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SYNERGISTIC ANTI-BIOFILM ACTIVITY OF MEDICINAL PLANTS AGAINST BIOFILM FORMING *STREPTOCOCCUS PYOGENES* FROM PHARYNGITIS PATIENTS

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ABSTRACT

Upper respiratory tracts infections caused by *Streptococcus pyogenes* is one among the predominant infections. Biofilm formation is the major cause of bacterial pathogenesis and antibiotic resistance. Medicinal plants are widely studied for their inhibitory effect against biofilm forming *S. pyogenes*. The present study involved the antibiofilm activity of synergistic methanolic extracts of *Leucas aspera*, *Vitex negundo* and *Gymnema sylvestre* (LVG) against *S. pyogenes* isolated from pharyngitis patients. Minimum Inhibitory Concentration (MIC), Biofilm Inhibitory Concentration (BIC), antibacterial activity, growth curve, light microscopy and GC-MS analysis were performed. Results revealed significant (p<0.05) biofilm inhibition percentage (94.2%) against *S. pyogenes* at sub-MIC. The GC-MS analysis revealed the presence of thirty tree compounds among which n-hexadecanoic acid, phytol isomer, and octadec-9-Enoic acid were predominant. Microscopic analysis showed dose dependant reduction in the biofilm architecture as compared to control. To our knowledge this study is the first report on the synergistic antibiofilm activity of *Leucas aspera*, *Vitex negundo* and *Gymnema sylvestre* emphasizing on its importance as an alternative medicine. However, it remains for the further study to elucidate the active principles of the combined plant extracts against biofilm forming *S. pyogenes*.

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INTRODUCTION

Upper respiratory tract infection is a common illness caused by *Streptococcus sp* [1], with varying symptoms ranging from runny nose, sore throat, cough, pharyngitis, breathing difficulty, and fatigue. In recent years pathogenic microorganisms have developed multiple drug resistance [2]. These pathogens have unique character of surviving in environment with biotic and abiotic stresses by forming clusters in which bacterial cells adhere with one another [3]. This cluster is initiated by a single or a multispecies community of bacterial species called biofilm, a phenomena that protects pathogens from the environmental stress and antibiotics [4]. The emergence of antibiotic resistant bacteria has become exponential to the development of novel antibiotics used in treatment [5]. Although, antibiotics show effective inhibition against most of the pathogens, the fact that the microbes generate resistance against frequent administration of antibiotics cannot be denied. Plant compound aid in treating microbial infection effectively without the risk of developing drug resistance. Medicinal plant rich with unique phytochemical substances has become the focus of research for an alternative medicine in the treatment against *S. pyogenes* infections [6]. In the last few decades the indigenous medicines have been used as new therapeutic agents in prevention and treatment of diseases and infections [7]. A variety of plants such as *Mikania glomerata* [8], *Psidium guajava* L[9], *Syzygium aromaticum* (L) [10], *Allium sativum* L [11], *Zingiber officinale* Roscoe [12], *Cymbopogon citratus* (DC) Stapf [13], *Mentha piperita* L [14], and *Baccharis trimera* (Less.) [15], *Piper longum* L. and *Piper nigrum* L. [6], *Leucas aspera* L. and *Vitex negundo* [16], have been reported to exhibit significant inhibition against different microbial communities. However, the research on the synergism of the medicinal plants is limited and less reported [17, 18, and 19]. Synergistic effect of medicinal plants surpasses the individual efficacy of a phyto compounds without the risk of developing resistance among pathogens [20]. *Leucas aspera* (Lamiaceae), *Vitex negundo* (Verbinaceae) and *Gymnema sylvestre* (Ascelpiadaceae) are indigenous medicinal plants that are reported for their medicinal properties such as anti-inflammatory, hepato protective, analgesic activity and are used in the treatment of cough, cold, sore throat, diabetes [21, 22, and 23]. The present study evaluates the *in vitro* synergism of three plants *Leucas aspera*, *Vitex negundo* and *Gymnema sylvestre* (LVG) extracts for its antibiofilm activity against the biofilm forming *Streptococcus pyogenes*.

MATERIALS AND METHODS

Plant collection and solvent extraction

The leaves of *Leucas aspera*, *Vitex negundo and Gymnema sylvestre* were collected from Coimbatore and Kolli Hills, Namakkal, Tamil Nadu, India. 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 leaves were washed, air dried and powdered using the blender. About 5 g of the each dried powder was soaked in 50 ml (1:10) of methanol for seven days. After soaking, the methanol extracts were collected and dried at 55 °C for 1 hour using rotary vacuum evaporator (Buchi Type, India). After vacuum evaporation the plant extracts were mixed in equal ratio (1:1:1 w/w) and resuspended in Di-Methyl Sulphoxide (DMSO). The extract was stored at 4 °C for further analysis.

Bacterial strain and culture conditions

About 30 throat swab samples were obtained 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* was isolated from the throat swab samples using Streptococcus Selection Agar (SSA) (Himedia, India). All the isolates were tested for their biofilm forming characteristics by observing the slime formation in routine media and β-haemolysis using Blood Agar (Himedia, India). For routine propagation, all isolates were cultivated 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.

GC–MS Analysis

Bioactive compound analysis of LVG extract was determined by GS-MS analysis using Shimadzu Gas chromatography (Model: QP2010 Plus, Japan), consisting of a 30 m× 0.25 mm RTX-5MS low bleed column with a thickness of 0.25 µm. The carrier gas was helium maintained at a column flow of 1.0 ml/min (at a pressure of 53.5 kPa). 1 µl of sample from a 1 mg/ml stocks was injected by split injection (1:20) at 260°C. The column temperature was maintained at 50 °C/3 min followed by temperature programming at 10 °C/min to 150 °C / 8 min. The temperature was further increased to 250 °C/ 2 min at the rate of 8 °C/min, and finally to 280 °C/5 min at a rate of 10 °C/min. The Mass Spectrometric Detector (MSD) was used in the ACQ Scan mode with an m/z ranging from 40-600 for the sample. Screening of volatile and semi volatile compounds was performed using automatic screening software. The spectrum of the unknown components was compared with the spectrum of known components available in Wiley Online Library (Wiley08), NIST08 library. The name of phyto compound, molecular weight, and structure of the sample were determined.

Antibacterial activity assays

Determination of Minimal Inhibitory Concentration (MIC)

The p-iodonitrotetrazolium violet (INT) micro plate assay was used to determine the MIC [24]. Briefly, 100 µl of sterile medium was aliquoted into 96 well microtitre plate. 100 µl of the prepared plant extract at different concentration (8 - 0.0625 mg/ml) was incorporated into the appropriate wells. Then, 10 µl of *S. pyogenes* MTCC 1924 and isolates (1.0 x 10^5 CFU/ml) were added to the wells. The plates were sealed with aluminum foil and incubated at 37 ^{o}C for 24 h. After, incubation, the MIC of the plant extract was determined. To visualize the bacterial growth, 40 µl of INT (0.04 mg/ml) was added to each well and the plates were incubated at room temperature for 3 h. MIC was determined by the visualizing the well that exhibited red color.

Agar well diffusion assay

The antibacterial activity of LVG 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 (CLSI) [25]. Briefly, 100 µl of test bacterial suspensions with the cell density equivalent to 0.5 McFarland standard units (1 x 10^5 CFU/ml) were uniformly spread over the surface of MHA plates. The plates were kept undisturbed for 10 min for the absorption of excess moisture. Then, different volumes of LVG extract (25 µl and 50 µl) prepared from 1 mg/ml stock solution were added to wells and the plates were incubated at 37 °C. DMSO without plant extract was used as negative control and streptomycin (0.03 mg/ml) was used as positive control. The zone of inhibition was measured after 24 h.

Growth curve analysis

Growth curve analysis was performed according to Packiavathy et al. [26]. Briefly, 1 % of overnight test pathogens (0.5 OD at 600 nm) were inoculated in 50 ml of LB broth separately, supplemented with 2 mg/ml (sub MIC) of LVG extract. The flasks were incubated at 37 °C with 170 rpm agitation in a rotatory shaker (Orbitek – LT, India). Cell density was measured using UV-visible spectrophotometer (Shimadzu UV-3600 Plus, Japan) at interval of one hour for 12 h.

Biofilm biomass quantification assay

The effect of plant extract on the biofilm formation of test pathogen was determined by quantifying the biofilm biomass through MTP assay [27]. Overnight culture of *S. pyogenes* was incubated on 24 well microtitre plate containing 1 ml of Todd-Hewitt Broth (THB) with LVG extract at different concentration (1-0.0625 mg/ml). Aliquots without the plant extracts were used as control, plates were incubated without agitation at 37 °C for 24 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 resuspended in 1 ml of 80 % ethanol and the absorbance was observed at 620 nm. The amount of stain absorbed is directly proportional to the biomass of biofilm on the surface. Thus, higher the absorbance reading, the greater the cell biomass.

***In situ* visualization of biofilm Light microscopic analysis**

Visualization of biofilm by light microscopy was performed according to Nithya et al. [28]. Briefly, the biofilm were allowed to grow on glass pieces (1x1 cm^2) placed in 24-well polystyrene plates supplemented with solvent extracts of LVG extract at different concentration (8-0.0625 mg/ml) and incubated for 24 h at 37 °C. The slides were stained using 0.4 % crystal violet and observed under light microscopy at magnification of ×400. Visible biofilms were documented with an attached digital camera (Nikon eclipse Model: E200).

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

Spectral Analysis

GC-MS analysis was used to identify the phytoconstituents of volatile matter, long chain, branched chain hydrocarbons, alcohols acids, esters etc. The GC-MS analysis of combined plant extracts revealed thirty three compounds. The identification of the phytochemicals was confirmed based on the peak area, height percentage and chemical abstracts service (CAS) number. The active principles with retention time, peak area and CAS number are presented in (Table 1 and Fig 1). Based on the height (%), n-hexadecanoic acid (21.08%), phytol isomer (15.45%), octadec-9-enoic acids (17.54%) were observed in large quantities. The phytochemicals identified through GC-MS analysis showed many biologically active compounds relevant to this study.

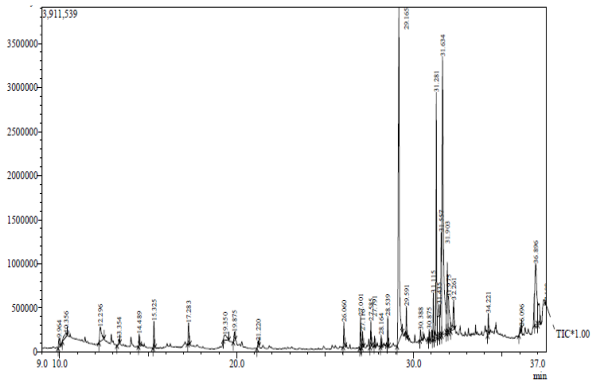

Fig.1- Chromatogram showing the GC-MS spectrum of LVG methanol extract.

Table 1- Phytochemical composition of LVG methanol extract determined using GC-MS analysis.

S. No	Compound name	Area percentage %	Chemical Formula	Height %	CAS No
1	1-Hexadecene	0.58	$C_{16}H_{32}$	0.57	629-73-2
2	Caryophyllene	0.72	$C_{15}H_{24}$	0.23	87-44-5
3	Phenol, 2,4-Bis(1,1-Dimethylethyl)-	1.72	$C_{14}H_{22}$	0.93	96-76-4
4	1-Nonadecene	0.15	$C_{19}H_{38}$	0.28	18435-45-5
5	2-Hexadecen-1-Ol, 3,7,11,15-Tetramethyl	0.40	$C_{20}H_{40}$	0.76	150-86-7
6	2-Pentadecanone, 6,10,14-trimethyl	1.08	$C_{18}H_{36}O$	1.67	502-69-2
7	(1e)-1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1-penten-3-one	1.03	$C_{14}H_{22}O$	1.32	127-43-5
8	Neophytadiene	0.94	$C_{20}H_{38}$	0.33	504-96-1
9	7a-Isopropenyl-4,5-dimethyloctahydroindene-4-carboxylic acid	0.50	$C_{15}H_{24}O_2$	0.54	0-00-0
10	Hexadecanoic acid, methyl ester	0.33	$C_{17}H_{34}O_2$	0.36	112-39-0
11	n-Hexadecanoic acid	1.04	$C_{16}H_{32}O_2$	1.53	57-10-3
12	n-Nonadecanol-1	1.29	$C_{19}H_{40}O$	2.00	1454-84-8
13	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)-	0.67	$C_{20}H_{34}O_2$	1.00	7220-78-2
14	9-Octadecenoic Acid (Z)-, Methyl Ester	1.02	$C_{19}H_{36}O_2$	1.62	112-62-9
15	Phytol	0.34	$C_{20}H_{40}O$	0.62	150-86-7
16	Stearic acid, methyl ester	0.43	$C_{19}H_{38}O_2$	0.73	112-61-8
17	9,12-Octadecadienoic acid (Z,Z)-	1.02	$C_{18}H_{32}O_2$	1.84	60-33-3
18	2,3-Bis[(9E)-9-octadecenoyloxy]propyl (9E)-9-octadecenoate	21.28	$C_{57}H_{104}O_6$	21.08	537-39-3
19	Octadecanoic acid	0.96	$C_{18}H_{36}O_2$	1.79	57-11-4
20	17-Octadecen-14-yn-1-ol	0.48	$C_{18}H_{32}O$	0.64	18202-28-3
21	1-Heptacosanol	0.37	$C_{27}H_{56}O$	0.70	2004-39-9
22	5-Methyl-5-(4,8,12-trimethyltridecyl)dihydro-2(3H)-furanone	1.96	$C_{21}H_{40}O_2$	2.90	96168-15-9
23	Phytol isomer	10.17	$C_{29}H_{50}O$	15.45	83-46-5
24	1-(Phenylsulfanyl)-4-(2,2,6-Trimethylbicyclo[4.1.0]Hept-1-Yl)-2-Butanone.	2.91	$C_{20}H_{28}OS$	2.01	0-00-0
25	Heptadecane.	5.72	$C_{17}H_{36}$	6.49	629-78-7
26	Octadec-9-Enoic Acid	23.47	$C_5H_{10}O_4$	17.54	106-61-6
27	Acetin, mono	4.25	$C_5H_{10}O_4$	4.42	26446-35-5
28	2-Methoxy-4-Vinylphenol	2.74	$C_9H_{10}O_2$	2.19	7786-61-0
29	1-Octen-3-yl-acetate	2.12	$C_{10}H_{18}O_2$	1.88	2442-10-6
30	n-Pentadecanol	0.80	$C_{15}H_{32}O$	1.34	629-76-5
31	Bicyclo[2.2.2]octane, 1,2,3,6-tetramethyl-	0.21	$C_{12}H_{22}$	0.16	62338-45-8
32	2-[(4-Methylphenyl)sulfonyl]-2,3-dihydro-4(1H)-isoquinolinone	7.02	$C_{16}H_{15}NO_3S$	3.87	125159-93-5
33	Tetrapentacontane	2.28	$C_{54}H_{110}$	1.20	5856-66-6

Determination of Minimal Inhibitory Concentration (MIC)

A total of 15 isolates of *Streptococcus sp*. were obtained from the throat swab samples. Among the 15 isolates, 6 isolates were identified as *S. pyogenes*, based on their characteristics growth on the streptococcus selection agar and beta haemolytic activity. Further, it was observed that 2 isolates among six *S. pyogenes* strain namely SP-1 and SP-2 were moderate to strong biofilm formers as the strains produced slime colonies on the blood agar plates. Further, MIC was determined for the LVG extract against the test pathogens. The MIC of LVG extract is represented in Fig 2. The MIC of *S. pyogenes* MTCC 1924 and isolates (SP-1 and SP-2) was recorded from 1-4 mg/ml.

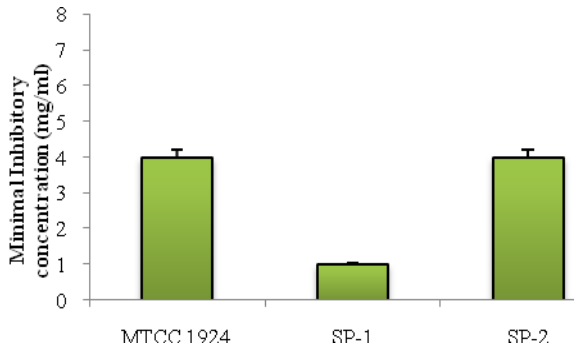

Fig.2-Minimal Inhibitory Concentration of LVG extract against *S. pyogenes.*

Determination of agar well diffusion assay for extracts

LVG extract was studied for the antibacterial activity using agar well diffusion method. Table 2 represents the antibacterial activity result of LVG against different strains of *S. pyogenes*. The combination of extracts exhibited antibacterial activity against the tested bacteria with inhibition zones ranging from 22-24 mm (in diameter), against SP-1, SP-2 and MTCC 1924. The results of the antibacterial susceptibility assay was compared with positive control Streptomycin (Table 2).

Table 2. Antibacterial activity of LVG extracts against test pathogens.

S. No	Microorganism	LVG Methanol extract	
		25µl	50µl
1.	MTCC 1924	+	++
2.	SP-1	+++	+++
3.	SP-2	+++	+++
4.	Streptomycin (Positive Control)	+++	+++
5.	DMSO (Negative Control)	-	-

+; weak inhibition, ++; medium inhibition, +++; strong inhibition, - ; no inhibition.

Growth curve analysis

The LVG extract was analyzed for their ability to inhibit *S. pyogenes* growth at a sub MIC concentration (2 mg/ml) using the growth curve analysis. The results revealed that the growth rate as well as the maximum cell densities of LVG extract treated samples did not differ as compared to the control (Fig 3).

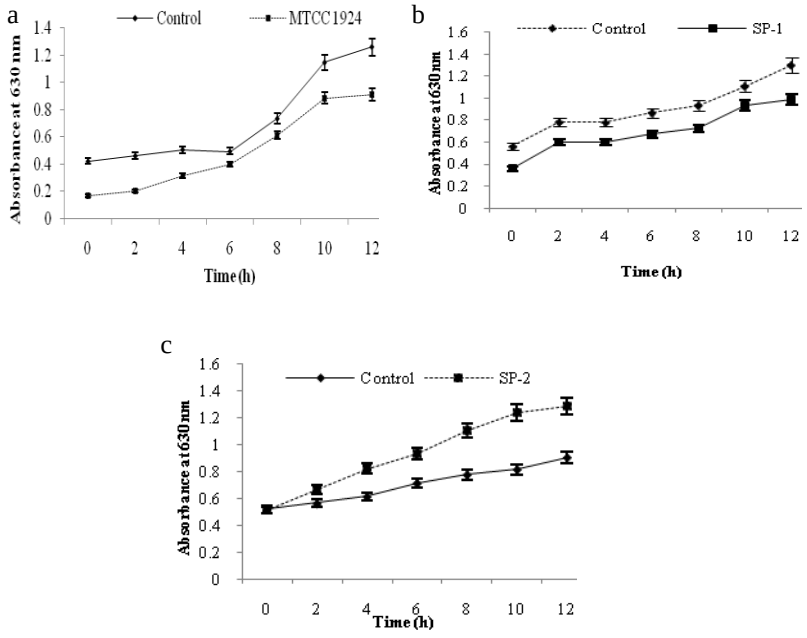

Fig.3-Effect of LVG extract, on the growth of *S. pyogenes* a) MTCC 1924; b) SP-1; c) SP-2 respectively.

Biofilm biomass quantification assay

This assay was performed to determine the concentration at which LVG extract showed significant biofilm inhibition. It was observed that the biofilm inhibitory concentration of the combined plant extracts were dose dependent and highest biofilm inhibition percentage (94.2%) was observed at 1 mg/ml concentration against SP-2 (Fig 4). However the biofilm inhibition percentage decreased with the decrease in the concentration of plant extracts.

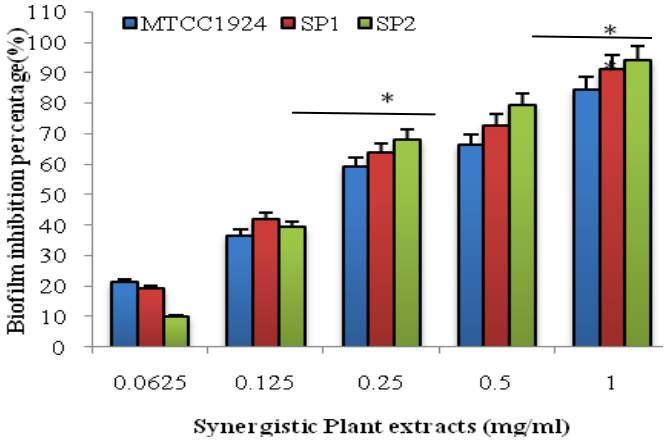

Fig.4- Biofilm inhibition percentage of LVG methanol extract at different concentrations against test isolates. Values are expressed as percentage inhibition. Values indicated with *; $p<0.05$ and **; $p<0.01$ are significantly different from control.

***In situ* light microscopic observation of biofilm**

The LVG extract showed significant ($p<0.05$) anti-biofilm activity at minimal concentration as even as 0.0625 mg/ml. Interestingly, our results showed a dose dependant inhibition of biofilm by synergistic extracts. It was observed that the biofilm architecture was well established in control (untreated samples) as compared to test sample (Fig 5). Further biofilm inhibition was evident from distortion of biofilm architecture in the treated samples which was proportional to the concentration of LVG extract.

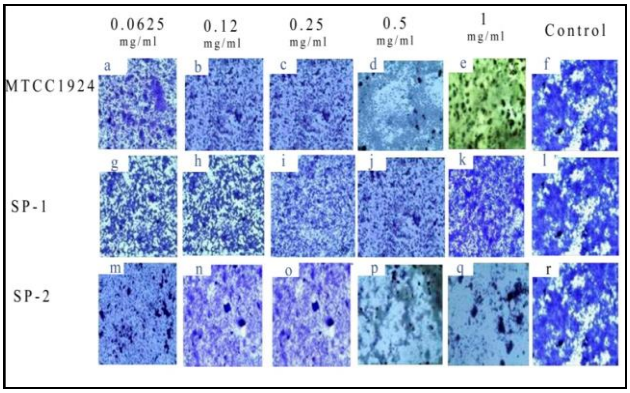

Fig 5 - Light microscopic images of a-f) *S. pyogenes* MTCC 1924; g-l) *S. pyogenes* SP-1; and m-r) *S. pyogenes* SP-2 respectively in the presence and/or absence of LVG extract. All images were observed at ×400 magnification using Nikon eclipse E-200 series Microscope.

DISCUSSION

Biofilms play an important role in most of the human infections caused by bacteria [29]. The biofilms of *S. pyogenes* have been observed in skin, pharyngitis, necrotizing fasciitis and root canal infections [30, 31, 32, and 6]. Biofilm formation leads to increased resistance against antimicrobial treatments and host defenses which favor the growth of microorganisms in suboptimal environments [33]. Generally, higher concentration of the antibiotics are required to kill bacteria in the biofilm phase than their planktonic counterparts [34]. Phytochemicals are used in the treatment of infections caused by bacterial biofilms as the compounds suppress the expression of genes responsible for pathogenesis by interfering with bacterial biofilm formation [35].

In our previous study, it was substantiated that, methanol extracts of *L. aspera* (LA) and *V. negundo* (VN) interfered with the biofilm formation of *S. pyogenes* at a MIC ranging between 2-4 mg/ml, but our present study on the LVG extract revealed an MIC of 1-4 mg/ml against test pathogens, and so it could be hypothesized that the synergistic extract exhibited significantly higher activity than the respective individual plant extracts. Antibacterial activity by agar well diffusion method (Table 2) showed high inhibition of test bacteria with zone ranging from 22-24 mm. Growth curve analysis revealed that, at the tested sub- MIC of LVG the growth of the test pathogens did not differ significantly with the respective control. The LVG extract significantly ($p < 0.05$) interfered with the

biofilm formation at sub-MIC (0.625-1 mg/ml) and inhibited the initial adherence on the substratum which was evident from our results (Fig. 4 and 5). In addition, our previous studies on the individual plant extract of *L. aspera* and *V. negundo* elucidated that the antibiofilm activity of extracts individually inhibited the biofilms at the concentration ranging from 0.5 to 2 mg/ml [16].

Specific information on the phyto compounds could be obtained by quantitative analysis of plant extracts using gas-chromatography coupled with mass spectrometry (GC-MS) [36]. In the present study, the GC-MS analysis of LVG plant extracts revealed the presence of thirty three compounds among which n-hexadecanoic acid (21.08%), phytol isomer (15.45%), octadec-9-enoic acid (17.54%) were predominant. n-hexadecanoic acid have been reported for its biological activities such as antioxidant, hypocholesterlemic, nematicide, pesticide and lubricant [37]. Inoue *et al.,* [38] reported that Phytol isomer showed anti-bacterial activities against group A pathogens causing damage to cell membranes as a result of leakage of potassium ions from bacterial cells. Phytol and phenol, 2, 4-bis (1-phenylethyl) have been studied for medicinal properties [39]. 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. The main components present the current study were n-hexadecanoic acid, octadec-9-enoic acid and phytol isomer. These compounds were found to have potential antioxidant and anticancer activities. Kadiyala Gopia et al. [40] reported the biological significance of the compounds such as stearic acid and linoleic acids in *L. aspera.* In addition, Linoleic acid was reported for antibacterial activity against different pathogens [41]. The present study has revealed the presence of poly unsaturated fatty acids in the LVG extract. Poly unsaturated fatty acids synthesized by plants are reported to influence the metabolic, immunological functions of host there by serving as an alternative medicine against bacterial pathogen [42]. The study conducted by Praveen kumar et al. [37], revealed the presence of medically important compounds such as phytol, stearic acid, linoleic acid, and hexadecanoic acid in *V. negundo*. Moreover, the presence of antibacterial compounds in *G. sylvestre* was elucidated by Parimala Devi et al. [43]. These reports are in accordance with the result of our study which evidently showed the effective antibiofilm activity of the LVG extract than their individual counterparts. These characteristics may be attributed to the enhanced and combined efficacy of the phyto compounds leading to a significant synergistic activity against *S. pyogenes*.

CONCLUSION

Phytochemicals continue to play a vital role in human health. Plants are a rich source of secondary metabolites with interesting biological activities. These secondary metabolites have different structural configuration and properties that can be exploited as an alternative medicine to treat different diseases. The presence of medically important phyto chemicals and anti-biofilm activity of the combination of medicinal plants in the current study revealed the significance of synergistic activity of plant extracts against *S. pyogenes*. Further studies are needed to determine the mechanism involved in the anti-biofilm property of the synergistic extracts at molecular level and evaluate their biological functions.

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Competing Interests

The authors declare no conflict of interest.

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