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Synergistic effect of ethno medicinal plants against biofilm forming *Streptococcus pyogenes* isolated from upper respiratory tract infection

Manimekalai Kanagarajan[1] , Teepica Priya Darsini Deivamarudachalam[1*], Srinivasan Ponnuraj[1] , Dineshbabu Jaganathan[1]

***Corresponding author:**

Teepica Priya Darsini Deivamarudachalam
[1]Laboratory of Clinical Biotechnology and Herbal Medicine, Department of Biotechnology, Karpagam Academy of Higher Education, Coimbatore – 641 021

Abstract

The present study is an attempt to determine the synergistic anti-biofilm efficacy of three Indian medicinal plants namely *Ocimum tenuiflorum*, *Plectranthusamboinicus*, and *Tylophora indica* (OPT) against the biofilm forming *Streptococcus pyogenes*of upper respiratory tract infection. The leaves were collected and subjected to methanol extraction. The combined extract (OPT) was subjected to preliminary phytochemical and GC-MS analysis. About,ten isolates of *S. pyogenes* were obtained from the throat swabs samples and studied for biofilm forming potential. The strong biofilm forming isolates were subjected to Minimal Inhibitory Concentration (MIC), Biofilm Inhibitory Concentration(BIC)assays and light microscopic analysis the biofilm inhibition. The results of GC-MS revealed the presence of 39 biologically active phyto-compounds attributed the medicinal properties. The MIC and BIC assay results OPT extracts exhibited anti-biofilm activity at minimal concentration ranging from0.0156 mg/ml to1.0 mg/ml. Further,themicroscopic observationsconfirmed the altered biofilm architecture of S. *pyogenes*treated with OPT extract. The findings expound the sturdy synergistic anti-biofilm efficacy of the OPT against biofilm forming *S. pyogenes*.

Keywords: *S. pyogenes,* synergism, anti-biofilm activity, medicinal plants

Introduction

Plants used for traditional medicine contain wide range of substances that are used to treat chronic and communicable diseases [1]. Phytomedicine remains as an important source in developing countries, to combat serious diseases [2]. The bioactive phytochemical substances such as alkaloids, flavonoids, tannins and phenols serve as an important component against microbial infections[3]. The phytochemicals have been reported to possess anti-bacterial, anti-fungal and anti-inflammatory activities which play an essential role in developing of newer drugs because of their effectiveness, less side effects and relatively low cost as compared toantibiotics [4].

Streptococcus pyogenes is a major upper respiratory tract pathogen causing bacterial pharyngitis that leads to serious complications [5]. *S. pyogenes* is one of the most important human pathogens associated with extensive human morbidity worldwide [6]. It is classified as Group-A Streptococcus (GAS), Gram-positive, facultative anaerobic bacterium and is associated with primary infections of skin, throat and mucosal surfaces. Streptococcal biofilms are aggregations of cells surrounded by the matrix of extracellular polymeric substance (EPS) produced by the cells within the biofilm, which show high antibiotic resistance [7, 8].

Although, antibiotics exhibit their inhibitory activity against *S. pyogenes*, the concentration required to inhibit the biofilms is very high. The increasing phenomenon of antibiotic resistance among microorganisms is attributed its indiscriminate use [9]. *S.pyogenes* are characterized by multiple drug resistance leading to increasing global health threat [10]. Hence, it has become essential to find an alternative medicine which effectively inhibits pathogens without developing resistance [11]. Unlike antibiotics, phytochemicals are not associated with side effects and have vast therapeutic potential against infectious diseases [12].

The genus *Ocimum*, has long been acclaimed for its diversity. The major phenolic compounds founds in plants are secondary metabolites, possessing high antioxidant activity and is wide spread in the species belonging to Lamiaceae. Leaves from *Ocimum tenuiflorum* (Thulasi) species are used as a culinary condiment [13] and for insect control [14]. Various effects of *Ocimum* spp. including anti-inflammatory, anti-oxidative, anti-ulcer, anti-diarrheal, chemo preventive, anti-diabetic, and radioactive protection have also been reported [15]. Tulasi is used in siddha medicine for the treatment of myriad of illness like common cold, cough, headache, flu, sore throat, bronchitis, asthma, and malaria fever.[16]

Plectranthus is a large genus, with more than 300 species from the family Lamiaceae. It is a large succulent aromatic perennial herb found

throughout India and Sri Lanka [17].Many pharmacological properties have been reported in *P. amboinicus* commonly known as Karpooravalli in Tamil. The leaves of this plant is used in cases of chronic cough, asthma, headache, fever, epilepsy and dyspepsia including anti-urolithiasis, antiepileptic, anti-tumorogenic, anti-mutagenic, radio-protective effect, anti-viral, antifungal and neuro-pharmocological properties [18, 19 and 20]. *Tylophora indica* (Nencaruppan) belongs to Ascelpiadaceae family is a slender twiner shrub and a medicinal plant commonly known as Antamul or Indian ipecac. The leaves extracts were internally used as effective antidote for poisonous case and poisonous bite and treatment to bronchial asthma, bronchitis, rheumatism, allergies, inflammation and whooping cough. The roots and leaves extract of the plant is used in treatment of asthma, dermatitis and rheumatism. The plant has been described as bronchodilator, emetic, expectorant and diaphoretic. Additionally, used to treat dysentery and joint pain. [21-24].

Although, the phytochemicals are extensively studied for their therapeutic potential against upper respiratory pathogens with significant anti-infective results [25],medicinal plants with unique phytochemical substances has been continuously researched for an alternative source of novel compounds for the treatment of *S. pyogenes* upper respiratory tract infections [26].A variety of plants such as *Alliumsativum* [27], *Zingier officinale* Roscoe [28], *Leucasaspera* L. and *Vitex negundo* [29], *Limonia acidissima L.* [30] have been reported to exhibit significant inhibition against different microbial communities.However, it is also Therefore, the synergistic activity may be due to interaction of bioactive phyto components which is more effective in the inhibition of microorganisms either by inhibiting the cell wall synthesis.The present study evaluates the *in vitro* synergism of three plants *Ocimum tenuiflorum*(L), *Plectranthusamboinicus*(Lour) Spreg. and *Tylophora indica* (Burm. f.) Merr., methanol extract for its anti-biofilm activity against the biofilm forming *Streptococcus pyogenes*.

Materials and Methods

Plant collection and solvent extraction

The leaves of *Ocimum tenuiflorum*, *Plectranthusamboinicus* and *Tylophora indica* (OPT) were collected from Vellingiri hillsand Kolli Hills, Tamil Nadu. The samples were taxonomically identified and authenticated at Botanical Survey of India (BSI), Southern Circle, Tamil Nadu Agricultural University (TNAU), Coimbatore and voucher specimen was deposited.

The collected plants were washed, air dried and powdered. About 25 g of the each dried plant powder was soaked in 100 ml of methanol (1:4) for seven days with periodic soaking methanol extracts were collected and dried at55 C for 1 h using rotary vacuum evaporator (Buchi Type, India). After vacuum evaporation the plant extracts were mixed in equal ratio (1:1:1 w/w) and re-suspended in 80% Di-Methyl Sulphoxide (DMSO) (Himedia, India).

Phytochemical analysis

The OPT extract was subjected to phytochemical screening for the presence of alkaloid, carbohydrates, tannins, saponin, flavonoid, steroids, terpenoid, glycosides and phenol. Phytochemical analyses of the plant extracts were carried out and the bioactive compounds were determined by the standard methods [31].

GC–MS analysis

Bioactive compound analysis of OPT extract was determined by Shimadzu Gas chromatography (QP2010 Plus, Japan), with a 30 m 0.25 mm RTX-5MS low bleed column with a thickness of 0.25 m [32]. The spectrum of the unknown phytocomponents were compared with the spectrum of known components available in Wiley Online Library (Wiley08), NIST08 library. The name of phytocompounds, molecular weight, and structure of the sample were determined.

Bacterial strain and culture conditions

About 10 throat swab samples were collected from pharyngitis patients, attending Karpagam Faculty of Medical Sciences and Research, Coimbatore, Tamil Nadu, India. S. pyogenes MTCC 1924 (IMTECH, Chandigarh, India) was used as reference strain. S. Pyogenes were isolated from the throat swab samples using streptococcus selection agar and blood agar medium (Himedia, India). Isolates were tested for their biofilm forming characteristics by observing the slime formation in routine media. For routine propagation, all isolates were cultured and maintained in Todd Hewitt's broth at 37 C. The biofilm forming isolates alone were used for further studies. Glycerol stock was maintained at -20 C for further use.

Minimal Inhibitory Concentration (MIC) assay

Micro plate assay was used to determine the lowest concentration of an extract that inhibited growth of the test pathogen[33]. Briefly, 100 µl of sterile nutrient broth was aliquoted into each wells of the 96 well micro titre plate (Tarsons, India). Then, 50 µl of the OPT methanol extract at different concentration(0.0625 mg/ml- 8 mg/ml) was incorporated into the appropriate wells. Then, 10 µl of S. pyogenes MTCC 1924 and isolates (1.0 x 105 cfu/ml) were inoculated into the wells. The plates were sealed with aluminum foil and incubated at 37 oC for 24 h. After, incubation, the MIC of the OPT extract was determined. To visualize the bacterial growth, 40 µl of The p-iodonitrotetrazolium violet (INT) (0.04 mg/ml) was added to the wells and microtitre plates were incubated at room temperature for 3 h. MIC was determined as the least concentration of the OPT extract that showed complete reduction in the colour indicating the inhibition of microbial growth.

Agar well diffusion assay

The anti-bacterial activity of OPT extract was performed through agar well diffusion method using Muller-Hinton agar (MHA) (Himedia, India) following the method specified by Clinical and Laboratory Standards Institute [22]. Briefly, 100 µl of test bacterial suspensions with the cell density equivalent to 0.5 McFarland standard units (1 x 105cfu/ml) were uniformly spread over the surface of MHA plates. The plates were kept undisturbed for 10 min for the absorption of excess moisture. 1mg/mlof OPT extract was added to wells and the plates were incubated at 37 C. Solvent without plant extract was used as negative control and standard antibiotic streptomycin (0.03 mg/ml) was used as positive control. The zone of inhibition was measured after 24 h.

Biofilm biomass quantification assay

Biofilm Inhibitory Percentage = [(A_{620} of Untreated Control - A_{620} OPT extract treated sample) (A_{620} of Untreated Control -A_{620} of Positive control)] 100

The effect of plant extracts on the biofilm formation of bacterial pathogens was determined by quantifying the biofilm biomass through microtitre plate (MTP)assay [34]. Overnight culture of *S. pyogenes* was incubated on 24 well microtitre plate containing 1 ml of Todd-Hewitt Broth (THB) with OPTextract at different concentration (0.0156 mg/ml to 1 mg/ml). Aliquots without the plant extracts were used as control, plates were incubated without agitation at 37 C for 18 h. After incubation, planktonic cells and spent media were discarded, the adherent cells on the slide were gently rinsed twice with deionized water and air dried. The biofilm was stained with 0.4% crystal violet solution for 5 min and then rinsed twice with deionized water. Finally, it was destained using 80% ethanol and the absorbance was observed at 620 nm. The biofilm inhibitory percentage was calculated using the following formula

Light microscopic analysis

Visualization of biofilm by light microscopy was performed according to[35].Briefly, the biofilm were allowed to grow on glass pieces (1x1 cm^2) placed in 24-well polystyrene plates supplemented with solvent extracts of OPTat different concentration (0.0156 mg/ml to 1 mg/ml) and incubated for 24 h at 37 C. The slides were stained using 0.4 % crystal violet and were placed with biofilm facing upwards. The slides were observed under light microscopy at magnification of 400. Visible biofilms were documented with an attached digital camera (Nikon eclipse E200, Japan).

Statistical analysis

All experiments were performed in triplicates and the data obtained from the experiments were presented as mean values ± Standard Error. Students-t test was used to determine the significance between control and test samples.

Results and Discussion

Phytochemical screening

Traditional medicine comprises of medical knowledge systems that had been developed over generations within various societies before the era of modern medicine. They were prepared from a single plant or as a concoction of more than one plant. Qualitative phytochemical analysis of the plant extract revealed the presence of various secondary metabolites like alkaloids, glycosides, tannins, saponin, flavonoids, steroid, triterpenes and phenol. The presence of biologically active phytochemicals are responsible for medicinal activity of plants[36-38].This results correlate and evidently suggested that the phytochemical properties of OPT extract was suitable for treating microbial infections as it had essential phytochemicals with anti-inflammatory, anti-microbial, anti-oxidant properties [Table 1]. Moreover, these bioactive compounds synthesized by plants as secondary metabolites are taxonomically and structurally diverse.

Table 1. Phytochemical analysis of using OPT extracts

S. No	Bioactive compounds	OPT methanol extract†
1	Alkaloids	+
2	Carbohydrates	+
3	Flavonoids	+
4	Saponin	+
5	Phenols	+
6	Glycosides	+
7	Steroids	+
8	Terpenoids	+

-;Absence, and +; Presence of the respective phytochemical.

GC-MS analysis

The active compounds identified in the methanol leaf extract of OPT using GC-MS analysis are represented in Figure 1. Totally thirty nine compounds were detected from GC-MS analysis based on retention time, molecular formula, molecular weight and peak area. The active compounds with their retention time (RT), molecular formula, molecular weight (MW) and concentration (peak area %) are presented in Table2.The major compounds present in the leaves were 9,12-

Octadecadienoic acid (Z,Z)- (CAS) (15.80%),2-Furanmethanol, tetrahydro-(CAS) (11.33%), Benzenepropanol, .alpha.-methyl-.beta.-nitro-, (11.49 %) and Ethyl (trimethylsilyl)acetate (10.58%).The results of GC-MS analysis led to the identification of number of phytochemical compounds from the OPT methanolic extract with different chemical structures. The presence of various bioactive compounds elucidated the medicinal property of OPT extract. However, study on the individual phytochemical constituents might enabled the determination of key compound of the medicinal plants with potential phototherapeutic properties.

The major compounds present in the OPT extract were 9,12-Octadecadienoic acid (Z,Z) (15.80%), 2-Furanmethanol, tetrahydro (11.33%), Benzenepropanol, .alpha.-methyl-.beta.-nitro-, (R*,R*)-(.+-.)- (11.49 %) and Ethyl (trimethylsilyl)acetate (10.58%) etc., other compounds present were, 9,12-Octadecadienoic acid (Z,Z)- is one among the phytocompounds in leaf (8.36%). Similar compounds were reported in the stem and bark (18.81%) of *P. alatum* were found to have potential anti-cancer, anti-inflammatory and anti-arthritic activities. In addition, 9,12-Octadecadienoic acid (Z,Z)- in Croton tiglium seeds and Euphorbia longan leaves were reported to have potential anti-oxidant and anti-cancer activity [39,40].n-Hexadecanoic acid (1.53%), hexadecanoic acid, ethyl ester (0.11%)- Palmitic acid have the property of anti-oxidant, hypocholesterolemic, nematicide, pesticide, lubricant activities and hemolytic 5- alpha is a reductase inhibitors. Phytol was extensively studied for promising novel class of pharmaceuticals in the treatment of rheumatoid arthritis and possibly other chronic inflammatory diseases [41].

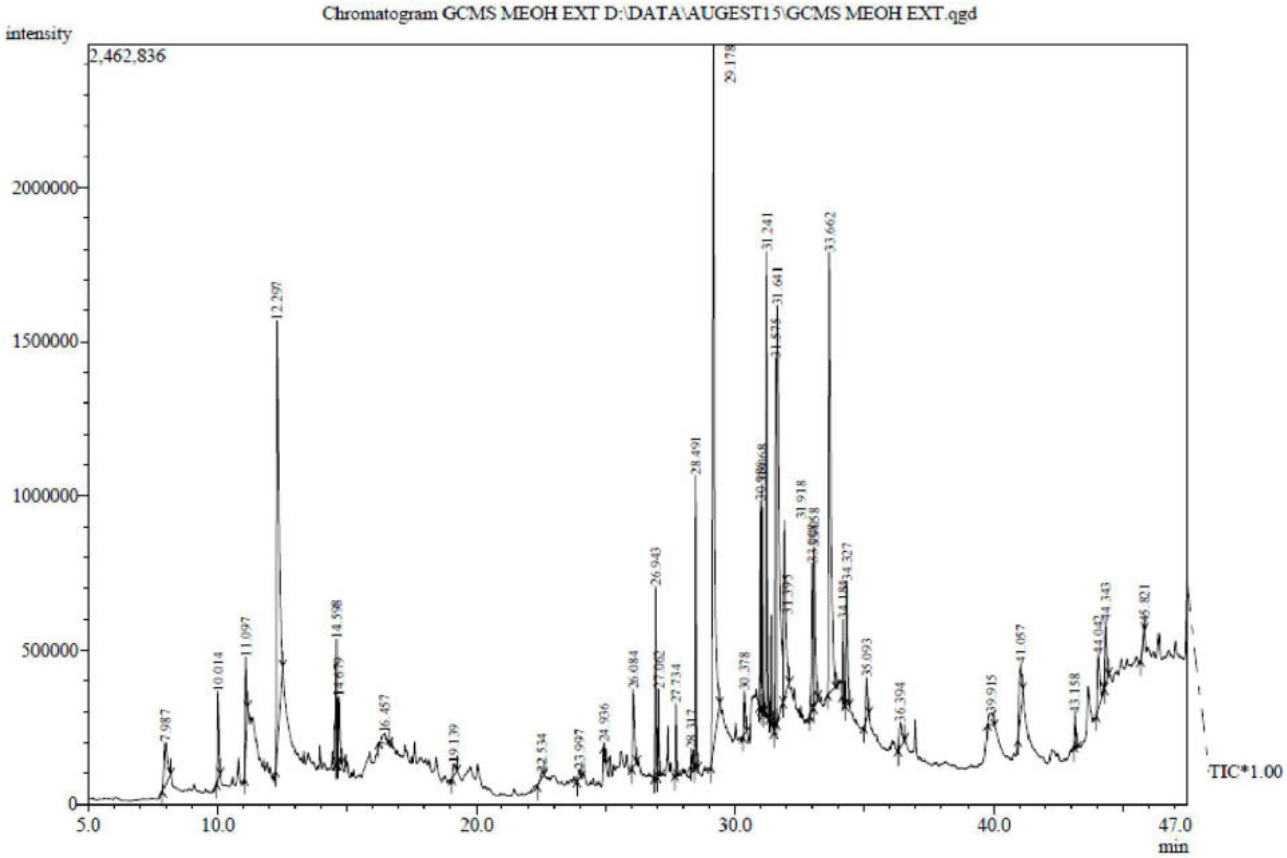

Figure 1. GC-MS chromatogram of OPT methanol extract

Table 2. Phytochemical components identified in the methanol extract of OPTby using GC-MS showing their RT, peak area percentage, molecular weight, molecular formula.

S.No	RT	Peak area (%)	Name of the compound	Molecular Formula	Molecular Weight	CAS No.
1	7.987	1.45	Isovaleric acid, isopentyl ester	$C_{10}H_{20}O_2$	172	659-70-1
2	10.014	1.38	Benzene, 4-pentynyl	$C_{11}H_{12}$	144	1823-14-9
3	11.097	1.27	4-Phenyl-2-Butanone	$C_{10}H_{12}O$	148	2550-26-7
4	12.297	**11.33**	2-Furanmethanol, tetrahydro-	$C_5H_{10}O_2$	102	97-99-4
5	14.598	1.19	Di(1-methyl-1-silacyclobutyl)amine	$C_8H_{19}NSi_2$	185	7266-78-6
6	14.679	1.16	2-Cyclohexen-1-one, 4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl)-	$C_{13}H_{18}O_3$	222	7070-24-8
7	16.457	0.45	Neophytadiene	$C_{20}H_{38}$	278	504-96-1
8	19.139	0.39	2-Pentadecanone, 6,10,14-trimethyl-	$C_{18}H_{36}O$	268	502-69-2
9	22.534	0.15	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296	102608-53-7
10	23.997	0.22	Cyclopentadecanone	$C_{15}H_{28}O$	224	502-72-7
11	24.936	0.11	Hexadecanoic acid, Methyl ester	$C_{17}H_{34}O_2$	270	112-39-0
12	26.084	1.53	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	57-10-3
13	26.943	2.02	1-Phenylpyrrolidine	$C_{10}H_{13}N$	147	4096-21-3
14	27.062	1.00	Linoleic acid, methyl ester	$C_{19}H_{34}O_2$	294	112-63-0
15	27.734	0.77	9-octadecenoic acid, Methyl ester	$C_{19}H_{36}O_2$	296	112-62-9
16	28.317	0.41	Phytol	$C_{20}H_{40}O$	296	150-86-7
17	28.491	2.89	Octadecanoic acid, Methyl ester	$C_{19}H_{38}O_2$	298	112-61-8
18	29.178	**15.80**	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280	60-33-3
19	30.378	0.73	Oleic acid	$C_{18}H_{34}O_2$	282	112-80-1
20	30.986	1.87	Octadecanoic acid	$C_{18}H_{36}O_2$	284	57-11-4
21	31.068	2.31	3-Chlorophenyl-beta phenylpropionate	$C_{15}H_{13}ClO_2$	260	23522-74-9
22	31.241	**4.89**	Z,Z-6,28-Heptatriacontadien-2-one	$C_{37}H_{70}O$	530	133530-21-9
23	31.395	0.98	1H-Imidazole, 4,5-dihydro-2-(phenylmethyl)	$C_{10}H_{12}N_2$	160	59-98-3
24	31.575	**5.28**	2H-Pyran-2-one, tetrahydro-6-nonyl	$C_{14}H_{26}O_2$	226	2721-22-4
25	31.641	**11.49**	Benzenepropanol, alpha-methyl-beta-nitro.	$C_{10}H_{13}NO_3$	195	96040-24-3
26	31.918	3.96	Geranylgeraniol	$C_{20}H_{34}O$	290	24034-73-9
27	33.008	2.15	Boroxin, Ethyldipropyl	$C_8H_{19}B_3O_3$	196	0-00-0
28	33.058	2.61	Octatriacontyltrifluoroacetate	$C_{40}H_{77}F_3O_2$	646	0-00-0
29	33.662	**10.58**	Ethyl (trimethylsilyl)acetate	$C_7H_{16}O_2Si$	160	4071-88-9
30	34.184	0.80	1,3-Propanediol, 2 (hydroxymethyl)-2-nitro	$C_4H_9NO_5$	151	126-11-4
31	34.327	2.05	2H-Pyran-2-one, 5,6-dihydro-4-(2,3-dimethyl-2-buten-1-yl)	$C_{11}H_{16}O_2$	180	0-00-0
32	35.093	0.70	1,2-Benzenedicarboxylic Acid, Diethyl Ester	$C_{12}H_{14}O_4$	222	84-66-2
33	36.394	0.68	7-Oxabicyclo[4.1.0]heptane, 1-methyl-4-(2-methyloxiranyl)	$C_{10}H_{16}O_2$	168	96-08-2
34	39.915	0.58	13-cis-Retinal	$C_{20}H_{28}O$	284	116-31-4
35	41.057	1.54	Loliolide	$C_{11}H_{16}O_3$	196	5989-02-6
36	43.158	0.47	Benzenepropanol, alpha-methyl-beta nitro	$C_{10}H_{13}NO_3$	195	96040-24-3
37	44.042	1.50	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	330	23470-00-0
38	44.343	1.22	Benzene, (2-Decyldodecyl)	$C_{28}H_{50}$	386	55334-72-0
39	45.821	0.10	Ethyl homovanillate	$C_{11}H_{14}O_4$	210	60563-13-5

Antibacterial assays

A total of 10 isolates were obtained and identified as *S.pyogenes*. Among 10 isolates five isolates (SP-1 to SP-5) exhibited strong biofilm forming potential. Whereas, SP-6 and SP-10 did not form biofilms. Strong biofilm forming isolates were selected and subjected to further

studies. MIC was determined for Methanol extract of OPT against the test pathogens. The MIC of OPT methanol extract is represented in [Figure 2]. The methanol extract of OPT inhibited the growth of *S.pyogenes* isolates at an MIC at 1 mg/ml (SP-4), whereas a MIC ranging from 1-2 mg/ml was observed for other isolates. Due to the increase in complexity of most microbial infections and the resistance to conventional therapy, researchers have been prompted to identify alternatives for the treatment of infections. Plant extracts and other biologically active compounds isolated from plants have gained widespread interest in this regard as they have been known to cure diseases and illness since ancient times. The methanol extract of *Ocimum tenuiflorum, Plectranthusamboinicus and Tylophoraindica* when subjected to individual assays, interfered with the biofilm formation of *Streptococcus pyogenes* at a MIC ranging between 2-4 mg/ml, but our present synergistic study revealed that the methanol extracts OPT exhibited a MIC of 1-2 mg/ml against test pathogens, which elucidate that synergistic extract exhibited significantly higher activity than the respective individual plant extracts.

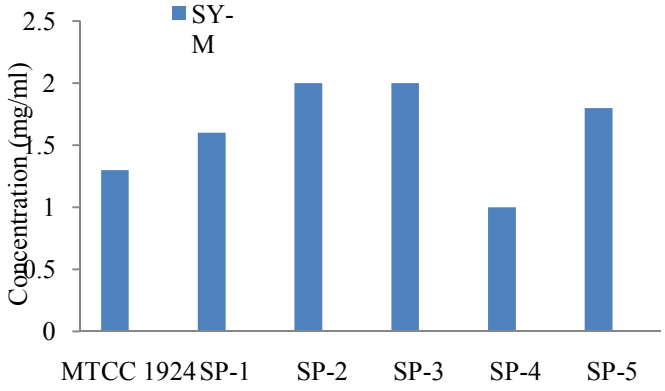

Figure 2. Minimum Inhibitory concentration assay of OPT synergistic methanol extract against *Streptococcus pyogenes* isolates and MTCC 1924.

The synergistic antibacterial activity of OPT methanol extract against S. pyogenes isolates were evaluated at a random single point sub-MIC level and the zone of inhibition were compared. Results elucidated no antibacterial activity of selected isolates by the OPT methanol extracts at the tested concentration [Table 3]. The results of *Ocimum tenuiflorum,Plectranthusamboinicus,* and *Tylophora indica* (OPT) single plant extracts has been previously reported to have potential antibacterial activities against various pathogens including S. pyogenes[36,37,38, and 42]which correlated with our results of the MIC assay which elucidated that the synergistic extracts inhibited the test pathogens at the higher tested concentration. However, in the present study the three plant extracts in their combination did not show antibacterial activity at the tested sub-MIC level (1 mg/ml) which was evident from the absence of inhibition zone in the results and also evidently showed that the extracts were not cytotoxic to the pathogen. According to Vattemet al.,[43] medicinal plants with well-known antibacterial properties could also significantly possess anti-pathogenic activities, which may not be related to growth inhibition of microorganism, which is well matched with our results.

Table 3. Anti-bacterial activity of OPTmethanol extracts against *S. pyogenes*.

Extract	Concentration (mg/ml)	Zone of inhibition (mm)					
		Sp-1	Sp-2	Sp-3	Sp-4	Sp-5	MTCC1924
OPT	1	-	-	-	-	-	-
Streptomycin (Positive Control)	0.3	28	24	20	28	28	25
DMSO (Negative control)	-	-	-	-	-	-	-

Biofilm Inhibition assay

The results of biofilm inhibition assay of the OPT methanolextract showed significant (p 0.05) inhibition of the biofilm when assessed spectrophotometrically. The lowest and most effective concentration that caused the reduction in the biofilm adherence index was dose dependent and observed from15.6 µg/ml to 1 mg/ml. Moreover, isolates treated with methanol extracts of OPTshowed significant (p 0.05) decrease in the biofilm formation when compared to the untreated control [Figure 3].The biofilm inhibitory concentration (BIC) was observed at 0.0625 mg/ml for MTCC 1924, SP-1and SP-4 whereas the BIC for SP-2, SP-3 and SP-5 was observed at 1 mg/ml concentration.

In the present study, anti-biofilm effect of plant extracts against Streptococcus pyogenes has been studied adopting biofilm inhibition spectrophotometric assay. Our results correlated with the reports of

Choi et al., [44] who reported that interactions of nanosilver with biofilm forming cells leading to significant inhibition.The plant extract inhibited biofilm in a dose dependent manner. Anti-biofilm effect of various plant extracts against biofilm of human pathogenic bacteria has been reported by Carneiroet al.,[45].The matrix is one of the most distinctive features of a microbial biofilm. The composition of the matrix varies according to the nature of the organism and reduction in the biochemical composition of the biofilm matrix leads to weakening of the biofilm thus facilitating the entry of the drugs [46]. Purification and identification of the phytochemicals responsible for the anti-biofilm effect and the coating of the bioactive compound will lead to development of effective anti-microbial against harmful pathogenic microorganism.

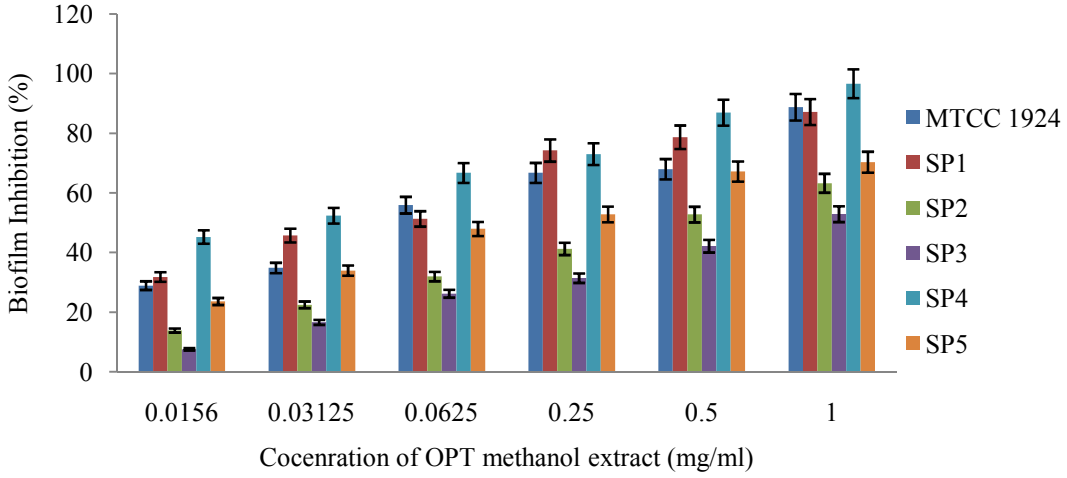

Figure 3. Percentage inhibition of biofilm formation of *S. pyogenes* by varying concentrations (0.0156 mg/ml to 1 mg/ml) of OPT methanol extract. Mean values of triplicate independent experiments and S.E are shown.

Light Microscopic Analysis

Analysis of *S. pyogenes* biofilm treated with OPT methanol extract at BIC showed significant biofilm reduction which was evident from loosened architecture of the biofilms in the treated sample [Fig – 4 a,b and c]. The results of the present study elucidated that the OPT methanol extract might have prevented the preliminary adhesion of the bacterial cells on to the substratum which then prevented the biofilm formation from progressing. Similar results were observed by Teepica et al.,[26] against biofilm forming *S. pyogenes* clinical isolates treated with solvent extracts of *Piper longum* and *Piper nigrum* in which the biofilm matrix was disrupted significantly which was evidently indicated by decreased biofilm matrix density in treated samples as compared to control. However, the biofilm distribution observed in the present study, showed better reduction in the biofilm density which could be attributed to the synergistic efficacy of the medicinal plants used. According to Carneiro et al., [45] the antibiofilm property of the medicinal plants were dependent on dose, and the combination of plant extracts used which well correlated with our results. As only BIC levels were used to study the biofilm inhibition it could be postulated that the significant reduction in the biofilm densities by the OPT extract might attributed to the synergism existing between the phytochemicals of the plant extract used.

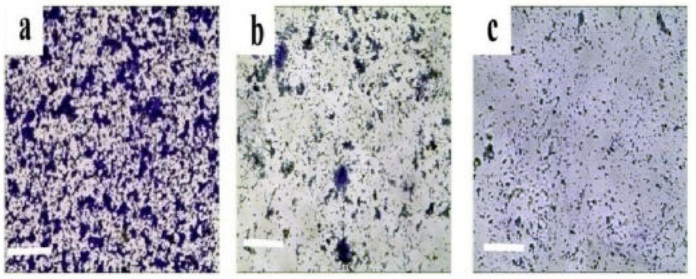

Figure 4. Representative light microscopic images of *S. pyogenes* SP-4(strong biofilm former) a) control, b) OPT methanol extract treated at 0.625 mg/ml (BIC) and c) OPT methanol extract treated at 1 mg/ml.

Conclusion

Natural products have been used as an alternative treatment because they have been considered safe for treating disease without side effects. Many research has been exclusively focused on the planktonic forms and less attention has been given to microbial biofilms. Our present study reported the preliminary results on the synergistic anti-biofilm property of three Indian medicinal plants *Ocimum tenuiflorum*, *Plectranthusamboinicus*, and *Tylophora indica* against the biofilm forming *S. pyogenes* clinical isolates. As the synergistic effect of the tested plants showed significant anti-biofilm property, these medicinal

plants could be studied with advanced techniques to identify the principal component responsible for inhibiting the biofilms.

Authors' Contribution

MK and DJ performed the experiments, SP and MK wrote the article, TPDD scrutinized and gave her suggestion in editing the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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