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Influence of *Tylophora indica* against biofilm forming *Streptococcus pyogenes* isolated from Pharyngitis patients

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Abstract

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

Methods: The plant extracts (methanol, ethyl acetate, chloroform and petroleum ether) 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 exhibited the strong phytochemical components. GC-MS analysis of the methanol extract showed a total of thirty seven 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. 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 *T. indica* leaves consisted of pharmacologically active components and could be used as an anti-biofilm agent at minimal concentrations thereby successfully preventing the formation of biofilms.

Keywords: *Tylophora indica*, GC-MS, Anti-biofilm activity, Light Microscopy, *S. pyogenes*.

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Introduction

Bacterial infections can occur in almost every part of the human body, which indicates that bacteria have adapted to survive in physiologically specific anatomical locations. To facilitate this process, an organism must express the proper growth and virulence factors at the convenient time, endure a probably harsh surrounding chemical environment, and prevent a host's immune defenses. Several bacterial species use structures referred to as biofilms to combat these hazards. A bacterial biofilm is described as a sessile community of organisms encased in a matrix of extrapolymeric substances and attached to a substratum, interface, or to each other. Biofilms tend to exhibit an altered phenotype with respect to growth rate and gene transcription [1]. Biofilms are responsible for a large medical burden throughout the world. According to the US National Institute of Health, biofilms account for over 80% of microbial infections in the human body [2]. Biofilms pose a significant health risk because they are inherently tolerant to host defenses and are up to a thousand times more resistant to conventional antibiotics [3]. *Streptococcus pyogenes* (group A streptococcus) is a Gram-positive pathogen that is responsible for a wide variety of human disease. Diseases range from relatively mild clinical illnesses such as pharyngitis, cellulitis, and impetigo, to life-threatening conditions, such as puerperal sepsis, myositis, toxic shock syndrome, and necrotizing fasciitis (flesh-eating disease). *S. pyogenes* infections can also lead to post-infectious sequelae, such as acute rheumatic fever, rheumatic heart disease, and acute post-streptococcal glomerulonephritis [4].

S. pyogenes has been shown to form biofilms both in vitro and *in vivo*. Biofilm resistance is due to several reasons, like restricteddiffusion of antibiotics into biofilm matrix, expression ofmultidrug efflux pumps, type IV secretion systems, decreasedpermeability, and the action of antibiotic-modifying enzymes [5]. The increased biofilm resistance to conventional treatmentsenhances the need to develop new control strategies [6]. Biofilm inhibition is considered as major drug target forthe treatment of various bacterial and fungal infections, andpharmacological development of this drugs is now extensively studied [7, 8]. A promising alternative is the search for naturallyoccurring compounds of plant origin capable of blocking biofilm formation [9]. Historically, plant extracts and theirbiologically active compounds have been a valuable sourceof natural products, which have played a central role in theprevention and treatment of diseases, helping to maintain human health [10]. Furthermore, they are widely accepted due to the perception that they are safe and have a long history of use in folk medicine to cure diseases and illnesses since ancient times [11].

Tylophora indica (Burm. F) Merill, commonly known as Indian Ipecse or Antmool belongs to family Asclepiadaceae. The plant is perennial, small, slander, a twining or climbing herb. Leaves are Ovate to elliptic (6.0-10.5*3.8-6.0 cm), petioles are up to 12 mm long. Flowers are minute (1-1.5cm across) and corolla is greenish yellow or greenish purple in color. Fruit is a follicle. Tylophora has been traditionally used for the treatment of bronchialasthma, jaundice and inflammation. It has antitumor, immunomodulatory, antioxidant, anti-asthmatic, muscle relaxant. Although the leaf and root ofthis plant are widely used for treating jaundice in northern Karnataka, there is a paucity of scientific evidence regarding its usage in liverdisorder. The other reported activities include immune-modulatoryactivity, anti-inflammatory activity, anticancer activity, antihistaminic andantirehumatic.The leaves and roots of Tylophora are used as a source of bioactive material [12]. It is reported to have laxative, expectorant, diaphoretic, purgative, stimulant, emetic and cathartic properties [13]. It has also beenused for the treatment of allergies, cold, dysentery, hay fever and arthritis.It has reputation as alterative and as a blood purifier often used inrheumatism. It is an expectorant and administered in respiratoryinfection, bronchitis and

whopping cough [14].The aim of this study was to examine the phytochemical, GC-MS analysis, Antibacterial and Antibiofilm activity aspects of various extracts of *Tylophora indica* against bacterial pathogens causing respiratory tract infections.

Materials and Methods

Collection of plants materials

Tylophora indica leaves were collected from area surrounding Kolli hill, Namakkal District, Salem, Tamil Nadu. The plant material was taxonomically identified and authenticated (Voucher No: 1113) by Botanical Survey of India, Southern Circle, Tamil Nadu Agricultural University (Govt. of India), Coimbatore, Tamil Nadu, India.The leaf (*Tylophora indica*) were thoroughly washed under tap water. The leaf was shade dried for a week, coarsely powdered using mixer grinder and stored in an air-tight container for further use.

Reagents

The reagents used for the study were of analytical grade as follows: Petroleum ether (Himedia), Chloroform (Himedia), Ethyl acetate (Himedia), Methanol (Himedia), di-methyl sulfoxide (Himedia).

Solvent extraction

25 g of the dried *T. indica* leaves powder was soaked in 100 ml of Petroleum ether, Chloroform, Ethyl acetate and Methanol (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 percentage yield was calculated. The dried *T. indica* methanol (TIM) , ethyl acetate (TIEA), chloroform (TICH) and Petroleum ether (TIPE) 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) [15]

Phytochemical analysis

The Petroleum ether, Chloroform, Ethyl acetate and Methanol of *T. indica* were subjected to preliminary phytochemical screening and the presenceof alkaloid, carbohydrates, tannins, Saponin, flavonoid, steroids, Terpenoid, glycosides, phenol, amino acids, and lipids according to Harborne [16].

Determination of bioactive compounds using GC-MS analysis

Bioactive compound analysis of *T. indica* methanol 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.*, (2011) [17]. The spectrum of the unknown phytocomponents was compared with the spectrum of known componentsavailable in Wiley Online Library (Wiley08), NIST08 library. The bioactive compounds name, molecular weight, and structure was determined.

Culture of pathogens

A total of 10 isolates were collected 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 standard culture. All the isolates were cultivated in Todd Hewitt's Broth (Himedia), for routine analysis. Glycerol stock was maintained at -20°C until further use.

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Screening of biofilm forming *S. pyogenes*

The isolates of *S. pyogenes* were screened for biofilm formation according to Srinivasan *et al.*, (2015) [8]. Briefly, 12 h cultures of isolates were 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 micro titer 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 recorded as strong (+++), moderate (++), weak (+), and negative (−) by visually comparing the thickness of the adherent layer, and the results were tabulated. The strong biofilm formingisolates were subjected for further studies.

Minimal inhibitory concentration (MIC) assay

The MIC assay of the plant extracts was performed according to Clinical and Laboratory Standards Institute guidelines [18]. The bacterial suspensions (1×10^6 CFU/ml) were added to THB supplemented with *T. indica* methanol (TIM) , ethyl acetate (TIEA), chloroform (TICH) and Petroleum ether (TIPE) 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.

Agar well diffusion assay

The antibacterial activity potential of *T. indica* methanol (TIM) , ethyl acetate (TIEA), chloroform (TICH) and Petroleum ether (TIPE) extract was performed byagar well diffusion method using Mueller-Hinton agar (MHA) (Himedia,India) by following the methods specified in Clinical and Laboratory Standards Institute [18]. Briefly, 1 % overnight culture of test pathogenwas swabbed uniformly over freshly prepared MHA plates and allowedto dry. Then, agar plugs were cut out using gel puncture. 30 µl of *T. indica* methanol (TIM), Ethyl acetate (TIEA), Chloroform (TICH) and Petroleum ether (TIPE) at sub-MIC (0.5 mg/ml) was incorporated onto the wells. Streptomycin (0.03 mg/ml) was usedas 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 [19] with slight modification. Briefly, 1 % overnight cultures (0.5 O.D at 600 nm) of testpathogens were added in 1 ml of fresh LB medium containing *T. indica* methanol (TIM), ethyl acetate (TIEA), chloroform (TICH) and Petroleum ether (TIPE) sub-MICs (0.5-2 mg/ml). The samples were incubated at37°C for 16 h. After incubation, MTPs were emptied of free-floatingplanktonic cells, and the wells were gently rinsed with sterile water.The sessile cells were stained with 0.4% CV (Himedia, India) solution. After 15 min, CV solution was discarded completely, and wells werefilled with 1 ml of 95% ethanol for de-staining. The biofilm biomasswas quantified by measuring the absorbance at 650 nm usingmulti-plate ELISA reader (Biotek-ELX-800, India).

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Microscopic observation of biofilm

In situ Light microscopic analysis

For visualization of biofilm by light microscopy [19], the biofilmswere allowed to grow on glass pieces (1 cm ×1 cm) placed in 24-well polystyrene plates supplemented with different solvent extracts of *T. indica* (1 mg/ml) and incubated for 24 h at 37 °C. Theslides were stained using CV and were placed on slides with biofilmpointing 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 21, Chicago, USA). Students T-test was used to calculate the significant difference. Values were considered significantly different if $p \leq 0.05$.

Results

Phytochemical analysis of *Tylophora indica*

Phytochemical contents of the *Tylophora indica* leaf were determined and presented in Table 1. The preliminary phytochemical screening of *Tylophora indica* revealed that the presence of terpenoids, alkaloids, carbohydrates, steroid, glycoside, saponin, phenol, flavonoid, fatty acid and fixed oil. Methanol extract were showed positive results for maximum amount of phytoconstituents when compared with ethyl acetate, petroleum ether and chloroform extract. Concurrently the phytochemical constituents like tannin were absent in these four extract (Table 1).

Table 1. Preliminary phytochemical analysis of different leaves extracts of *Tylophora indica*

Bioactive compounds	Methanol	Ethyl acetate	Chloroform	Petroleum ether
Alkaloids	+	+	−	−
Carbohydrates	+	+	−	−
Flavonoids	+	+	+	−
Phenol	+	+	+	+
Terpenoid	+	+	+	+
Tannins	+	+	+	+
Glycosides	+	−	+	−
Steroids	+	−	+	−
Saponin	+	−	−	−
Fixed oils and fats	−	−	−	−

+&− indicates that the compound was present and absent for the test respectively.

Determination of bioactive compounds using GC-MS analysis

The GC-MS analysis of *Tylophora indica* methanol extract revealed the presence of thirty two compounds that could contribute the medicinal quality of the plant (Table 2). The active principles with their retention time (RT), molecular formula (MF) and peak area in percentage are presented in Table 2 and Figure 1. It was found that main phytoconstituents of leaves parts are 9, 12-Octadecadienoic Acid (Z, Z)-, Methyl Ester (21.21%), 1-Heptacosanol (21.21%), Stearic acid (11.08%), Lanosterol (8.62%) Oxirane, hexadecyl- (7.73%).Our results showed the

presence of flavonoids, fatty acid, glycoside and phenol derivatives in the leaves part of *Tylophora indica*.

Table 2. GC-MS compound analysis of *T. indica* methanol extract

S.No	Compound name	RT(min)	(%)Area	Molecular formula
1.	Methylsulfonylmethane	8.137	10.83	$C_2H_6O_2S$
2.	Carvone	13.183	1.29	$C_{10}H_{14}O$
3.	N-hexadecanoic acid	9.965	1.33	$C_{16}H_{32}O_2$
4.	Phytol	11.047	1.10	$C_{20}H_{40}O$
5.	9-hexadecenal	12.333	2.74	$C_{16}H_3O$
6.	Nonadecanoic acid	13.187	11.18	$C_{19}H_{38}O_2$
7.	Neophytadiene	14.095	1.32	$C^{20}H_{38}$
8.	Eliminoxy	15.337	0.76	$C_{13}H_{16}O_2$
9.	Tocopherols	16.860	-0.15	$C_{29}H_{50}O_2$
10.	Campesterol	17.313	0.08	$C_{28}H_{48}O$
11.	Stigmasterol	18.903	0.16	$C_{29}H_{48}O$
12.	Squalene	19.894	0.47	$C_{30}H_{50}$
13.	Campesterol	23.031	0.12	$C_{28}H_{48}O$
14.	Tetramethyltricyclododec- 10(11)	26.068	0.47	$C_{16}H_{26}$
15.	Perilla acetate	27.006	0.33	$C_{12}H_{18}O_2$
16.	Undecane	28.855	1.02	$C_{11}H_{24}$
17.	1,3-diethoxy-1,1,3,3- tetramethyldisiloxane	29.149	4.09	$C_8H_{22}O_3SI_2$
18.	Phenol,2-methoxy(2-propenyl)-	31.287	8.68	$C_{10}H_{12}O_2$
19.	Caryophyllene	31.640	4.71	$C_{15}H_{24}$
20.	2,6-di-tert-butylphenol	31.949	1.72	$C_{14}H_{22}O$
21.	4-ethyl-1,2-dimethoxybenzene	32.594	1.72	$C_{10}H_{14}O_2$
22.	1-nonadecene	33.511	0.20	$C_{19}H_{38}$
23.	Neophytadiene	34.023	2.22	$C_{20}H_{38}$
24.	Benzenepropanoic acid	34.117	2.81	$C_{18}H_{28}O_3$
25.	Methyl commate c	36.410	0.12	$C_{31}H_{50}O_4$
26.	Benzene, (ethenyloxy)-	36.810	0.59	C_8H_8O
27.	Methane, chlorodifluoro	38.220	3.67	$CHCLF_2$
28.	Undecyl trichloroacetate	39.112	14.03	$C_{13}H_{23}CL_3O_2$
29.	Methyl 2-oxononanoate	39.960	2.96	$C_{10}H_{18}O$
30.	Cholest-22-ene-21-ol	40.821	16.25	$C_{33}H_{54}O_3$

S.No	Compound name	RT(min)	(%)Area	Molecular formula
31.	Octadecane-1,2-diol, bis(trimethylsilyl) ether	41.773	0.30	$C_{24}H_{54}O_2SI_2$
32.	1,2-benzenedicarboxylic acid	43.271	2.89	$C_{24}H_{38}O_4$

RT- Retention time

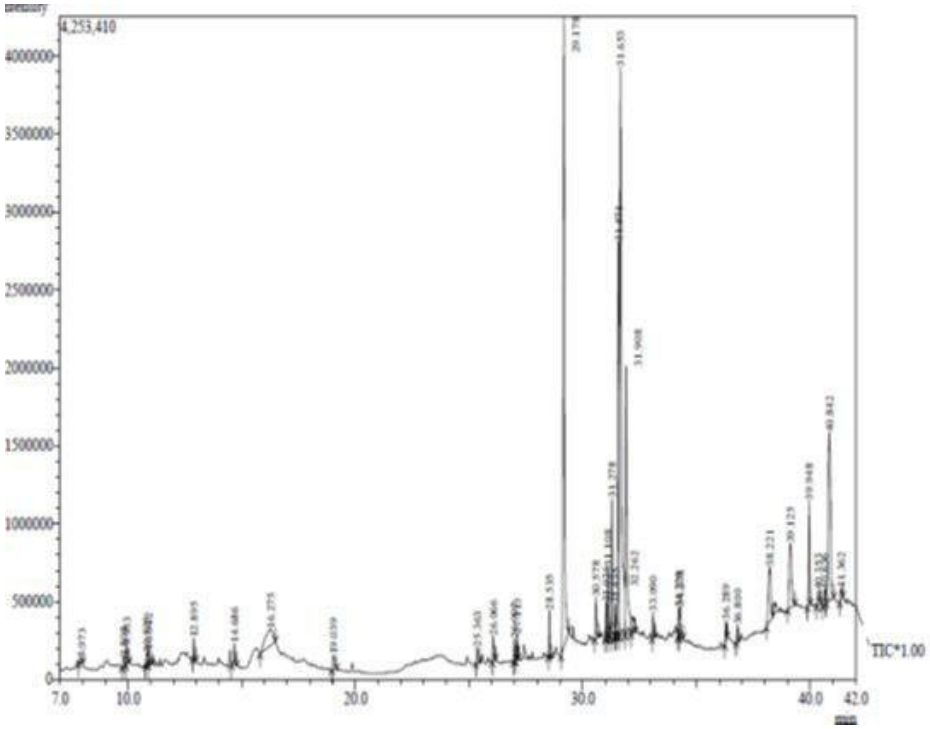

Figure 1. Chromatogram showing the GC-MS spectrum of *Tylophora indica* methanol extract.

Minimal InhibitoryConcentration

A total of ten isolates were isolated and identified as *S. pyogenes*. Among 10 isolates five isolates exhibited biofilm forming potential (SP1 to SP5). Whereas, isolates SP6 to SP10 did not form biofilms. Biofilm forming isolates were selected and subjected to further studies. MIC was determined for Methanol, ethyl acetate, Chloroform and Petroleum ether of *Tylophora indica* against the test pathogens. The MIC of *T. indica* extracts are represented in Figure 2. The methanol and ethyl acetate extract of *T. indica* inhibited the growth of *S. pyogenes* isolates at an MIC ranging from 2 mg/ml, whereas a MIC ranging from 4 mg/ml was observed for Chloroform and Petroleum ether extract

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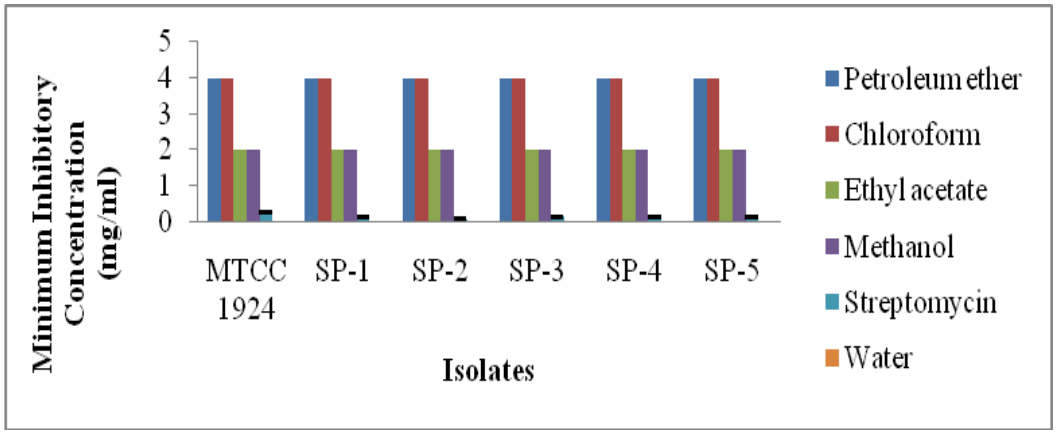

Figure 2. Minimal inhibitory concentration of *T. indica* solvent extracts against biofilm forming *S. pyogenes* isolates. Values are represented as mean ± standard error of the triplicate independent experiment

Antibacterial Activity

Antibacterial activity of different solvent extracts of *T. indica* leaves against *S. pyogenes* were evaluated and compared by zone of inhibition in disc diffusion method. The methanol, ethyl acetate, chloroform, and petroleum ether extract exhibit significant antibacterial activity. The activities of various extracts were comparable to antibacterial agent of Streptomycin. The petroleum ether and chloroform extracts exhibit less antibacterial activity compared with other extracts. The Petroleum ether extracts exhibited maximum activities of against five of the strains; SP1 (8 mm), SP2 (8 mm), SP3 (6 mm), SP4 (6 mm), SP5 (8 mm) and MTCC 1924 (15mm). The chloroform extracts showed SP1 (12 mm), SP2(10 mm), SP3 (10 mm), SP4 (10 mm), SP5 (11 mm) and MTCC 1924 (12 mm).The ethyl acetate extract of maximum activities against five of the strains; SP1 (16 mm), SP2(14 mm), SP3 (16 mm), SP4 (18 mm), SP5 (12 mm) and MTCC 1924 (16 mm). The methanol extracts shows showed the maximum Zone of Inhibition against SP1 (15 mm), SP2 (18 mm), SP3 (16 mm), SP4 (26 mm), SP5 (18 mm) and MTCC 1924 (22 mm). The result of antibacterial activities is presented in Tables - 3.

Table 3. Antibacterial activity of methanol, ethyl acetate, chloroform and petroleum ether extract of *T. indica* at different concentrations.

Extract	Concentration	Zone of inhibition (mm)					
		SP1	SP2	SP3	SP4	SP5	Mtcc-1924
Petroleum ether	4mg/ml	8	8	6	6	8	15
Chloroform	4mg/ml	12	10	10	12	11	12
Ethyl acetate	2mg/ml	16	14	16	18	12	16
Methanol	2mg/ml	15	18	16	26	18	22
Streptomycin	0.1mg/ml	28	24	20	28	28	25
Water		-	-	-	-	-	-

Biofilm Inhibition assay

The effect of *T. indica* extracts (methanol, ethyl acetate, chloroform and petroleum ether) on *S.pyogenes* biofilm formation was evaluated by crystal violet (CV) assay. Biofilm formation was checked by taking absorbance (OD 600) of stained biofilm cells. Inhibition assay showed all the solvent extracts of *T. indica* had biofilm inhibition property at Sub-MIC concentration ranging from 0.5-2mg/ml. All extracts ($p \leq 0.05$) inhibited biofilm formation as compared to control but biofilm inhibition activity of methanol extract was more as compared to other extract (Figure. 3). *T. indica* Methanol extract 2 mg/ml dislodged the biofilm biomass by SP1-94%, SP2-91%, SP3-87%, SP4-95%, SP5-94% and MTCC 1924 – 88%. Ethyl acetate biofilm biomass ranging at SP1-88%, SP2-86%, SP3-82%, SP4-90%, SP5-86% and MTCC 1924 – 82%. Whereas chloroform biofilm biomass ranging at SP1-50%, SP2-52%, SP3-55%, SP4-63%, SP5-57% and MTCC 1924 – 44%. Petroleum ether biofilm biomass ranging at SP1-36% , SP2-44%, SP3- 42%, SP4-48%, SP5-43% and MTCC 1924 – 24%.

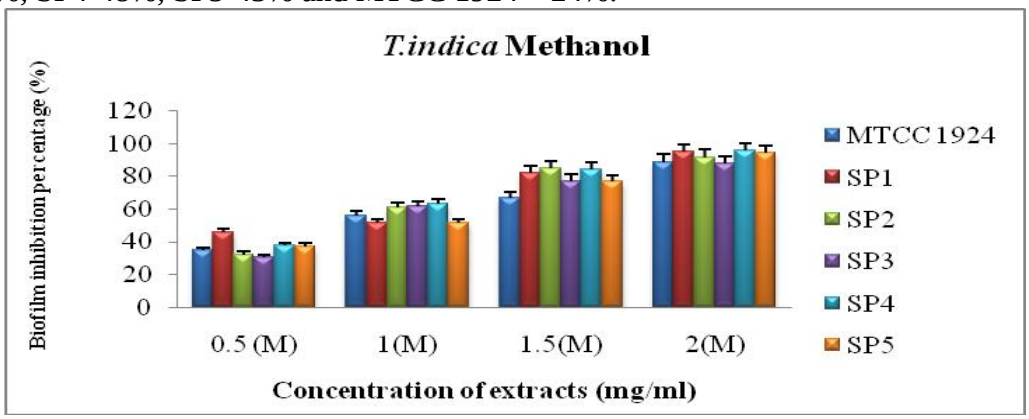

Figure 3.a. Percentage inhibition of biofilm formation of *S. pyogenes* by varying concentrations (0.0156 mg/ml to 1 mg/ml) of methanol extract. Meanvalues of triplicate independent experiments and S.E are shown.

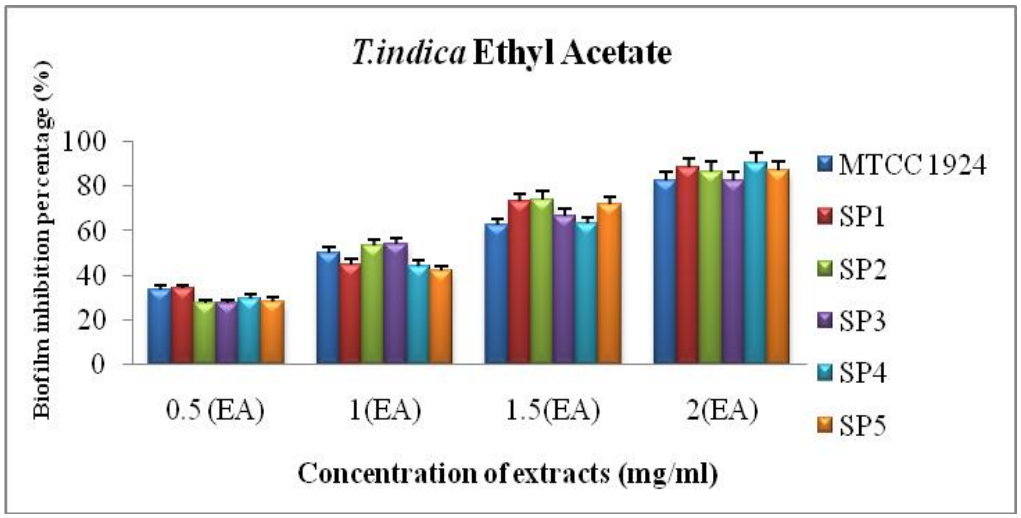

Figure 3.b. Percentage inhibition of biofilm formation of *S. pyogenes* by varying concentrations (0.0156 mg/ml to 1 mg/ml) of Ethyl acetate extract. Mean values of triplicate independent experiments and S.E are shown.

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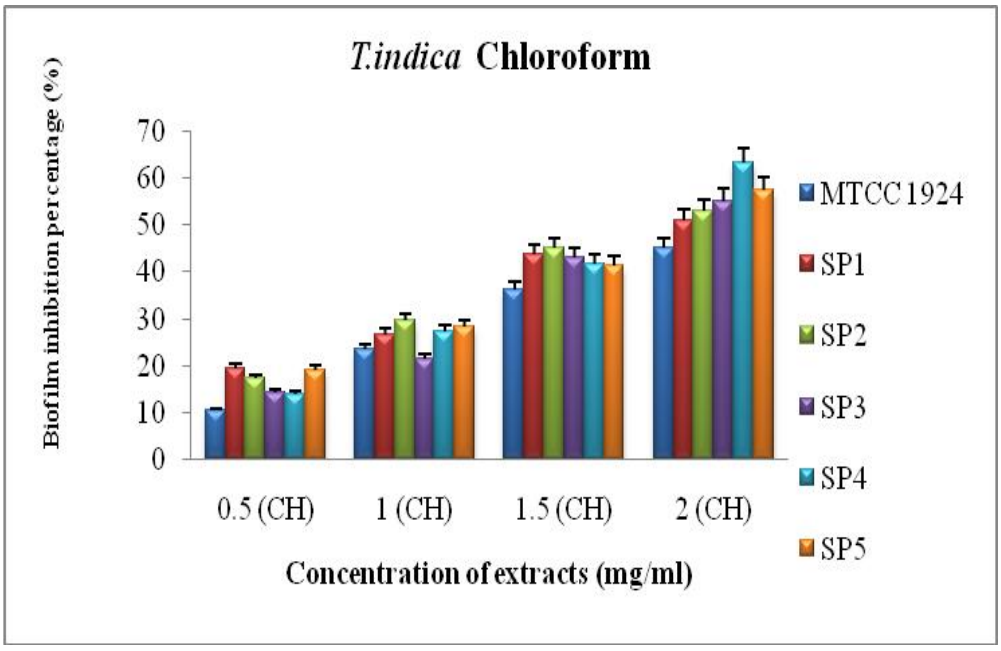

Figure 3.c. Percentage inhibition of biofilm formation of *S. pyogenes* by varying concentrations (0.0156 mg/ml to 1 mg/ml) of Chloroform extract. Mean values of triplicate independent experiments and S.E are shown.

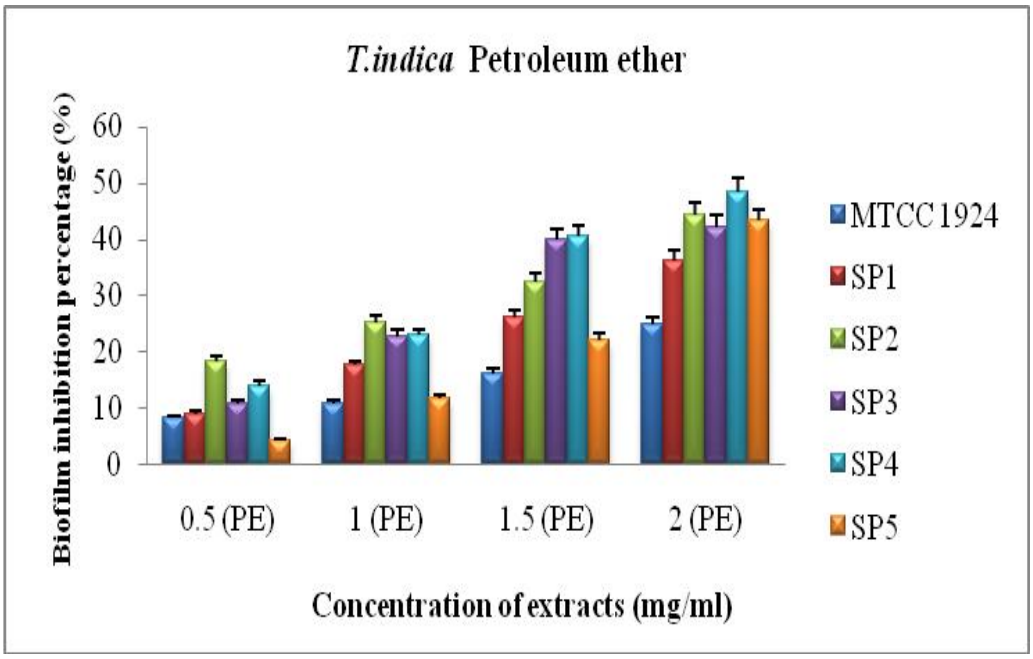

Figure 3.d. Percentage inhibition of biofilm formation of *S. pyogenes* by varying concentrations (0.0156 mg/ml to 1 mg/ml) of Petroleum ether extract. Mean values of triplicate independent experiments and S.E are shown.

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Light Microscopic Observation of Biofilm

In the control coverslips, a thick layer of biofilm was formed and stained easily with CV stain. However, *T. indica* different extracts-treated coverslips exhibited concentration dependent inhibitory activity, which showed a consistent reduction in biofilm formation of *Streptococcus pyogenes* hospital isolates with increasing concentrations. Results of microscopic analysis revealed methanol and ethyl acetate extract shows maximum level of reduction in number of microcolonies at the concentration of 2 mg/ml against all tested pathogens (Figure 4). Whereas chloroform and petroleum ether negligibly inhibited the biofilm architecture.

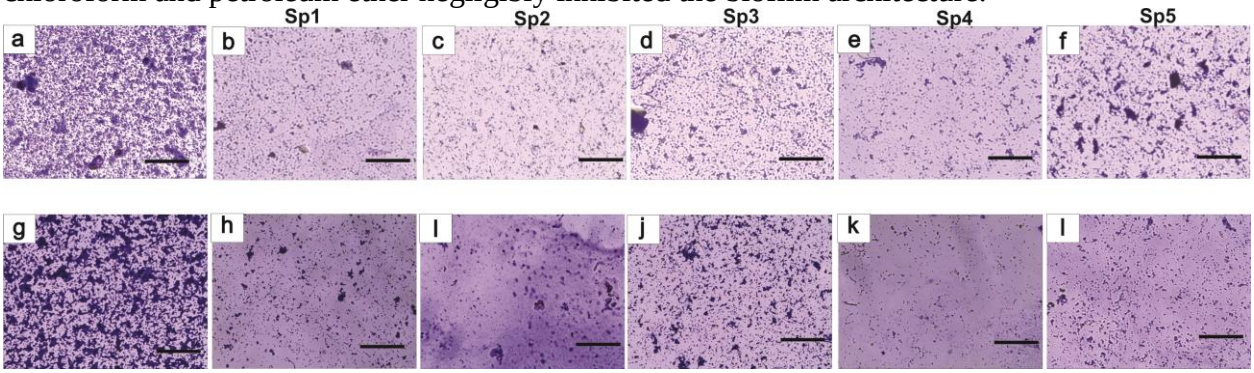

Figure 4. Light microscopic images of *S. pyogenes* isolates treated with *T. indica* b-f) methanol extract and h-i) ethyl acetate extract. a and g) Untreated control. (Scale bar – 20 µm; magnification 20x).

Discussion

Plant extracts are potential sources of antimicrobial compounds especially against bacterial pathogens [20]. A medicinal herb can be viewed as a synthetic laboratory as it produces and contains a number of chemical compounds. Earlier report [21] which recorded all the microorganisms to be more susceptible to the higher concentration of the extracts, is in support of the present investigation. The present study has authenticated the medicinal values of *T.indica*. Preliminary qualitative analysis of bioactive compounds revealed the presence of alkaloids in methanol extract. The presence of terpenoids and tannin present in all extracts. [22] reported that tannins have astringent properties, healing of wounds and inflamed mucous membranes. Tannins are reported to exhibit antiviral, antibacterial, anti-tumor activities and was also reported that certain tannins are also able to inhibit HIV [23] replication selectively and is also used as anti-diuretic [24]. Alkaloids shown only in methanol and ethyl acetate extract but absent in petroleum ether and chloroform extract indicated the analgesic, anti-spasmodic and bactericidal effects.

Alkaloids are known to be effective for the treatment of syphilis and other venereal disease [25] had earlier reported that saponins have antibiotic properties and so help the body to fight infections and microbial invasion. Also, it is used as a mild detergent and in intracellular histochemistry staining to allow antibody access to intracellular proteins, also reported in hyperchlolestrolaemia, hyperglycaemia, antioxidant, anticancer, anti- inflammatory and weight loss and have antifungal properties [23]. The GC-MS analysis of *T. indica* leaves revealed the presence of thirty seven compounds. The identified compounds possess many biological properties. For instance, 9,12,15-Octadecadienoic acid a conjugated linoleic acid possesses anti-inflammatory, insectifuge , hypocholesterolemic, cancer preventive, nematicide,

hepatoprotective, antihistaminic, antieczeemic, antiacne, 5-alpha reductase inhibitor,antiandrogenic, antiarthritic and anticoronary properties. Hexadecanoic acid – palmitic acid can be an antioxidant, pesticide, lubricant activities. Phytol- diterpene is an antimicrobial, anticancer, anti-inflammatory and diuretic agent [26]. 9, 12, 15-Octadecatrienoic acid, methyl ester, (Z, Z, Z)-, n-Hexadecanoic acid were present in *Caesalpinia sappan* ethanol extract [27] Hexadenoic acid has earlier been reported as a component in alcohol extract of the leaves of *Kigelia pinnata* [15] and *Melissa officinalis* [28]. Similar types of compounds were identified among the thirty seven compounds of this present study. Phytol is one among the compounds of present study. Similarly [29] observed the presence of phytol in the leaves of *Lantana camara* and [30] in *Mimosa pudica*leaves. Similar result was also observed in the leaves of *Lantana camara* [31]. Phytol was observed to have antibacterial activities against *Staphylococcous aureus* by causing damage to cell membranes as a result there is a leakage of potassium ions from bacterial cells [32]. Phytol is a key acyclic diterpene alcohol that is a precursor for vitamins E and K1. It is used along with simple sugar or corn syrup as a hardener in candies. Mangunwidjaja *et al*., [33] reported the main components of 9, 12 octadecadienoic acid, Octadec- 9enoic acid and 9, 12-actadecadienoic acid present in *Croton tigliums*eed. These compounds were found to have potential antioxidant and anticancer activities. Devi *et al*., [34] reported that *Euphorbia longan*l eaves mainly contained n-hexadecanoic acid and 9, 12-Octadecadienoic acid. These reports are in accordance with the result of this study.

Plants are important source of potentially useful component for the development of new chemotherapeutic agents. Many reports are available on the antiviral, antibacterial, antifungal, anthelmintic, antimolluscal and anti-inflammatory properties of plants. Some of these observations have helped in identifying the active principle responsible for such activities and in the developing drugs for the therapeutic use in human beings.The findings of the present study revealed that *Tylophora indica*, contain potent antimicrobial property against tested microbes. Herbal medicine is still the mainstay of about 70-80% of world population, mainly in the developing countries, for primary health care because of better cultural acceptability, better compatibility with human body with lesser side effects. The World Health Organization (WHO) has listed more than 21,000 plants, which are used for many medicinal purposes around the world. It also estimates that 4 billion people presently use herbal medicine for health care.

In the present study, antibacterial activity of different extracts was observed against five hospital isolates of *Streptococcus pyogenes*. The study showed that antibacterial activity of medicinal plant between methanol, ethyl acetate, chloroform and petroleum ether extract of *Tylophora indica* was found to be effective against all gram positive organisms. Methanolic extract of *T. indica* was found to be more effective against SP4 (26 mm), followed by SP1 (15 mm), SP2 (18 mm), SP3 (16 mm), SP5 (18 mm) and MTCC 1924 (22 mm) . Ethyl acetate extract of *T. indica* was found to be more effective against, SP1 (16 mm), SP3 (16 mm), SP4 (18 mm), and MTCC 1924 (16 mm) while least was shown by SP2 (14 mm), SP5 (12 mm). Petroleum ether and chloroform shows low antibacterial activity. Similarly results were reported by Wai *et al.*, [53] which elucidated that a significant degree of activity was observed against all the test bacteria in case of *Acacia nilotica.* The zone of inhibition in case of *Acacia nilotica* varied between 9 mm to 35.5 mm highest degree of zone of inhibition was observed against *Staphylococcus aureus.* Another report showed that the methanol leaf extracts of *Acacia nilotica, Sidacordifolia* showed the activity against *E. coli, S. aureus* [35]. Methanol extracts of *A. nilotica* exhibited good activity in the range of 18.75-75.0 µg/ml had been reported by Dabur *et al.*, [36]. Hence, these medicinal plants can be used to discern bioactive natural products and new

pharmaceutical molecules that serve in the development of unmet therapeutic needs. Another experiment showed that *C. roseus* have shown a good zone of inhibition. *Staphylococcus aureus* at 10 µg/ml formed a zone of 9 mm in diameter and 20 µg/ml formed a zone of 16 mm in diameter. Vijayalakshmidevi *et al*., [37] examined that these extracts may not find a therapeutic use in immediate future but definitely it can be used as aprophylactic agent in regions where certain diseases can occur as endemic if not in pandemic scale.

The lack of efficacy in antibiotic treatment in the eradication of susceptible organisms has recently induced microbiologists to hypothesize the presence of bacteria ordered in communities, attached to surfaces, identified as biofilms [38]. It has been investigated that *S. pyogenes* was able to form biofilm as an alternative method to escape antibiotic treatment and host defenses leading to recurrent infections [39]. A biofilm is a colony of single or multiple bacterial species embedded in a self-producing polymeric matrix, this matrix guarantees better survival and protection from macrophage action, antibiotics, temperature and pH fluctuations [38]. One of the best known biofilm-specific properties is antibiotic resistance, which can be up to 1000-fold greater than that seen with planktonic cells [40]. So biofilm-associated infections are difficult to eradicate by routine antibiotic doses in compare with planktonic form of bacteria. They need thousands times of doses used for non-biofilm infections [41]. As in biofilm formed by *Streptococcus pyogenes* in pharyngitis patients, which evading high antibiotic concentrations greater than 10-folds minimum inhibitory concentration (MIC) for planktonic *S. pyogenes* [40]. Focus on treating established biofilms may need to shift from antibiotic to non-antibiotic therapy to effectively eradicate established biofilms. Among these modalities, were physical (laser-produced pressure waves, pulsed ultrasound) and chemical methods such as using of surfactants, which may soon replace traditional surgical techniques [42].

Researchers have also demonstrated ENT biofilm (ear, nose, and throat) prevention using many various techniques that have been shown to disrupt established biofilms, including the use of probiotics and surfactants [43]. New strategies to inhibit biofilm formation or find modern class of antibiotics are the only options left for researchers worldwide. Using plant products to inhibit biofilm and increase antibiotic efficacy may prove to be a viable alternative. Phytochemicals are known to have antimicrobial and antibiofilm activity against the broad spectrum of pathogenic organisms. Their multitargeted mechanism may play an important role in reducing the emergence of drug resistance [44]. Workers have reported that phytochemicals also increases susceptibility of the organism to various antibiotics [45, 46, 47 and 48]. Our study has evaluated effect of *T.indica* on biofilm formed by *S. pyogenes* isolated from patients. In the present study, anti biofilm effect of plant extracts against *Streptococcus pyogenes* has been studied adopting biofilm inhibition spectrophotometric assay.

The plant extracts tested inhibited biofilm as dose dependent manner. *T. indica* methanol extract shows maximum inhibition when compared to other extracts 2mg/ml dislodged the biofilm biomass by SP1-94% , SP2-91%, SP3- 87%, SP4-95%, SP5-94% and MTCC 1924 – 88%. The present data corroborates well with the findings of Vattem *et al.*, [49] who reported the inhibition of violacein pigment production in *C. violaceum* by certain spices such as thyme, ginger and turmeric to the level of about 41%. Similarly, the extracts from Vanilla planifolia confer a 98% reduction in violacein production [50]. This evidence that biofilm formation was possibly inhibited at the beginning of the attachment stage itself. This is in agreement with previously published report of You *et al*. [51], wherein, the extract of *Streptomyces albus* significantly inhibited biofilm formation of Vibrio sp. by preventing their initial adherence. Therefore, it is envisaged that the active principle present in *T.indica* extract could possibly

interfere with the expressions of genes responsible for initial attachment, which facilitates the observed reduction in the biofilm formation. It has been already reported that production of EPS is essential for the development of biofilm architecture and maturation [52]. It was reported that over production of EPS leads to alterations in biofilm architecturethat correlate with an increased resistance of cells to osmotic and oxidative stresses as well as killing by biocides such as chlorine [53]. Thus, if *T. indica* loosens the architecture of the matured biofilm by inhibiting the secretionof EPS, it is possible that the resistance of the sessile cells toantibiotics would be reduced.

Conclusion

In conclusion, the methanolic extract obtained from *Tylophora indica* efficiently inhibited biofilm formation in the tested bacterial pathogen. Furthermore, *T. indica* leaves are well known for its medicinal properties and being used regularly as one of the food ingredients in South Indian dishes from time immemorial. Though, it is known for its bioactive potentials such as antioxidant, antibacterial, anti-inflammatory, antispasmodic, diuretic properties, the present study appends further note on its QSI and antibiofilm potentials.

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

MG and SP designed the work, RM, SP and MG executed the work, MG, RM and DJ drafted the manuscript.

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