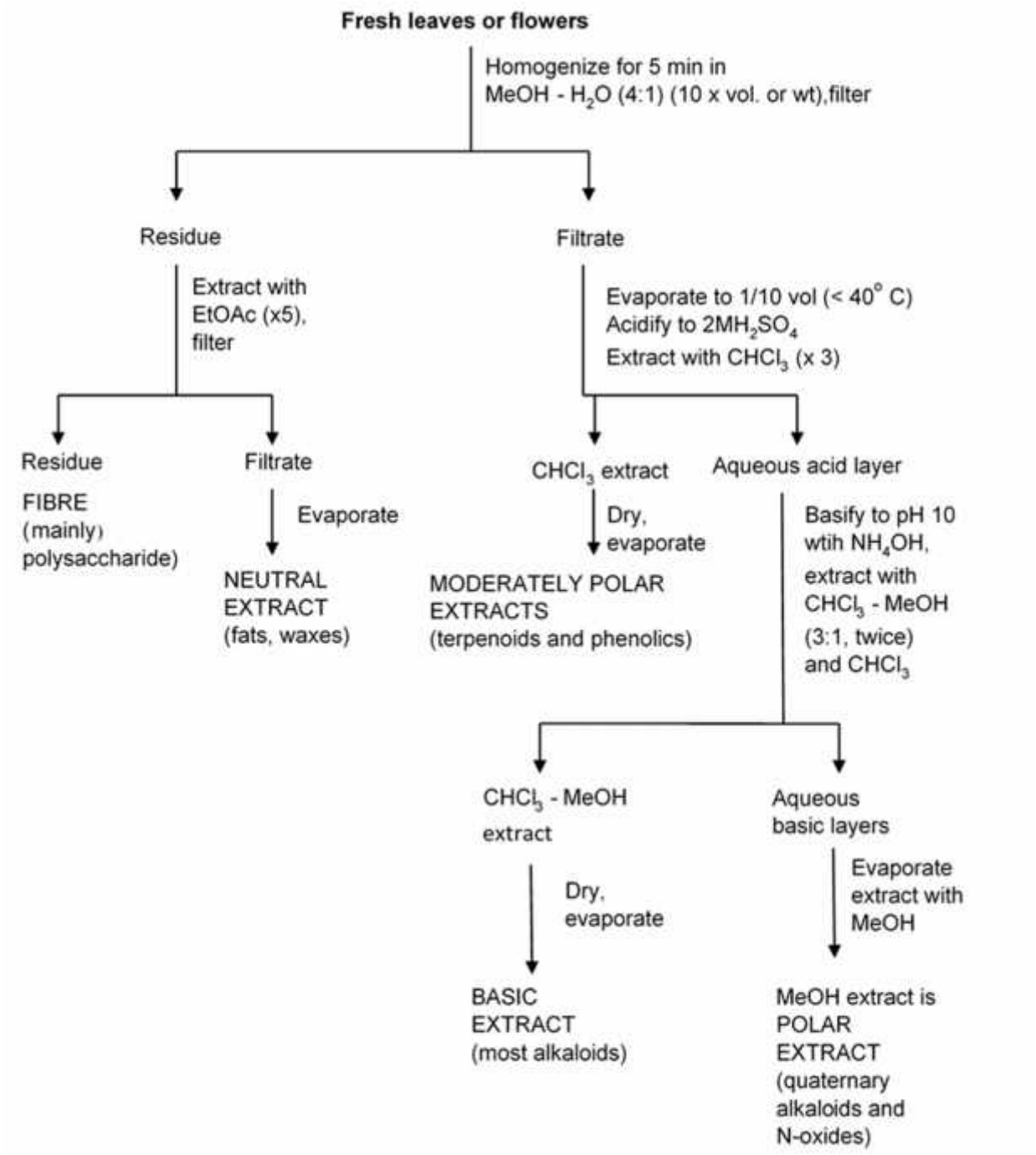


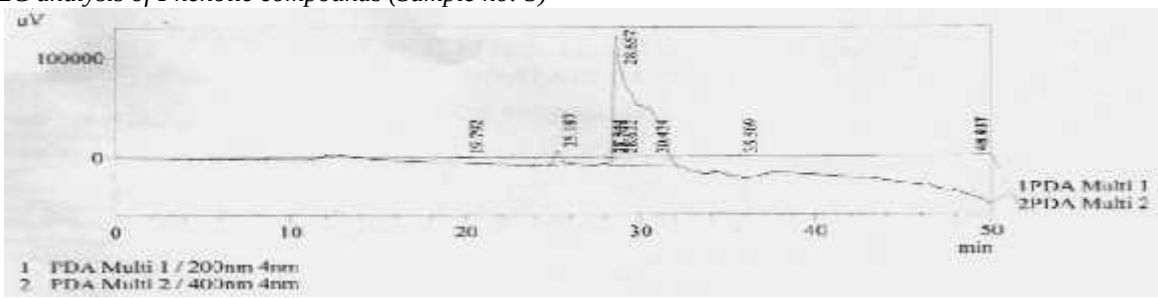
Fig 4: A general procedure for extracting fresh plant tissues and fractionating into different classes according to polarity[22]

vulgaris when compared to control. However in *Staphylococcus epidermidis* slight decrease in beta-lactamase activity was found only at higher concentration. In *Klebsiella pneumonia* and *Bacillus sps* the extract could not influence the beta-lactamase activity rather there was an increased beta-lactamase activity.

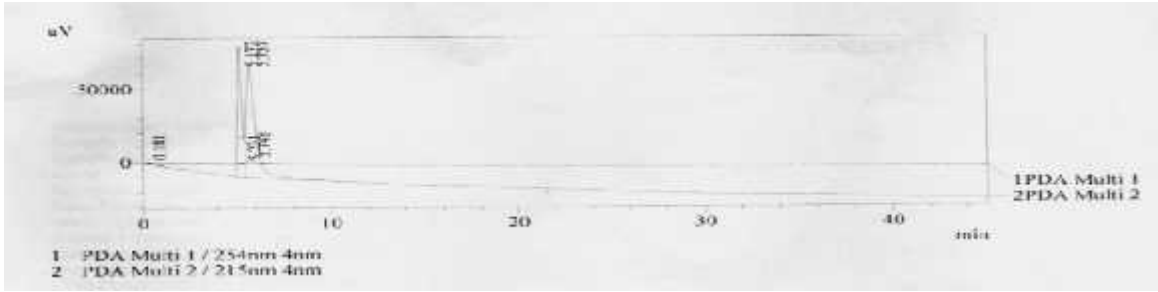
DISCUSSION

Preliminary evaluation of QSI activity of certain medicinal plants of Indian origin were tested against an established pathogen of *Pseudomonas sps*. Out of the plant extracts tested *Calotropis* leaf extract had the potential QSI activity. *Calotropis* leaf extract could not affect the growth proving that it does not have any antibactericidal activity but at the same time it could reduce two out of three virulence characters like pyocyanin and protease activity. These two virulent factors are very important for the pathogen to invade the host.

a) HPLC analysis of Phenolic compounds (Sample no: 5)

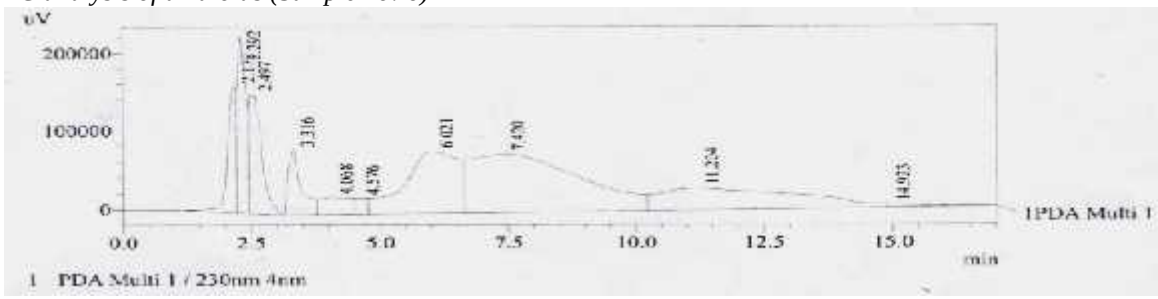


b) HPLC analysis of Flavonoids (Sample no: 5)



c) HPLC analysis of Terpenoids (Sample no: 5)

d) HPLC analysis of alkaloids (Sample no: 6)



e) HPLC analysis of alkaloids (Sample no: 7)

Fig. 5: HPLC analysis of Calotropis leaf extract

Universally QSI activity was confirmed by a bio-inidicator organism *C. Violecium* 12472 violacein inhibition assay. This test also established the fact that Calotropis leaf extract had potential QSI activity. As most of the QSI agents are known to effect the signal molecule AHL, the concentration of AHL molecule were tested in

treated cultures of *Pseudomonas* using CV026 as violacein induction assay. These experiments suggested that Fig 4. A general procedure for extracting fresh plant tissues and fractionating into different classes according to polarity[22]the AHL concentration in the treated samples were very low or insignificant for the colour induction. Hence we believe that *Calotropis* extract could decrease the levels of AHL molecules. Qualitative tests for phytochemicals of the extract had revealed that it has phenolic compounds, terpenoids, alkaloids and saponins. TLC and HPLC analysis of the fractionated phytochemicals proved that it has mixture of saponins, alkaloids, terpenoids and phenolics. *Calotropis* leaf extract can be further exploited as potential QSI agent as these phytochemicals could decrease the drug resistance of certain clinical bacterial isolates like *Enterococcus faecalis* and *Proteus vulgaris.* Further studies are being carried out for the identification of individual components of alkaloids, terpenoids and phenolics in the laboratory.

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