ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 9, Issue 4, 2016

Online - 2455-3891
Print - 0974-2441

Research Article

ANTI-BIOFILM EFFICACY OF *PLECTRANTHUS AMBOINICUS* AGAINST *STREPTOCOCCUS PYOGENES* ISOLATED FROM PHARYNGITIS PATIENTS

MANIMEKALAI K, SRINIVASAN P, DINESHBABU J, GUNA G, TEEPICA PRIYA DARSINI D*

Department of Biotechnology, Laboratory of Clinical Biotechnology and Herbal Medicine, Karpagam University, Coimbatore - 641 021, Tamil Nadu, India. Email: teepica@gmail.com

Received: 22 April 2016, Revised and Accepted: 03 May 2016

ABSTRACT

Objective: The objective of the study was to evaluate the anti-biofilm efficacy of Indian medicinal plant *Plectranthus amboinicus* extracts against the biofilm forming *Streptococcus pyogenes* isolated from pharyngitis patients.

Methods: The plant extracts (methanol and ethyl acetate) were screened for their preliminary phytochemical components. The solvent extract with higher phytochemical yield was subjected to quantitative analysis using the Gas Chromatography-Mass Spectrometry (GC-MS) technique. *In vitro* analysis of the anti-biofilm study was performed using the Minimal Inhibitory Concentration (MIC) assay, biofilm inhibitory concentration assay, growth curve analysis, anti-bacterial activity, and light microscopy analysis.

Results: The methanol extract showed the highest phytochemical content. GC-MS analysis of the methanol extract showed a total of thirty two phyto compounds among which most of the compounds were medicinally important. MIC assay showed that the inhibition of test pathogens was at an average concentration of 2 mg/ml. The agar well diffusion method elucidated that at sub-MIC the methanol and ethyl acetate extracts did not inhibit the growth of the test pathogen. Growth curve analysis was carried out at the concentration sub-MIC, in which the solvent extracts did not show any significant inhibition on the planktonic cells, whereas the biofilms of the test pathogens were significantly reduced and were dose dependent at sub-MIC levels as confirmed by the light microscopic analysis.

Conclusion: These preliminary results indicated that the methanol extract of *P. amboinicus* leaves consisted of pharmacologically active components and could be used as an anti-biofilm agent at minimal concentrations thereby successful preventing the formation of biofilms.

Keywords: *Plectranthus amboinicus*, GC-MS, Anti-biofilm activity, Light microscopy, *S. pyogenes*.

INTRODUCTION

The discovery of antibiotics and their application as chemotherapeutic agents emphasized that frequent administration would lead to the eradication of infectious diseases [1]. With the increase in the global emergence of multi-drug resistant bacterial strains, the effectiveness of anti-bacterial drugs has become limited leading to the failure in the treatment of infections [2]. Research has shown that majority of the bacterial cells form biofilm [3]. Biofilm formation protects the bacterial cells from environmental stress and antibiotic treatment by which they become more resistant. *Streptococcus pyogenes* is a major upper respiratory tract pathogen which causes bacterial pharyngitis that leads to serious complications and it is associated with extensive human morbidity worldwide [4,5]. The ability of this bacterium to form biofilms is one of the virulence-promoting factors as the bacteria are protected from the host immune system and antibiotics administered during the treatment period [6]. Biologically active compounds derived from herbal plants have always been a thrust area for the researchers working on infectious diseases and their control [7]. In India, a wide variety of aromatic plants are widely employed in traditional medicine in the treatment of infectious diseases as well as to extend the shelf life of foods [8,9].

The genus, *Plectranthus* consists of more than 300 species of plants and belongs to the family of Lamiaceae. Plants belonging to this family have rich ethnobotanical diversity with unique medicinal properties. One such medicinal plant in this genus is *Plectranthus amboinicus* [Lour.] Spreng, commonly known as Indian borage and it is widely used in the traditional medical systems of India [10]. The plant is a large, succulent, aromatic, perennial herb distributed throughout India and Sri Lanka [11]. *P. amboinicus* is medicinally used to treat urolithiasis, epilepsy, tumors and mutagens, neurological disorders, viral and fungal infections [12]. A decoction of its leaves are used to treat chronic cough and asthma and also used as an anti-spasmodic for, stomach ache, and for the treatment of a headache, fever, epilepsy, and dyspepsia [13]. It is also used in the treatment of skin ulcerations and urinary diseases, as well as to alleviate inflammation, kidney troubles and in conditions of congestive heart failure [14,15]. Although many reports have substantiated the various medicinal properties of *P. amboinicus*, the studies on the anti-biofilm properties of this plant are at a naïve stage. Moreover, based on the anti-bacterial efficacy of these plants against various bacterial pathogens, we hypothesize that the solvent extract would also aid in controlling the biofilm formation of *S. pyogenes*, which is one of the upcoming virulence factors for the pathogen. The present study is focused on the anti-biofilm properties of *P. amboinicus* solvent extracts against the biofilm forming Gram-positive pathogen *S. pyogenes*.

MATERIALS AND METHODS

Collection of plant materials

P. amboinicus leaves were collected from area surrounding Coimbatore, Tamil Nadu. Specimens of *P. amboinicus* (Voucher No: 1113) have been authenticated as *P. amboinicus* [Lour.] Spreng. and deposited in the Botanical Survey of India, Southern Circle, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

Solvent extraction

The leaves were washed, shade dried, and powdered. About 25 g of the each dried plant powder was soaked in 100 ml of methanol and ethyl acetate (1:4) for 7 days with periodic soaking and then filtered using Whatman filter paper No. 1. The filtrate was then dried at 55 °C for 1 h using rotary vacuum evaporator (Buchi Type, India) and the yield percentage yield was calculated. The dried *P. amboinicus* methanol (PAM) and ethyl acetate (PAEA) extracts were then aliquoted using

80 % di-methyl sulfoxide (DMSO) (Himedia, India) to prepare stock (20 mg/ml) and working solution (0.0625-8 mg/ml).

Phytochemical analysis

The methanol and ethyl acetate extracts of *P. amboinicus* were subjected to preliminary phytochemical screening and the presence of alkaloid, carbohydrates, tannins, saponin, flavonoid, steroids, terpenoid, glycosides, phenol, amino acids, and lipids were observed according to the methods of Harborne [16].

Spectral analysis using Gas Chromatography-Mass Spectrometry (GC-MS)

Bioactive compound analysis of *P. amboinicus* extract was determined by Shimadzu Gas chromatography (QP2010 Plus, Japan), with a 30 mm × 0.25 mm RTX-5MS low bleed column with a thickness of 0.25 µm according to Rijo *et al.* [17]. The spectrum of the unknown phyto components was compared with the spectrum of known components available in Wiley Online Library (Wiley08), and NIST08 library. The name of phyto compounds, molecular weight, and structure of the sample was determined.

Bacterial strains and culture conditions

A total of 10 isolates were obtained from throat swabs of pharyngitis patients at hospitals in and around, Coimbatore, Tamil Nadu. All the isolates were confirmed for *S. pyogenes* using Streptococcus selection agar (Himedia, India). *S. pyogenes* MTCC 1924 (IMTECH, Chandigarh) was used as reference strain. All the isolates were cultivated in Todd Hewitt's Broth (THB) for routine analysis. Glycerol stock was maintained at −20°C until further use.

Screening of biofilm forming *S. pyogenes*

The isolates of *S. pyogenes* were screened for biofilm formation according to Srinivasan *et al.* [18]. Briefly, 12 h culture was prepared and gently resuspended in 1 ml THB (pH 7.3±0.2) (Himedia, India) medium and adjusted to an optical density of 1.0 at 620 nm. Then, the bacterial suspensions were aliquoted (100 µl) in each well of polystyrene 96 well flat-bottomed microtiter plates (Tarsons, India) and incubated for up to 48 h at 37 °C without shaking. After incubation, the isolates were analyzed for biofilm formation.

The planktonic cells were discarded, and attached cells were gently washed twice with 1X phosphate-buffered saline, and fixed with 2 % glutaraldehyde for 15 min at room temperature and stained with 0.4 % (w/v) crystal violet (CV) (Himedia, India) for 10 min at room temperature. Then, the CV stained cells were solubilized with 1 ml of ethanol-acetone solution (8:2, v/v). The biofilm formation ability was scored as strong (+++), moderate (++), weak (+), and negative (−) by visually comparing the thickness of the adherent layer, and the results were tabulated. The isolates capable of strong biofilm formation were subjected for further studies.

Minimal inhibitory concentration (MIC) assay

The MIC assay of the plant extracts was performed according to Wayne [19]. The bacterial suspensions (1×10^6 CFU/ml) were added to THB supplemented with PAM and PAEA at different concentrations ranging from 0.0625 mg/ml to 8 mg/ml and incubated at 37 °C for 24 h. The lowest concentration that produced inhibition of visible growth after overnight inhibition was recorded as MIC value.

Growth curve analysis

Growth curve analysis was performed according to Srinivasan *et al*. [18] Briefly, 1 % of overnight test pathogens (0.5 OD at 600 nm) was inoculated in 50 ml of luria broth (LB) broth separately, supplemented with 0.5 mg/ml (sub-MIC) of *P. amboinicus* extracts. The flasks were incubated at 37 °C with 170 rpm agitation in a rotatory shaker (Orbitek - LT, India). Cell density was measured using ultraviolet (UV)-visible spectrophotometer (Shimadzu UV-3600 Plus, Japan) at every 1 h interval up to 12 h.

Agar well diffusion assay

The anti-bacterial activity of PAM and PAEA extracts performed by agar well diffusion method using Mueller-Hinton agar (MHA) (Himedia, India) by following the methods specified in Clinical and Laboratory Standards Institute [19]. Briefly, 1 % overnight culture of test pathogen was swabbed uniformly over freshly prepared MHA plates and allowed to set. Then, agar plugs were cut out using gel puncture and wells were cut onto the medium. 30 µl of PAM and PAEA at sub-MIC (0.5 mg/ml) was incorporated onto the wells. Streptomycin (0.03 mg/ml) was used as positive control, and DMSO was used as negative control.

Quantification of biofilm biomass inhibition

Quantification of biofilm biomass was performed using 24 well microtiter plate (MTP) assay Dineshbabu *et al*. [20] with slight modification. Briefly, 1 % overnight cultures (0.5 O.D at 600 nm) of test pathogens were individually added to 1 ml of fresh LB medium containing PAM and PAEA sub-MICs (0.5-2 mg/ml). The samples were incubated at 37 °C for 16 h. After incubation, MTPs were emptied of free-floating planktonic cells, and the wells were gently rinsed with sterile water. The sessile cells were stained with 0.4 % CV (Himedia, India) solution. After 15 minutes, CV solution was discarded completely, and wells were filled with 1 ml of 95 % ethanol for de-staining. The biofilm biomass was then quantified by measuring the absorbance at OD 650 nm using multi-plate ELISA reader (Biotek-ELX-800, India).

Microscopic observation of biofilm

Light microscopic analysis

For visualization of biofilm by light microscopy [21], the biofilms were allowed to grow on glass pieces (1 cm ×1 cm) placed in 24-well polystyrene plates supplemented with different solvent extracts of *P. amboinicus* (1 mg/ml) and incubated for 24 h at 37 °C. The slides were stained using CV and were placed on slides with biofilm pointing upward. The slides were observed under light microscopy at magnification of ×400. Visible biofilms were documented with an attached digital camera (Nikon Eclipse Model: E200).

Statistical analysis

Statistical analysis was performed using SPSS software (Version 16, Chicago, USA). Students T-test was used to calculate the significant difference. Values were considered significantly different if $p\leq0.05$.

RESULTS

Percentage yield

The percentage yield of the methanol and ethyl acetate fractions of *P. amboinicus* is summarized in Table 1. Among the two extracts, methanol extract showed the maximum yield percentage.

Preliminary phytochemical screening of solvent extracts

The results of the qualitative phytochemical screening are represented in Table 2. The present study showed positive results for the presence of alkaloids, flavonoids, carbohydrates, saponins, phenols, glycosides, terpenoids, and carbohydrates in the methanol of *P. amboinicus*. However, sterols were not present in the methanol extract. Similar results were observed for the ethyl acetate extract except for the absence of saponins.

GC-MS analysis of methanol extract

A total of 32 compounds were observed from the GC-MS analysis, and they were classified according to the retention time, molecular formula, molecular weight, and peak area (Fig. 1). The name of the compounds

Table 1: Yield percentage of the *P. amboinicus* solvent extracts

S. No	Bioactive compounds	Weight of crude extract (g)	Yield (%)
1.	Methanol	7.5	30.0
2.	Ethyl acetate	2.4	9.60

and their molecular formula are tabulated (Table 3). The major compounds present in the leaves were methylsulfonylmethane (10.83), nonadecanoic acid (11.18), phenol,2-methoxy(2-propenyl)-(8.68), Undecyl trichloroacetate (14.03), and Cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy-, pivalate (16.25). In addition, compounds such as hexadecanoic acid, stigmasterol, and propanaoic acid.

Screening of biofilm forming isolates

A total of 10 strains were isolated and identified as *S. pyogenes*. Among 10 strains, 5 strains exhibited biofilm forming potential as mentioned in Table 4. Interestingly, SP-1 and SP-2 isolates formed moderate to strong biofilm, whereas SP-5, SP-9, and SP-10 formed weak biofilms, which were evident from the surface topology of the sessile cells.

Minimum inhibitory concentration assay

MIC was determined for the planktonic cells of the biofilm forming *S. pyogenes* isolates SP-1, SP-2, SP-5, SP-9, and SP-10 treated with different concentrations of PAM and PAEA extracts using the MTP method. Results revealed that the MIC of PAM was 1 mg/ml, whereas the PAEA extract was 2 mg/ml which was evident from the absence of bacterial growth at the designated concentrations (Fig. 2).

Table 2: Preliminary phytochemical analysis of *P. amboinicus* using different solvents

S. No	Bioactive compounds	Methanol	Ethyl acetate
1.	Alkaloids	+	+
2.	Carbohydrates	+	+
3.	Flavonoids	+	+
4.	Saponin	+	-
5.	Phenols	+	+
6.	Glycosides	+	+
7.	Steroids	-	+
8.	Terpenoids	+	+

+: Present, - : Absent

Growth curve analysis

The results of the growth curve analysis are presented in Fig. 3. Results elucidated that at the tested sub-MIC level (0.5 mg/ml) PAM and PAEA extracts did not show significant inhibition of the bacterial cells as compared to the untreated control which is evident from the growth curve pattern of the test pathogens compared to control.

Anti-bacterial activity assay

Antibacterial activity of PAM and PAEA extracts at their sub-MIC (0.5 mg/ml) against *S. pyogenes* isolates SP-1 and SP-2 were evaluated and compared by a zone of inhibition using agar well diffusion method. The methanol and ethyl acetate extract did not show antibacterial activity at the tested concentration which was observed from the minimal or no zone formation around the well in the MHA plates (Table 5).

Quantification of biomass inhibition

PAM and PAEA extracts at sub-MIC (0.0625-0.5 mg/ml) disrupted the biofilm formation in a dose-dependent manner. The biofilm inhibitory concentration (BIC) (≥50%) of PAM was observed at 0.25 mg/ml against the test pathogens, whereas the BIC of the PAEA was observed at 0.5 mg/ml against the test pathogen elucidating that methanol extract was significant in reducing the biofilm formation as compared to ethyl acetate extract (Fig. 4). The highest biofilm inhibition by methanol extract was observed in MTCC-1924 (81.3%) followed by SP-1 (79.15%) and SP-2 (77.45%), wherein the ethyl acetate extract showed the highest biofilm inhibition against SP-1 (73.52%) followed

Table 3: Phytochemicals identified in PAM extract using GC-MS analysis

S. No	Rt	Area (%)	Compound name	Molecular formula
1.	8.137	10.83	Methylsulfonylmethane	$C_2H_6O_2S$
2.	13.183	1.29	Carvone	$C_{10}H_{14}O$
3.	9.965	1.33	n-hexadecanoic acid	$C_{16}H_{32}O_2$
4.	11.047	1.10	Phytol	$C_{20}H_{40}O$
5.	12.333	2.74	9-hexadecenal	$C_{16}H_{30}O$
6.	13.187	11.18	Nonadecanoic acid	$C_{19}H_{38}O_2$
7.	14.095	1.32	Neophytadiene	$C_{20}H_{38}$
8.	15.337	0.76	Eliminoxy	$C_{13}H_{16}O_2$
9.	16.860	−0.15	Tocopherols	$C_{29}H_{50}O_2$
10.	17.313	0.08	Campesterol	$C_{28}H_{48}O$
11.	18.903	0.16	Stigmasterol	$C_{29}H_{48}O$
12.	19.894	0.47	Squalene	$C_{30}H_{50}$
13.	23.031	0.12	Campesterol	$C_{28}H_{48}O$
14.	26.068	0.47	Phytol	$C_{20}H_{40}O$
15.	27.006	0.33	Perilla acetate	$C_{12}H_{18}O_2$
16.	28.855	1.02	Undecane	$C_{11}H_{24}$
17.	29.149	4.09	1,3-diethoxy-1,1,3,3-tetramethyldisiloxane	$C_8H_{22}O_3SI_2$
18.	31.287	8.68	Phenol,2-methoxy(2-propenyl)-	$C_{10}H_{12}O_2$
19.	31.640	4.71	Caryophyllene	$C_{15}H_{24}$
20.	31.949	1.72	2,6-di-tert-butylphenol	$C_{14}H_{22}O$
21.	32.594	1.72	4-ethyl-1,2-dimethoxybenzene	$C_{10}H_{14}O_2$
22.	33.511	0.20	1-nonadecene	$C_{19}H_{38}$
23.	34.023	2.22	Neophytadiene	$C_{20}H_{38}$
24.	34.117	2.81	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	$C_{18}H_{28}O_3$
25.	36.410	0.12	Methyl commate c	$C_{31}H_{50}O_4$
26.	36.810	0.59	Benzene, (ethenyloxy)-	C_8H_8O
27.	38.220	3.67	Methane, chlorodifluoro	$CHCLF_2$
28.	39.112	14.03	Undecyl trichloroacetate	$C_{13}H_{23}CL_3O_2$
29.	39.960	2.96	Methyl 2-oxononanoate	$C_{10}H_{18}O$
30.	40.821	16.25	Cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy-, pivalate	$C_{33}H_{54}O_3$
31.	41.773	0.30	Octadecane-1,2-diol, bis (trimethylsilyl) ether	$C_{24}H_{54}O_2SI_2$
32.	43.271	2.89	1,2-benzenedicarboxylic acid	$C_{24}H_{38}O_4$

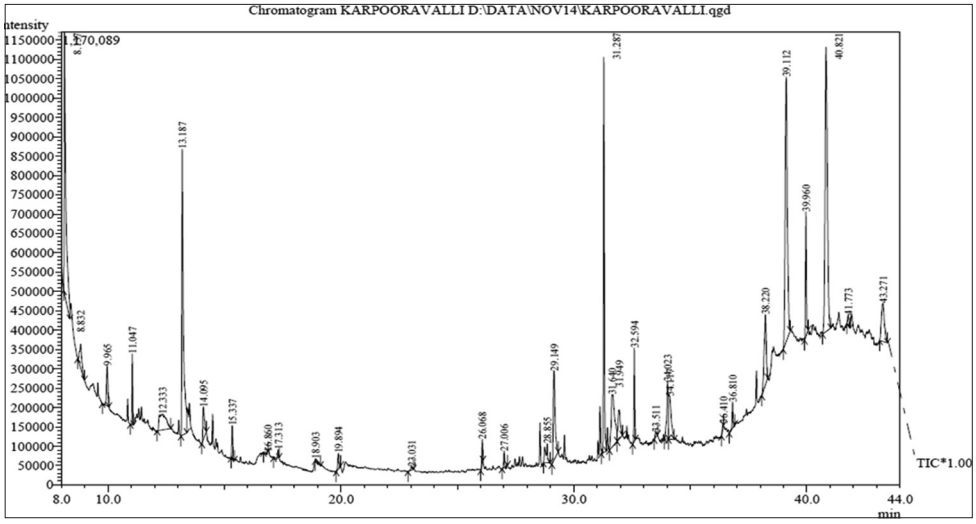

Fig. 1: Gas chromatography-mass spectrometry spectrogram of *Plectranthus amboinicus* methanol extract

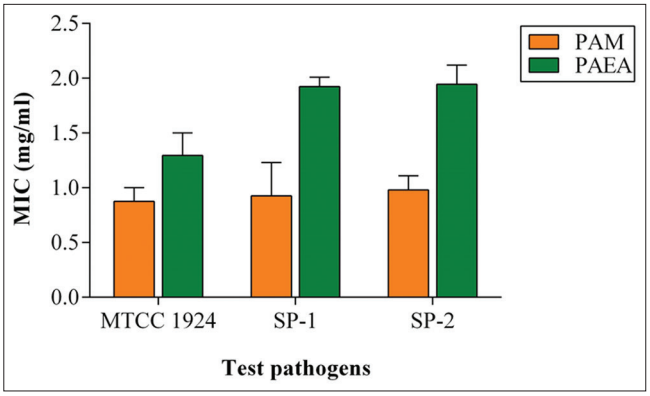

Fig. 2: Minimal inhibitory concentration of *P. amboinicus* solvent extracts against biofilm forming *S. pyogenes* isolates. Values are represented as mean±standard error of the triplicate independent experiment

Table 4: Screening of biofilm forming *S. pyogenes*

S. No	Isolate no	Biofilm formation
1.	SP-1	+++
2.	SP-2	++
3.	SP-3	-
4.	SP-4	-
5.	SP-5	+
6.	SP-6	-
7.	SP-7	-
8.	SP-8	-
9.	SP-9	+
10.	SP-10	+

Strong (+++); Moderate (++); weak (+) and negative (-) biofilm formers.

Table 5: Anti-bacterial activity of *P. amboinicus* against *S. pyogenes* isolates

Extract	Concentration	Zone of inhibition (mm)		
		SP-1	SP-2	MTCC-1924
Methanol	0.5 mg/ml	6	8	4.5
Ethyl acetate		4	3.5	2
Streptomycin	0.1 mg/ml	24.5	21	28
DMSO	80 %	-	-	-

by SP-2 (71.52%) and MTCC 1924 (70.2%) at 1 mg/ml concentration. The results also elucidated that the biofilm reduction decreased with the decrease in the concentration of the plant extract.

Light microscopic observation of biofilm

Light microscopy analysis was performed to determine the anti-biofilm property of PAM and PAEA under *in situ* conditions at BIC levels. A thick coating of biofilms was observed in controls, whereas a visible reduction in biofilm densities was observed in the *S. pyogenes* biofilms treated with plant extracts. Methanol extract treated samples showed deteriorated biofilm structure at BIC (0.25 mg/ml). On the other hand, ethyl acetate extract (0.5 mg/ml) treated samples showed a partial reduction in the biofilm architecture thereby elucidating that methanol extract was significant in controlling the biofilm formation (Fig. 5).

DISCUSSION

In the present study, the methanol and ethyl acetate extracts of *P. amboinicus* was evaluated for its anti-biofilm activity against biofilm forming *S. pyogenes* SP-1 and SP-2 isolated from pharyngitis patients. Nowadays, screening of biologically active phyto compounds from plants plays a significant role in the herbal medicine research, and reports have elucidated that only about 10 % of the medicinal plants have been studied in detail for their medicinal properties [22]. Moreover, more than half of the pharmaceutical products in the pharmaceutical industries are synthesized from the medicinal plants owing to their rich and diverse phyto compounds [23]. In the present study, the preliminary qualitative phytochemical screening of methanol (PAM) ethyl acetate (PAEA) extracts of *P. amboinicus* revealed the presence of biologically active compounds such as phenols, flavonoids, steroids, terpenoids, carbohydrates, and saponins. These compounds are well established for their medicinal values. Furthermore, the medicinal properties of the genus *Plectranthus* could be elucidated from the presence of such vital phyto compounds [24,25]. The results of the present study revealed the presence steroids which plays a key role in the regulation of sex hormones [26]. The presence of alkaloids and saponins in the methanol and ethyl acetate extracts were observed which are significant in the treatment of venereal diseases [17]. Flavonoids are well known for their rich antioxidant properties, whereas terpenoids exhibit significant antimicrobial activity [27], and both of these compounds were observed in the plant extracts used in this study. The presence of terpenoids could be attributed to the medicinally property of this plant in the treatment of skin diseases as terpenoids are known to enhance the wound healing process and preventing microbial infections of the skin. In addition, terpenoids have been reported to exhibit antioxidant [28], antifungal [29], antiurolithiasis [30,31], antiepileptic [32], antitumor and antimutagenic [33], antineurodegenerative [34], radioprotective

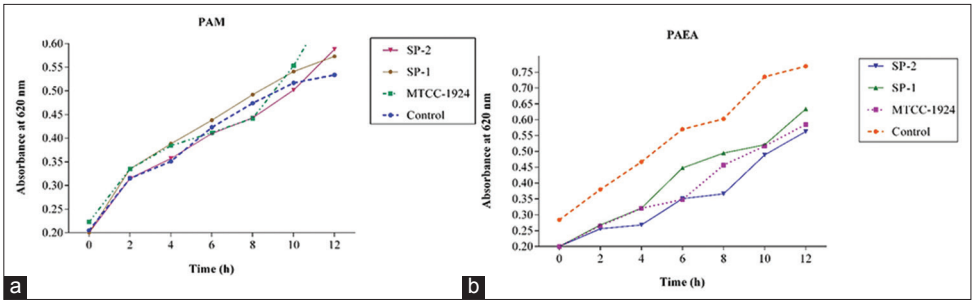

Fig. 3: (a and b) Effect of the *P. amboinicus* methanol (PAM) and ethyl acetate (PAEA) extracts on the growth of *S. pyogenes*

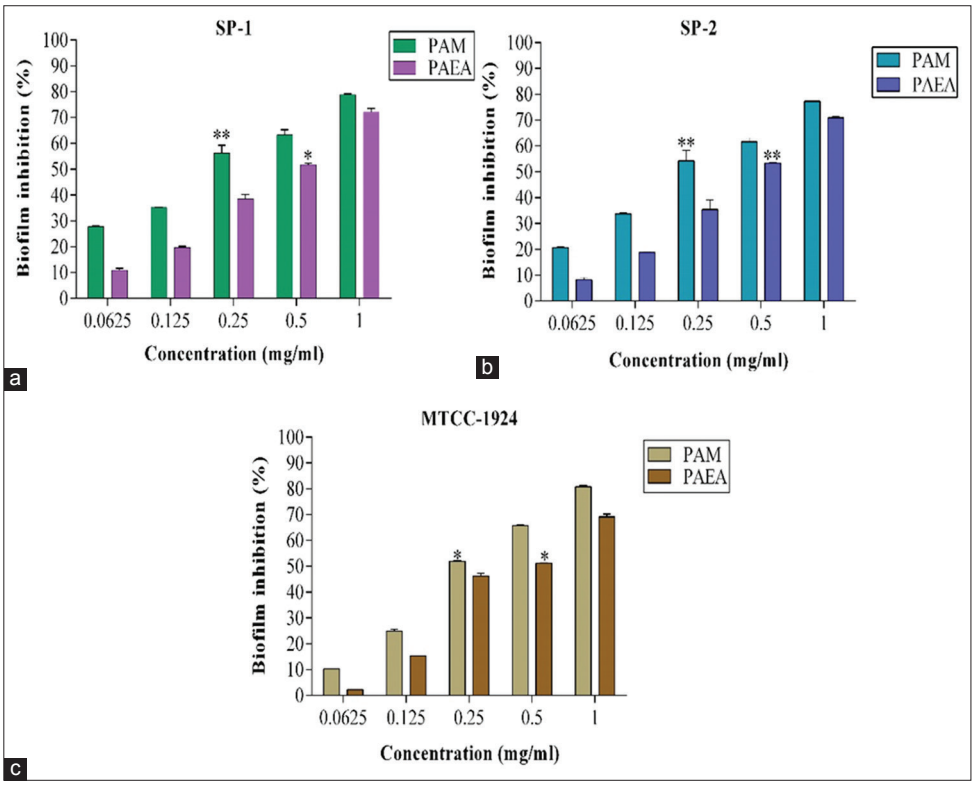

Fig. 4: (a,b,c) Effect of *P. amboinicus* solvent extracts on the biofilm biomass of *S. pyogenes* at sub-MIC levels. Values are expressed as mean percentage biofilm inhibition±standard error. Values with *p<0.05 and **p<0.01 are significantly different from control

effect [35], and antimicrobial [36,37]. Although earlier reports have substantiated the biologically active compounds in *P. amboinicus* using quantitative analysis, we have performed GC-MS analysis of the methanol extract in the present study to further validate the medical importance of this plant. The GC-MS analysis of PAM in the present study revealed the presence of many biologically active compounds such as hexadecanoic acid, nonadecanoic acid, phyto, carvol, stigmasterol, tocopherol, and undecanoic acid. According to Das *et al.,* [38] fatty acids synthesized by plants influence the metabolic and immunological functions of the host. Moreover, plant fatty acids are reported to exhibit anticancer and immune gene expression in the upper respiratory tract of the host during infections [39,40]. Stigmasterol is well known for its antimicrobial anti-biofilm properties [41]. In addition, Manickam *et al*. [42] have elucidated that the crude fatty acids have significant anti-biofilm activity against *S. pyogenes*.

The results of the MIC assay elucidated that the MIC for PAM (1 mg/ml) was lesser as compared to PAEA (2 mg/ml) which could be attributed to the rich biological activity of the methanol extract which enables an effective inhibition of the planktonic cells at much lower concentration. Our results were in agreement with Thaniarasu *et al*., [43], who elucidated the MIC of *Plectranthus bourneae* solvent extracts against different pathogens. To rule out the hypothesis that PAM and PAEA were bactericidal at a concentration below the MIC, we performed the growth curve analysis. Our results elucidated no significant difference between the absorbance of the control and the solvent extract treated samples at sub-MICs validating that the extracts were not antibacterial below MIC level. The results of the growth curve analysis were further validated using the agar well diffusion method, in which the results evidently showed no zone formation in the agar plates against the test pathogens elucidating that at the minimal concentration the PAM and PAEA extracts were not bactericidal but bacteriostatic. From the result obtained, it could also be elucidated that Gram-positive bacteria such as *S. pyogenes* in spite of being more susceptible to inhibition by plant extracts when compared to their Gram-negative counterparts [44] were not inhibited by PAM and PAEA which could be attributed to the bacteriostatic activity of *P. amboinicus* solvent extracts below sub-MIC levels. Our results corroborate with the results of other medicinal plants belonging to the same family of *P. amboinicus,* namely, *Vitex negundo* and *Leucas aspera* solvent extracts against the biofilm forming *S. pyogenes* [20].

Biofilm formation in *S. pyogenes* is regulated by multifactorial cell functions, which includes cell signaling systems enabling the bacteria to attach to the host substratum and establish infection [21].

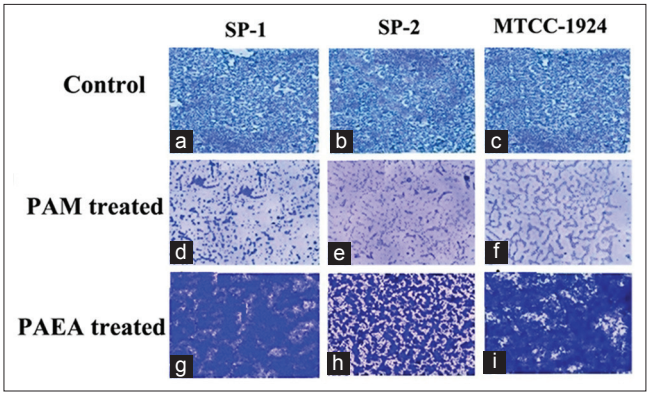

Fig. 5: (a-i) Light microscopy images (×400) of *S. pyogenes* biofilms SP-1, SP-2, and MTCC-1924 treated with *P. amboinicus* methanol (PAM) and and ethyl acetate (PAEA) extracts at BIC (PAM-0.25 mg/ml and PAEA-0.5 mg/ml). Untreated samples served as control

Furthermore, many biofilm-forming pathogens are capable of eliciting chronic infections by creating protective barrier against antibiotics and host immune cells [45]. Since PAM and PAEA were observed to contain antimicrobial compounds, we further hypothesized that these extracts may also affect the biofilm formation of *S. pyogenes*. Our results supported the hypothesis in which the extracts inhibited the biofilm biomass significantly ($p<0.05$) in a dose-dependent manner at sub-MIC levels without affecting the growth of the bacteria thereby reducing the chance of developing resistance. Also the results of the biofilm biomass inhibition assay in the present study elucidated a dose dependent significant inhibition of *S. pyogenes* isolates by PAM and PAEA as compared to the untreated control. Further, the biofilm inhibitory percentage was higher in PAM-treated samples than PAEA-treated test pathogens which may be attributed to the rich bioactive molecules present in the methanol extract of *P. amboinicus*. Our results were in agreement with previous studies on bacterial biofilm inhibition by plant extracts [20,46,47].

Mature biofilms represent a source of chronic infection that is highly resistant to antibiotics, whereas planktonic cells are susceptible to a wide range of antibiotics [42]. The concentration-dependent inhibition of mature biofilms was visually analyzed by employing the light microscopic analysis of *S. pyogenes* biofilms in the presence and absence of the plant extract. Our study revealed well defined and tightly packed biofilm formation in the untreated control samples after 48 h, whereas treated cells exhibited disorganization of biofilm stages with increasing concentration of PAM and PAEA extracts. Therefore, it is expected that treatment of SP-1, SP-2, and MTCC 1924 with sub-MICs of PAM and PAEA resulted in the reduction in the surface charge intensity leading to reduction in the microcolony formation. However, complex processes related to genetic and ecological parameters are involved in the biofilm development and establishment mechanisms [48].

CONCLUSION

In conclusion, the methanol and ethyl acetate extract of *P. amboinicus* showed a dose-dependent inhibition against the biofilm forming *S. pyogenes* isolated from the pharyngitis patients. Among the solvent extracts, the methanol extract showed significant activity against the test pathogens at minimal concentration. The crude extracts of the PAM extracts are rich in biologically active phytochemicals which might have interacted directly or indirectly in the process of biofilm formation by *S. pyogenes*. In addition, these results are the first of its kind in elucidating the role of a well-known medicinal plant *P. amboinicus* in controlling the biofilm formation of the upper respiratory tract pathogen *S. pyogenes*. However, further studies are necessary to evaluate the pharmacological properties of *P. amboinicus* solvent extracts at the molecular level in reducing the biofilms formation of *S. pyogenes*.

ACKNOWLEDGMENT

The authors thank Karpagam University, Coimbatore, for providing infrastructure facility to perform the experiments.

REFERENCES

1. Al Masoudi SB, Aly MM, Al Humidi NQ, Halwani M. Incidence and prevalence of *Acinetobacter baumannii* in King Fahd General Hospital, Saudi Arabia. Life Sci J 2013;10(4):1702-10.
2. Rangan C, Barceloux DG. Food additives and sensitivities. Dis Mon 2009;55(5):292-311.
3. Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, *et al.* Bacterial biofilms in nature and disease. Annu Rev Microbiol 1987;41:435-64.
4. Cunningham MW. Pathogenesis of Group A streptococcal infections. Clin Microbiol Rev 2000;13(3):470-511.
5. Bisno AL, Gerber MA, Gwaltney JM, Kaplan EL, Schwartz RH. Practice guidelines for the diagnosis and management of Group A streptococcal pharyngitis. Clin Infect Dis 2002;35(2):113-25.
6. Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, *et al.* Gene expression in *Pseudomonas aeruginosa* biofilms. Nature 2001;413(6858):860-4.
7. Samy RP, Ignacimuthu S. Antibacterial activity of some medicinal plants from Eastern ghats, South India, Solai Bull. Ethanopharmacology 2000;72(1):39-41.
8. Ahmad I, Mehmood Z, Mohammad F. Screening of some Indian medicinal plants for their antimicrobial properties. J Ethnopharmacol 1998;62(2):183-93.
9. Fu Y, Zu Y, Chen L, Shi X, Wang Z, Sun S, *et al.* Antimicrobial activity of clove and rosemary essential oils alone and in combination. Phytother Res 2007;21(10):989-94.
10. Kirtikar KR, Basu BD. Indian Medicinal Plants. Vol. 3. Dehradun: International Book Distributors; 1999. p. 1970-1.
11. Warrier PK, Nambiar VP, Ramankutty C. Indian Medicinal Plants: A Compendium of 500 Species. Vol. 4. Chennai: Orient Longman; 1996. p. 315.
12. Nadkarni KM. Indian Materia Medica. 3rd ed. Mumbai: Popular Prakashan; 2002. p. 371-2.
13. Patel R, Mahobia NK, Gendle R, Kaushik B, Singh SK. Diuretic activity of leaves of *Plectranthus amboinicus* (Lour) Spreng in male albino rats. Pharmacognosy Res 2010;2(2):86-8.
14. Khory RN, Katrak NN. Materia Medica of India and Their Therapeutics. New Delhi: Komal Prakashan; 1999. p. 380.
15. Lukhoba CW, Simmonds MS, Paton AJ. Plectranthus: A review of ethnobotanical uses. J Ethnopharmacol 2006;103(1):1-24.
16. Harborne JB. Methods of plant analysis. In: Phytochemical Methods. Netherlands: Springer; 1973. p. 1-32.
17. Rijo P, Rodríguez B, Duarte A, Fatima Simoes M. Antimicrobial properties of *Plectranthus ornatus* extracts, 11-acetoxyhalima-5, 13-dien-15-oic acid metabolite and its derivatives. Nat Prod J 2011;1(1):57-4.
18. Srinivasan P, Dineshbabu J, Manimekalai K, Castro J, Priya Darsini DT. Influence of *Limonia acidissima* L. against the biofilm forming *Aeromonas hydrophila* isolated from fresh water fishes. J Biochem Technol 2015;6(1):910-21.
19. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. Wayne, PA: Clinical and Laboratory Standards Institute; 2007. p. 17.
20. Dineshbabu J, Darsini DT, Srinivasan P, Everlyne IM, Manimekalai K. Synergistic anti-biofilm activity of medicinal plants against biofilm forming *Streptococcus pyogenes* from pharyngitis patients. Indo Am J Pharm Res 2015;5(8):2598-606.
21. Darsini DT, Srinivasan P, Guna G, Manimekalai K, Dineshbabu J. *In vitro* anti biofilm activity of *Piper longum* and *Piper nigrum* against clinical isolates of *Streptococcus pyogenes* isolated from pharyngitis patients. Int Res J Pharm 2015;6(2):122-32.
22. Sandberg F, Bruhn JG. Screening of plants for biologically active substances. In: Soforowa EA, editor. African Medicinal Plants. Lagos: University of Ife Press; 1979. p. 119.
23. Raskin I, Ribnicky DM, Komarnytsky S, Ilic N, Poulev A, Borisjuk N, *et al*. Plants and human health in the twenty-first century. Trends Biotechnol 2002;20:522-31.
24. Okwu DE. Evaluation of chemical composition of indigenous species and flavoring agents. Glob J Pure Appl Sci 2001;7(3):455-60.
25. Egwaikhide PA, Gimba CE. Analysis of the phytochemical content and anti-microbial activity of *Plectranthus glandulosis* whole plant. Middle

East J Sci Res 2007;2(3-4):135-8.
26. Sofowora EA. Medicinal Plants and Traditional Medicine in Africa. New York: John Wiley and Sons Ltd.; 1982. p. 150-3.
27. Oyedemi SO, Afolayan AJ. Antibacterial and antioxidant activities of hydroalcoholic stem bark extract of *Schotia latifolia* Jacq. Asian Pac J Trop Med 2011;4:952-8.
28. Sharma. Dravyaguna Vijnana. Vol. II. Varanasi, India: Chaukhamba Bharati Academy; p. 461-3. Available from: http//www.wikipedia.Org/wiki/*Plectranthus amboinicus*. 1995. [Last accessed on 2012 Jun 10].
29. Perumal G, Subramanyam C, Natrajan D, Srinivasan K, Mohanasundari C, Prabakar K. Antifungal activities of traditional medicinal plant extracts: A preliminary survey. J Phytol Res 2004;17:81-3.
30. Baskar R, Varalaksmi P. Amsaveni R. Changes in tissue enzymes produced by *Coleus aromaticus* experimental urolithiasis. Indian Drugs 1992;29(6):254-8.
31. Jose MA, Janardhanan S. Modulatory effect of *Plectranthus amboinicus* Lour. on ethylene glycol-induced nephrolithiasis in rats. Indian J Pharmacol 2005;37(1):43.
32. Buznego MT, Pérez-Saad H. Antiepileptic effect of *Plectranthus amboinicus* (lour.) Spreng. (French marjoram). Rev Neurol 1999;29:388-9.
33. Annapurani S, Priya R. Antimutagenic, antitumourogenic and antigenotoxic effects of polyphenol extracts of selected medicinal plants. Indian J Nutr Diet 1999;36:431-5.
34. Pérez Saad H, Buznego MT, Llanio Villate M, Fernández Perez M, Menéndez R. Neuropharmacological profile of *Plectranthus amboinicus* (Lour.) Spreng. (Indian borage). Rev Neurol 2003;36:98-9.
35. Rao BS, Shanbhoge R, Upadhya D, Jagetia GC, Adiga SK, Kumar P, *et al.* Antioxidant, anticlastogenic and radioprotective effect of *Coleus aromaticus* on Chinese hamster fibroblast cells (V79) exposed to gamma radiation. Mutagenesis 2006;21(4):237-42.
36. Alankararao GS, Baby P, Prasad YR. Leaf oil of *Coleus amboinicus* Lour: The *in vitro* antimicrobial studies. Parfümerie und Kosmetik 1991;72(11):744-9.
37. Deena MJ, Sreeranjini K, Thoppil JE. Antimicrobial screening of essential oils of *Coleus aromaticus* and *Coleus zeylanicus*. Int J Aromather 2002;12(2):105-7.
38. Das UN, Ramos EJ, Meguid MM. Metabolic alterations during inflammation and its modulation by central actions of omega-3 fatty acids. Curr Opin Clin Nutr Metab Care 2003;6:413-9.
39. Berquin IM, Min Y, Wu R, Wu J, Perry D, Cline JM, *et al*. Modulation of prostate cancer genetic risk by omega-3 and omega-6 fatty acids. J Clin Invest 2007;117:1866-75.
40. Zhao Y, Usatyuk PV, Gorshkova IA, He D, Wang T, Moreno-Vinasco L, *et al*. Regulation of COX-2 expression and IL-6 release by particulate matter in airway epithelial cells. Am J Respir Cell Mol Biol 2009;40(1):19-30.
41. Awolola GV, Koorbanally NA, Chenia H, Shode FO, Baijnath H. Antibacterial and anti-biofilm activity of flavonoids and triterpenes isolated from the extracts of *Ficus sansibarica* Warb. subsp. sansibarica (Moraceae) extracts. Afr J Tradit Complement Altern Med 2014;11:124-31. eCollection 2014.
42. Manickam R, Ponnuraj S, Francis PM, Rajendran S, Venkatesan BP. Crude fatty acid extracts of *Streptomyces* sps inhibits the biofilm forming *Streptococcus pyogenes* ATCC 19615. J Biochem Technol 2014;5(2):679-84.
43. Thaniarasu R, Kumar TS, Abubacker MN, Rao MV. Preliminary phytochemical screening and evaluation of antibacterial activity of different solvent extracts of *Plectranthus bourneae* Gamble (Lamiaceae). Asian J Pharm Clin Res 2015;8(1):79-82.
44. Ullah MO, Haque M, Urmi KF, Zulfiker AH, Anita ES, Begum M, *et al.* Anti-bacterial activity and brine shrimp lethality bioassay of methanolic extracts of fourteen different edible vegetables from Bangladesh. Asian Pac J Trop Biomed 2013;3(1):1-7.
45. Thurlow LR, Hanke ML, Fritz T, Angle A, Aldrich A, Williams SH, *et al. Staphylococcus aureus* biofilms prevent macrophage phagocytosis and attenuate inflammation *in vivo*. J Immunol 2011;186(11):6585-96.
46. Harjai K, Kumar R, Singh S. Garlic blocks quorum sensing and attenuates the virulence of *Pseudomonas aeruginosa.* FEMS Immunol Med Microbiol 2010;58(2):161-8.
47. Kavanaugh NL, Ribbeck K. Selected antimicrobial essential oils eradicate *Pseudomonas spp*. and *Staphylococcus aureus* biofilms. Appl Environ Microbiol 2012;78(11):4057-61.
48. Kjelleberg S, Molin S. Is there a role for quorum sensing signals in bacterial biofilms? Curr Opin Microbiol 2002;5(3):254-8.