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Research article

PHYTOCHEMICAL, ANTIMICROBIAL AND ANTI-ADHERENCE ANALYSIS OF PLANT AND AYURVEDIC EXTRACTS

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ABSTRACT: The incessant and vital need to discover new antimicrobial compounds with diverse chemical structures and novel mode of action is stimulated by the increasing failure of chemotherapeutics and expanded antibiotic resistance exhibited by pathogenic agents. In the present research work, antimicrobial activity of few plant extracts and ayurvedic samples were screened against bacteria. Phytochemical analysis of active plant extracts showed the presence of triterpenes, glycosides and flavonoids. Both aqueous and acetone extracts of *Holoptelia integrifolia* leaf and *Barringtonia acutangula* flower; and methanolic extract of *Glycyrrhiza glabra* and *Meera* powder exhibited activity against *S. aureus*. Aqueous mortar pestle and soxhlet extracts of *B. acutangula* flower exhibited activity against both *P. aeruginosa* and *E. coli*. Further, the *ability to* adhere and build biofilm was assessed in few biofilm formers at sub-MIC concentrations using the Microtiter plate assay and the Coverslip assay. Aqueous soxhlet and mortar pestle extract of *B. acutangula* flower and acetone overnight extract of *H. integrifolia* leaf exhibited antibiofilm activity against these organisms.

Keywords: Antibacterial activity, Phytochemical analysis, MIC, Biofilm, *Barringtonia acutangula*, cover slip assay

INTRODUCTION

Plant extracts provide unlimited opportunities for new drug development because of the unmatched availability of chemical diversity that plays an imperative role in curbing the new and re-emerging infectious disease (Vaghasiya Y et al., 2009). Of approx. 2,50,000 plant species on earth, more than 80,000 have medicinal properties. Primary and secondary metabolites (phytoconstituents) of plants are used for scientific investigation and as pharmaceutical compounds. The present study is an attempt to determine the antimicrobial and antibiofilm properties in *G. glabra*, *H. integrifolia* and *B. acutangula*. *Glycyrrhiza* (Family: *Leguminosae*) is widely used in bronchial problems and in the treatment of chronic inflammations. Its active constituents are glycyrrhizin, glycyrrhetinic acid, flavonoids, asparagine, iso-flavonoids, and chalcones (Khare, 2008). *H. integrifolia (*Family*: Ulmaceae)* bark and leaves are reported to be antiinflamatory, carminative, anthelmintic, depurative and urinary astringent; while *B. acutangula (*family *Barringtoniaceae*) is used in the folklore among India's for the treatment of *kapha* and *pitta* (Vinod N. V. et al.,2010), leprosy, arthralgia, dysmenorrhea, plumbago, skin diseases, diarrhea, inflammation, flatulence, hemorrhoids, and as an anthelmintic. The stem and bark of former plant contains the triterpenoidal fatty acid esters, holoptelin-A (*epi*-friedelinol palmitate) and holoptelin- B (*epi*-friedelinol stearate), friedelin and *epi*-friedelinol (Vinod N. V. et al., 2010), whereas *B. acutangula's* bark contains tannins and ellagic acid. The fruits contain triterpenoid and sapogenins (Mukhlesur M. et al., 2005).

Phytoconstituents with known antimicrobial properties are phenols, quinones, flavonoids, tannins, coumarine, terpenoids and alkaloids. The main antimicrobial mechanism of these phytochemicals may include enzyme inhibition, binding to bacterial cell membrane, disrupting microbial membrane, inactivating microbial adhesins, enzymes, cell envelope transport proteins etc or intercalating with DNA (Cowan M. M, 1999). Some bacterial species have an array of inherent defence mechanism making them invincible that "cumulates" into global health concern. *P. aeruginosa* is a common nosocomial contaminant and its persistence as a major cause of human disease is linked to its intrinsic resistance to many antibiotics (Loureiro, 2002).

In addition to this natural resistance, in many infections *P. aeruginosa* survives as a biofilm – adhered communities which themselves are more resistant to antibiotic therapy than free-living or planktonic organisms (Spoering A. L. *et al*., 2001). Given the decreased therapeutic options for this organism, there is a clear need for novel approaches to treat or prevent *P. aeruginosa* infection. Like *P. aeruginosa*, *S. aureus* also exhibit resistance to antibiotics and host defenses due to the ability of biofilm formation.

Bacterial biofilm is a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface, thereby protects growth and allows survival in hostile environment. They differ from their free-living counterparts in their growth rate hence control of biofilm formers are difficult due to a) increased resistance to biocides, antibiotics and antibodies by virtue of up regulation and/or down regulation of approximately 40% of their genes and b) less knowledge of mechanism of their formation and survival under sessile environments. Likely effective strategies for biofilm treatment include prevention of bacterial cell adhesion to the substratum, reduction of polysaccharide production and disruption of cell-to-cell communication involved in biofilm formation through physical, chemical, and biological approaches. Currently, much attention is focused on developing new ways to prevent biofilm formation on both industrial and medical surfaces. Quorum sensing plays such a substantial role in biofilm formation, hence by coating or embeding surfaces with compounds capable of interfering with related signaling mechanisms can be one of the mechanism of destruction of biofilm. Since no single method or chemical completely eliminates biofilm formers, a combination of various treatments are used for controlling biofilm formation (Romeo T, 2008). After preliminary screening of active components from various plants, the study further envisaged the ability of these plant extracts to reduce/stop biofilm formation by *S. aureus* and *P. aeruginosa* using the Microtiter plate and Cover slip assays (Khan S. A et al., 2001, Tram G. et al 2013).

MATERIAL AND METHODS

Antibacterial activity of the plant extracts

Plants used for antibacterial and antiadherence activity are listed in Tab 1. These were collected from Rani Jijamata Udhyan, Byculla, Mumbai. The ayurvedic samples and herbs were purchased from ayurvedic shops. Pure cultures (*S. aureus, S. lutea, B. subtillis, B. cereus, C. diphtheriae and S. pyogenes, S. dysenteriae, K. pneumoniae, P. vulagris, P. aeruginosa* and *E. coli)* were obtained from the School of Biotechnology and Bioinformatics, D. Y. Patil University, CBD Belapur, Navi Mumbai. While the biofilm formers viz. *P. aeruginosa* (ATCC 27853) and *S. aureus* (ATCC 25923) were procured from National Chemical Laboratory (NCL), Pune. Plant extracts were obtained using methods by Girish and Satish (2008), overnight extraction or solvent extraction protocol by Begum *et al.* (2006) and Adeiza *et al.* (2009). Preliminary antibacterial activity was determined by agar ditch plate method (Hinchliffle *et al*.; 1977,) followed by determination of MIC's by the agar cup diffusion method (Rose S. B. et al., 1939; Bauer A. W. *et al* 1966). Log phase cultures of overnight grown cultures were used for all estimation of antibacterial activity.

Antiadherence activity of the plant extracts:

Inoculum preparation and plant extract selection for determination of antibiofilm activity by the microtiter assays was similar to that of the previous technique. Each ELISA plate well loaded with 100µl of the plant extract at MIC and sub-MIC concentration. Measurement of absorbance was according to Meritt J. H. et al. (2005). In the Cover slip assay method for determination of the anti-biofilm activity, glass cover slip was placed in tube at an angle of 45° and was sterilized. The positive control contained culture only while solvent control consisted of 0.5M NaOH with 5% Sucrose with culture. Both the assays were performed in two sets of triplets. The tubes containing different extracts along with positive and solvent control were placed in vertical position and incubated for 24 hrs at 37ºC. The planktonic cells were decanted after incubation period from one set of tubes and the cover slips were gram stained to study the effect of anti- biofilm effect in presence of the plant extracts. In the second set of tubes absorbance was measured for 1: 10 dilution of the planktonic cells. The remaining planktonic cells in the same set of tubes were removed and 0.5M NaOH was added to the tubes containing coverslips. The tubes were vigorously shaken to remove the adhered cells and the absorbance was measurement at 600nm. Antiadherence activity was then compared to the positive control. Decrease in absorbance of tests containing plant extracts represents biofilm inhibition as compared to the positive control.

Phytochemical screening

Phytochemical screening for major constituents was undertaken using standard qualitative methods as described by Odebiyi and Sofowora (1990). The plant extracts were screened for the presence of biologically active compounds like glycosides, alkaloids, tannins, triterpenes, flavonoids and saponins. The HPLC apparatus consisted of a Water 6785 multi solvent delivery system, equipped with a UV dual detector. Data were processed using Waters Empower Software.

Chromatography was carried out using two solvents: i.e. 25% methanol in 1% acetic acid; and 30% methanol in 1% acetic acid in a linear gradient programme. The flow rate of the mobile phase was 0.75 ml/min. Peaks were detected at 280 and 360 nm absorption spectrum. A 20 µL volume of each plant extract was injected in triplicates onto HPLC column.

The secondary metabolites screened were tannic acid, cathechol, caffeic acid, vanillin, p-Coumeric acid and ferulic acid. The presence of phytochemical components was confirmed on comparison with graph obtained by running the standards of above mentioned components. The individual phytoconstituents collected according to their retention time and after comparison to the standards were checked for antibacterial activity against *S. aureus*, *P. aeruginosa* and *E.coli.*

RESULTS AND DISCUSSION

Antibacterial activity of the plant extracts

Phytoconstituents are produced by plants as protective measures against various predators and microorganisms. *In-vitro* evaluation for antimicrobial property is the preliminary step towards developing eco-friendly methods to control infectious diseases in humans. This study envisaged the effect of these phytoconstituents in different extractable form using the agar cup diffusion method on various bacteria. Plant extract obtained using different solvents and extraction methods (Table 1) were seen to be active mostly against Gram positive and few against Gram negative bacteria (Table 2). From the preliminary studies, *S. aureus* P. *aeruginosa* and *E. coli* were selected for further study. Comparatively high MIC for BFAS against S. aureus (140-150 mg/ml), BFAqS and BFAq against *P. aeruginosa* (220 - 225 mg/ml and 90 -95 mg/ml, Figure 2b) was observed. MIC of BFAq and BFAqS against *S. aureus* was in the range of 10 to 20 mg/ml, whereas MIC of BFAO was in range 30 to 35 mg/ml respectively. MIC of BFBAq was 60 to 65 mg/ml and MIC of HLAO, HLMO and HLAq against *S. aureus* were in range of 15 to 30 mg/ml while for *Meera* and *G. glabra* methanol overnight extracts had MIC in the range of 3 to 10 mg/ml and 35 to 40 mg/ml respectively. Similarly the MIC was also higher for BFAqS (215 - 225 mg/ml) than with the BFAq 95 mg/ml to 100 mg/ml with *E. coli.*

Previous study by Khan M. R. et al (2002) showed antibacterial activity of *Barringtonia racemosa* and antifungal activity of *Barringtonia asiatica*. In the present study the methanol overnight extract, gave MIC at much lower concentration (32 mg/ml) than for acetone soxhlet (160 mg/ml) against *S. aureus*. This difference in the activity could be attributed to the different phytoconstituents that get extracted by using different solvent system and techniques. Possibly some phytoconstituents have got destroyed during extracting by the Soxhlet method thereby increasing the MIC value. Recent studies by Padmavathi et al. (2012) and review by Kaur et al (2013) have indicated the antimicrobial activity of *Barringtonia acutangula* shade dried leaves. However this study cannot be compared with the present study as (a) Flower and flower bark were used instead of leaves (b) unlike present study their organic solvents used for extraction of phytoconstituents were ethyl acetate, ethanol and petroleum ether.

Antiadherence activity of the plant extracts:

A six fold decrease against *S.aureus* biofilm formation by cover slip assay was shown by both BFAqS with dilution of 25 mg/ml and HLAO with dilution of 15 mg/ml (Figure 2 a-c)**.** BFAqS with concentration of 20 mg/ml and HLAO with concentration of 10 mg/ml respectively showed an increase in biofilm formation as compared to BFAqS concentration of 25 mg/ml and HLAO with concentration of 15 mg/ml (Figure 3a). Both the extracts showed reduced growth of biofilm formers on coverslip as compared to positive control (Figure 2 a-c) Similarly decrease in biofilm formation for both the plant extracts compared to the positive control was observed in microtiter plate assay (Figure 3b).

An eight fold decrease in biofilm formation was shown by both BFAqS at 220 mg/ml and BFAq at 85 mg/ml against *P. aeruginosa* by coverslip assay (Figure 3a). BFAqS at 210 mg/ml showed an increase in biofilm formation as compared to BFAqS at 220 mg/ml however no such difference was observed for BFAq at 85 mg/ml and BAFAq at 90 mg/ml. The BFAq also showed an increase in the planktonic cells of *P.aeruginosa* thereby indicating that the plant extracts are exhibiting an antibiofilm activity. Also both the plant extracts showed reduced growth on the coverslip as compared to the positive control.

In spite of many studies reporting antibacterial, antihelmith (Bharathi R, V, et al., 2010, Padmavathi D, et al., 2011a, b), antioxidant and anticancer (LakshmiP. J, et al., 2013) properties of *Barringtonia*, however no such studies have been reported for effect of the extracts against biofilm producers. Biofilms formed by *P. aeruginosa* have long been recognized as a challenge. Cystic fibrosis, endocarditis, device-related infections, and ventilator-associated pneumonia are some of the diseases that are considerably complicated by the formation of bacterial biofilms, which are resistant to most current antimicrobial therapies (John L. P. et al., 2006).

Both the method used for determination of antiadherence and antibiofilm activity allows high screening of phytoconstituents as biocides. Use of Microtiter plate assay was attempted as it has high reproducibility with multiple biofilm grown in single experiment at a given time with similar condition and it uses less of plant extract as biocide.

The Cover slip assay method attempted was a miniaturized way of testing the formation of biofilm optically and microscopically. However both these methods tested the phytoconstituents antibiofilm property by the static model which is unrealistic with respect to biofilm formation due to no free flow of nutrients and waste products. However at MIC level extracts exhibited antibiofilm activity on glass slide under static condition but favoured conversion of biofilm formers to planktonic cells thereby increasing their concentration at sub MIC level.

Table 1: Plant extracts from different parts of plant using different solvents and methods.

Plant / part		Mortar pestle	Overnight samples		Soxhlet samples				
		Aq	Ac	Me	Ac	Me	Pe	Cf	Aq
Holoptelea integrifolia	Leaf	+	+	+	-	-	+	-	-
	Root	+	-	+	-	-	-	-	-
	Bark	+	+	+	+	+	-	-	-
	Seed	+	-	-	-	-	-	-	-
Barringtonia acutangula	Stem	+	+	+	+	+	+	-	-
	Seed	+	+	+	+	+	+	-	-
	Bark	+	+	+	+	+	+	-	-
	Flower	+	+	+	+	+	+	-	+
	Leaf	+	+	+	+	+	+	+	+
	Flo-bark	+	+	+	-	-	-	-	+
Plumeria rubra	Leaf	+	-	+	-	-	-	-	-
	Flower	+	-	+	-	-	-	-	-
Ficus glomerata	Leaf	+	-	+	-	-	-	-	-
	Bark	+	-	+	-	-	-	-	-
	Fruit	+	-	+	-	-	-	-	-
Bombax malbaricum	Leaf	+	-	-	-	-	-	-	-
	Bark	+	-	-	-	-	-	-	-
Catharanthus roseus	Flower	+	-	+	-	-	-	-	-
	Leaf	+	-	+	-	-	-	-	-
Senararia spp	Leaf	+	-	+	-	-	-	-	-
Cleom smilecifolia	Leaf	+	-	-	-	-	-	-	-
Glycerrhiza glabra	Root	-	-	+	-	-	-	-	-
Meera	Ayurvedic powder	-	-	+	-	+	-	-	-
Tribulus terrestris	Seed powder	-	-	+	-	-	-	-	-
Cinnamomum verum	Bark	+	-	+	-	-	-	-	-
Coriandrum sativum	Seed	-	-	+	-	-	-	-	-
Ocimum bacilicum	Seed	-	-	+	-	-	-	-	-
Fagonia arabica	Ayurvedic powder	-	-	+	-	-	-	-	-
Aegle marmelos	Leaf	-	-	+	-	-	-	-	-
Khas	Root	-	-	+	-	-	-	-	-

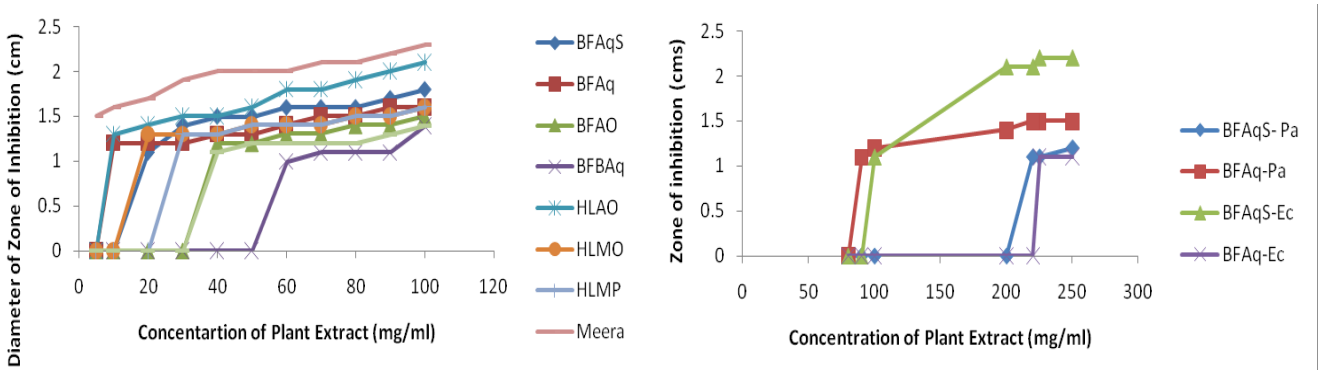
'Ac' (acetone): 'Pe' (Petroleum ether): 'Me' (methanol) :Aq' (aqueous) :'Cf' (Chloroform) :'+': (extract prepared)'-': extract not prepared

Table 2: Effect of different plant extracts on gram positive and negative bacteria

	Organisms used									
Plant extracts	***S. lutea***	***B.subtilis***	***C. diptheriae***	***S. pnemoniae***	***S. aureus***	***S. dysentriae***	***K. pnemoniae***	***P. vulgaris***	***P. aeuroginosa***	***E. coli***
BFAqS	-	+	-	+	+	+	-	-	+	-
BFMS.	-	+	-	-	-	-	-	-	-	-
BFAS	-	-	-	-	+	+	-	-	-	-
HLAO	+	-	+	-	+	-	-	-	-	-
HLAq	-	-	+	-	+	-	-	-	-	-
HLMO	-	+	-	+	+	-	-	-	-	-
BFAq	-	-	+	+	+	-	-	-	+	-
BBAS	+	+	+	+	-	-	-	-	-	-
BLAS	-	+	+	-	-	+	-	-	-	-
Meera MO	+	+	+	+	+	-	-	-	-	-
BBAq.	-	-	-	-	-	-	-	-	-	-

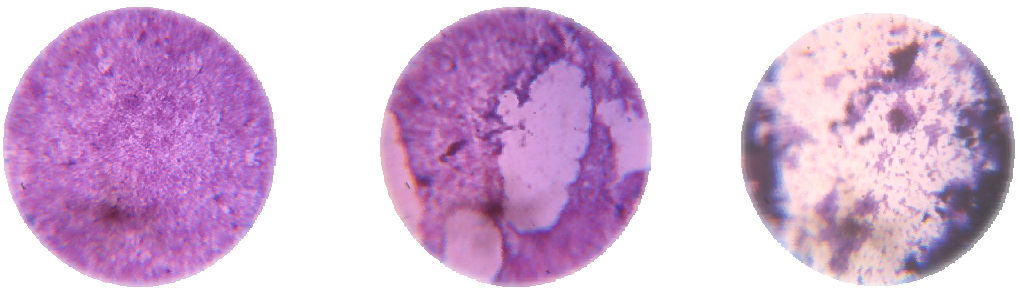
Key: "+": indicates Inhibition; & "-": indicates No inhibition

Abbreviation: BFAq – *B. acutangula* flower aqueous extract; BFAqS – *B. acutangula* flower aqueous Soxhlet extract; BFMS – *B. acutangula* flower methanol soxhlet; BFAS – *B. acutangula* flower acetone soxhlet extract; BFAO – *B. acutangula* flower acetone overnight extract; HLAO – *H. integrifolia* leaf acetone overnight extract; HLMO – *H. integrifolia* leaf methanol overnight extract; HLAq – *H. integrifolia* leaf aqueous extract; BLAS- *B. acutangula* leaf aqueous Soxhlet extract: BBAq - *B. acutangula* bark aqueous extract; BBAS- *B. acutangula* bark aqueous Soxhlet extract.



(a) (b)

Figure 1. Effect of different plant extracts against *S. aureus* (a) *P. aeuroginosa* and *E. coli* (b)



(a) (b) (c)

Figure 2: A view of a microscopic field for S. aureus Biofilm formed on coverslip (a) positive control (b) in presence of BFAqS-2 and (c) BFAq S-1

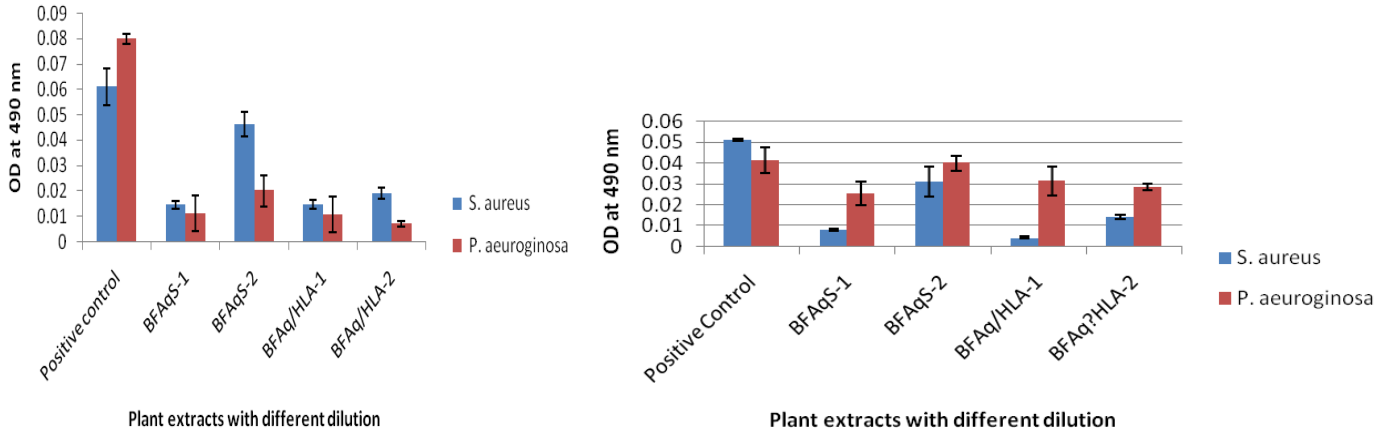


Fig 3: Effect of plant extract against biofilm formation using the coverslip assay (a) and Microtiter plate assay (b).

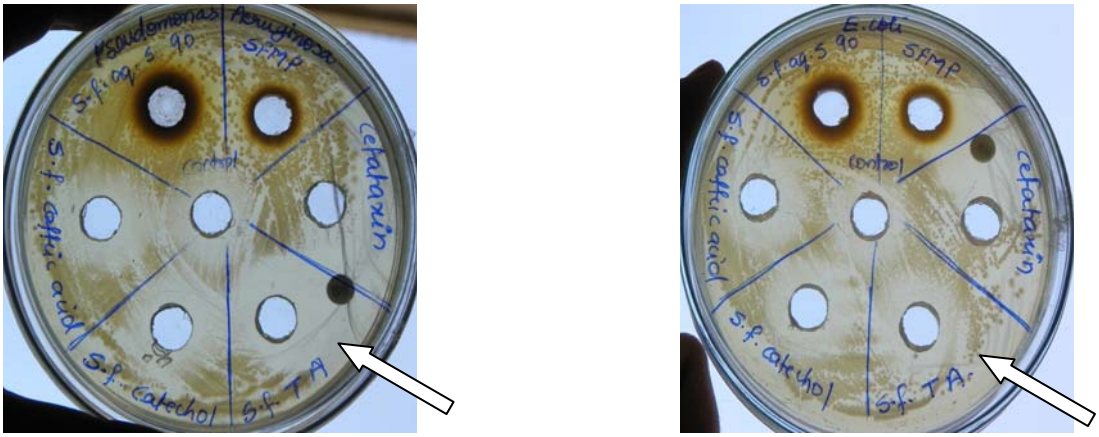


Figure 4: Effect of antibiotic Cefataxime, BFAqS (90 mg/ml), BFAq (100 mg/ml), *B. acutangula* flower Caffeic acid(CA), *B. acutangula* flower catechol and *B. acutangula* flower Tannic acid (TA) eluted samples against *P.aeruginosa* (a)and *E.coli* (b).

Phytochemical screening

Phytochemical analysis through qualitative method showed presence of various phytoconstituents, viz., saponins, tannins, triterpenes, alkaloids, flavonoids and glycosides, in plant samples. HPLC analysis of active plant extracts shows presence of various phytochemical components such as tannic acid, cathechol, caffeic acid, vanillin, and ferulic acid which may attribute to the antibacterial activity of plant extracts. Of all the extracts tested BFAqS and HLMO had significantly large amount of Tannic acids (2324 and 2058 ppm resp.) and Catechol (184 and 3.45ppm resp.). The ayurvedic powder Meera showed presence of Tannic acid (113.521ppm), catechol, caffeic acid and vanillin (1.414, 6.355 and 0.651ppm). Only GRMO showed presence of Ferrulic acid (21.732 ppm) while HLAq showed presence of Tannic acids and catechol, however HLAO showed presence of only Tannic acids. Some of these extracts were checked for antibacterial activity (Figure 4) against the three test organism. Elute containing Tannic acid gave a larger zone of inhibition for all the three organisms. The study can be further extended for *in vivo* evaluation of these antimicrobial extracts/elutes along with the toxicity studies in order to develop a novel broad spectrum antimicrobial herbal formulation.

CONCLUSION

The present study thus envisaged the antimicrobial and antibiofilm forming ability of the phytoconstituents obtained from different solvent extraction system in three of the pathogenic organism. The results obtained validated that some of the constituents may have promising effect in both of the direction.

However, the present study has limitations which need to be considered for future study. Firstly, extracts from fresh and dry plants parts may differ in phytoconstituents as some gets concentrated in dried parts while others such as tannins may get less extractable from dry plant material due to increased fixation. Secondly, some plant parts may get labile and may degrade when dried such as Cathinone. Further based on the seasonal condition and geographical location the phytoconstituents may change. Further studies may include checking the effect of phytoconstituents of active plant extract on the mechanism involved in destruction of *S. aureus* and *P. aeruginosa* biofilm. Utilizing the dynamic CBD (Caligary Biofilm Device) model (Santapoloa et al., 2012), the activity of extracts needs to be studied for antiadherence and antibiofilm activity.

Abbreviations: BFMO – *B. acutangula* flower methanol overnight extract; BFAq – *B. acutangula* flower aqueous extract; BFAS – *B. acutangula* flower acetone soxhlet extract; BFAO – *B. acutangula* flower acetone overnight extract; BFAqS – *B. acutangula* flower aqueous soxhlet extract; HLAO – *H. integrifolia* leaf acetone overnight extract; HLMO – *H. integrifolia* leaf methanol overnight extract; HLAq – *H. integrifolia* leaf aqueous extract; BFBAq - *B. acutangula* flower bark aqueous extract.

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