ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 9, Suppl. 1, 2016

Online - 2455-3891
Print - 0974-2441

Research Article

IN VITRO ANTIBIOFILM ACTIVITY OF *GYMNEMA SYLVESTRE* EXTRACT AGAINST BIOFILM FORMING *STREPTOCOCCUS PYOGENES* FROM UPPER RESPIRATORY TRACT

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

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

Received: 02 April 2016, Revised* and *Accepted: 09 April 2016

ABSTRACT

Objective: This study was aimed to evaluate the antibiofilm activity of *Gymnema sylvestre* solvent extracts against the biofilm forming *Streptococcus pyogenes* strains SP-1 and SP-2 isolated from pharyngitis patients.

Methods: The study involved the determination of minimum inhibitory concentration (MIC) and biofilm inhibitory concentration using microtiter plate method, growth curve analysis using spectrophotometry, antibacterial activity using agar well-diffusion method and light microscopic analysis of *S. pyogenes* biofilms treated with the plant extracts.

Results: Biofilm inhibition assay was showed that the *G. sylvestre* methanol and ethyl acetate extracts inhibited biofilms at sub-MIC which was also confirmed via light microscopy. Minimal inhibition concentration against the tested pathogens was observed at 4 mg/ml for both methanol and ethyl acetate extracts. The antibacterial and growth curve analysis showed no significant inhibition at the concentration below MIC.

Conclusion: This study is an attempt to highlight *G. sylvestre* as a novel antibiofilm source, as the plant is well known for its biologically active components. Identification and can develop a scientific base for the use in modern medicine.

Keywords: Antibiofilm, Biofilm inhibitory concentration, *Gymnema sylvestre, Streptococcus pyogenes.*

INTRODUCTION

Upper respiratory tract infections are the most common human infection, often caused by viruses and bacteria. *Streptococcus pyogenes* causes a wide variety of diseases such as runny nose, sore throat, cough, lethargy, skin infection, and upper throat tract infections [1]. The increasing phenomenon of antibiotic resistance among micro-organisms is attributed its indiscriminate use in the modern medical treatments [2]. *S. pyogenes* is characterized by multiple drug resistance leading to increasing global health threat [3]. Biofilms are formed by clumping of free living (planktonic) bacterial cells by adhering to a solid substratum in a common mucilaginous matrix [4]. Such cluster initiated biofilm forming is considered to be an important virulence potential which increases the resistance of the pathogen to the common antimicrobial treatment. Biofilm forming *S. pyogenes* has become one of the most important human pathogens associated with extensive human morbidity worldwide [5-7]. Plant biological active compounds used to be cure diseases and illnesses from ancient times [8]. Recently, many studies have been attempted to identify anti-microbial agents from natural products [9-11].

Traditional medicine currently occupies an important place in the treatment to disease. In traditional medicine, plants derived phytochemicals are used to protective mechanisms against microbial infections. *Gymnema sylvestre* (Asclepiadaceae) is one among the commonly used traditional medicines globally. The plant is a large tropical liana, native to central and Western India, tropical Africa and Australia [12]. *G. sylvestre* is known for its significant anti-diabetic properties. Gymnemic acid, the active ingredient of this plant, is extracted from leaves and used widely as an anti-diabetic [13], anti-sweetener [14], and as anti-hypercholesterolemia [15]. It is also used in the treatment diuretic, cough and enteric problems [16]. This study evaluates the medical property of *G. sylvestre* in a novel perspective, in which the antibiofilm property against the biofilm forming *S. pyogenes* is determined to identify an alternative natural source for the biofilm forming pathogens.

METHODS

Plant collection

The *G. sylvestre* leaves were collected from Kolli-hills, Tamil Nadu and authenticated (Voucher No. BSI/SRC/5/23/2016/Tech/132) The samples were taxonomically identified at Botanical Survey of India (BSI), Southern Circle, and Tamil Nadu Agricultural University (TNAU) Coimbatore.

Extraction procedure

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 and ethyl acetate (1:4) for 7 days with periodic soaking. The extracts were then filtered using Whatman No. 1 filter paper, and the filtrate was dried at 55°C for 1 hr using rotary vacuum evaporator (Buchi Type, India). After vacuum evaporation, the plant extracts were re-suspended in 80% dimethyl sulfoxide (Himedia, India) The extract was stored at 4°C for further analysis [17].

Test organisms

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 a reference strain and two biofilm forming isolates SP-1 and SP-2, obtained from throat swabs collected from pharyngitis patients were used in this study. All isolates were cultured on Todd Hewitt Broth (Himedia, India) for routine use [18].

Antibacterial activity assays

Minimum inhibitory concentration (MIC) of G. sylvestre extracts

MIC was determined by the broth dilution method [19]. Two milliliters of stock plant extract at different concentration (8-0.0625 mg/ml) was incorporated into the appropriate wells. Then, 10 µl of *S. pyogenes* MTCC 1924 (1.0×10^6 CFU/ml) was added to the wells. The plates were sealed with aluminum foil and incubated at 37°C for 24 hrs.

After, incubation, the MIC of the *G. sylvestre* methanol and ethyl acetate extract was determined. To visualize the bacterial growth, 40 µl of The p-iodonitrotetrazolium violet (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 *G. sylvestre* methanol and ethyl acetate extracts that showed complete reduction in the color indicating the inhibition of microbial growth and cell density was measured using ultraviolet (UV)-visible spectrophotometer (Shimadzu UV-3600 Plus, Japan).

Agar well-diffusion method

The antibacterial activity of *G. sylvestre* methanol and ethyl acetate extracts was determined by agar well-diffusion method [20]. Briefly, 100 µl of test bacterial suspensions with the cell density equivalent to 0.5 McFarland standard units (1×10^5 CFU/ml) were uniformly spread over the surface of Mueller-Hinton Agar plates. A cork borer (6 mm diameter) used to punch wells in solidified medium. The stock plant extracts were prepared in 2 mg/ml (25 µl and 50 µl) methanol, ethyl acetate final concentration of extracts. Sterile distilled water was used as negative control. The efficacy of extracts against test organisms was compared with broad spectrum antibiotic streptomycin as a positive control (30 µg/ml). The plates were incubated at 37°C for 24 hrs and the zone of inhibition was measured.

Growth curve analysis of plant extracts

According to Ponnuraj *et al*. [21], growth curve analysis was performed. Briefly, 1% of overnight test pathogens (0.5 optical density at 600 nm) was inoculated in 50 ml of Lysogeny Broth separately, supplemented with 1 mg/ml (sub-MIC) of *G. sylvestre* extracts. 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 every 1 hr interval up to 12 hrs.

Biofilm inhibition assay

Crystal violet staining of biofilm was done following the methodology described elsewhere [22] with slight modification in 24 well plate. Briefly, planktonic phase cells were aspirated and biofilm ring was washed with a continuous spray of 1X phosphate buffered saline (PBS; pH 6.8) and incubated at room temperature for 1 hr to fix the cells. Crystal violet (0.4% in isopropanol-methanol-1X PBS; 1:1:18) was poured into each well. Twenty-four well plate were incubated for 15 minutes at room temperature then washed thoroughly with 1X PBS (pH 6.8) until the buffer ran clear and gently rinsed twice deionized water. Finally, it was resuspended in 1 ml of 80% ethanol and the bacterial cells was measured at 620 nm.

Light microscopic analysis of biofilms treated with *G. sylvestre*

According to Ponnuraj *et al*. [21], visualization of biofilm by light microscopy was performed. Briefly, the biofilm was allowed to grow on glass pieces (1 cm × 1 cm) placed in 24-well polystyrene plates supplemented with solvent extracts of *G. sylvestre* at different concentration (8-0.0625 mg/ml) and incubated for 24 hrs at 37°C. The slides were stained using crystal violet and were placed on slides with biofilm pointing upward. The slides were observed under light microscopy at a 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. Student's t-test was used to determine the significance between control and test samples.

RESULTS AND DISCUSSIONS

MIC assay

The MIC of *G. sylvestre* solvent extracts is represented in Fig. 1. The methanol and ethyl extracts extract inhibited the growth of *S. pyogenes* MTCC 1924, SP-1 and SP-2 at a MIC of 1 and 4 mg/ml. Naidu *et al.,* [23] observed MIC values of methanol extract at 15.6 µg/ml against *Bacillus subtilis*, *Staphylococcus aureus* and 31.2 µg/ml against *Enterococcus faecalis*, *Micrococcus luteus*, *Streptococcus pneumoniae*, whereas chloroform extract showed low MIC against *S. aureus*, it was 62.5 µg/ml. This result correlated with the methanol extracts inhibited at 1 mg/ml.

Table 1: Antibacterial activity of *G. sylvestre* solvent extracts

Serial number	Organisms	*G. sylvestre* (µl)			
		Methanol		Ethyl acetate	
		25	50	25	50
1	*S. pyogenes* MTCC1924	++	+++	++	++
2	SP-1	++	+++	++	++
3	SP-2	+	+	+++	+++
4	Positive control (streptomycin)	+++	+++	+++	++
5	Negative control (water)	–	–	–	–

+: Weak inhibition, ++: Medium inhibition, +++: Strong inhibition, –: No inhibition, *S. pyogenes*: *Streptococcus pyogenes, G. sylvestre*: *Gymnema sylvestre*

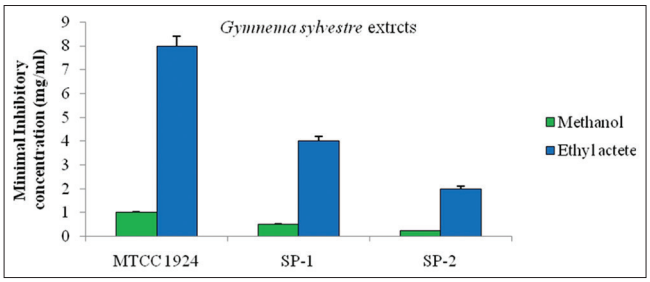

Fig. 1: Minimum Inhibitory concentration assay of two solvent extracts (0.0625-8 mg/ml) in two-fold dilution against *Streptococcus pyogenes* and clinical isolates

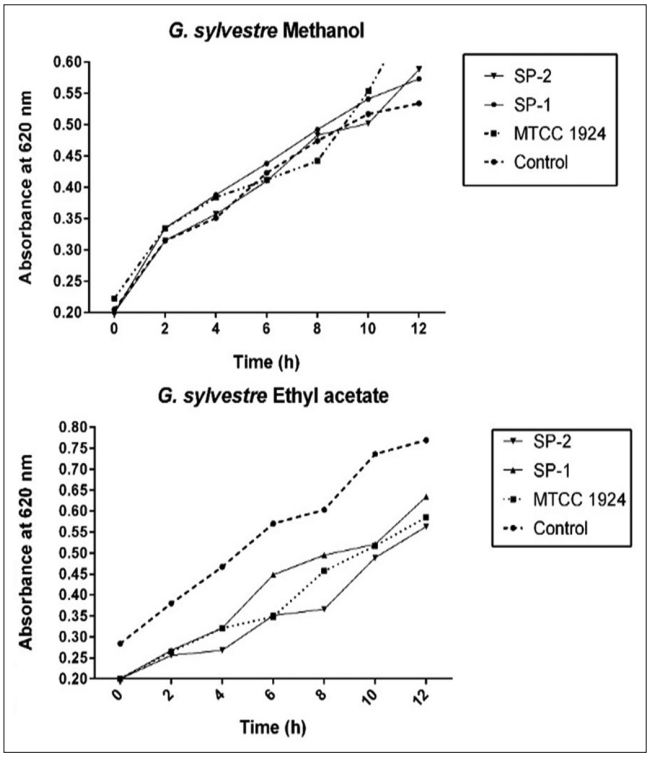

Fig. 2: Effect of *Gymnema sylvestre* extracts on the growth of *Streptococcus pyogenes* planktonic cells

Antibacterial activity

The *G. sylvestre* extracts were investigated for the antibacterial activity using agar well-diffusion method. Table 1 represents the antibacterial activity of *G. sylvestre* against the test pathogen. Exhibited antibacterial activity against the tested bacteria; among which the *G. sylvestre* showed maximum inhibition zone ranging from 22 to 24 mm in diameter, against SP-1, SP-2 and MTCC 1924 isolates in 2 mg/ml. Furthermore, the results of the antibacterial susceptibility assay were compared with antibiotic drug streptomycin as positive control (Table 1). *G. sylvestre* methanol and ethyl acetate extracts inhibited the *S. pyogenes* MTCC and isolates 22-24 mm. Dineshbabu *et al.* [17] reported that *Leucas aspera* and *Vitex negundo* solvent extracts (methanol and ethyl acetate) inhibited the test pathogens at above mic concentration of 2 mg/ml. In addition, Naidu *et al.* [23] reported that *G. sylvestre* methanol extracts showed the maximum zone of inhibition against *S. pyogenes* at a concentration of 2 mg/ml. Our results were also in agreement with [24] which is ethyl acetate extracts of the *G. sylvestre* also reported to have antibacterial effects against *Proteus vulgaris, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *S. aureus.*

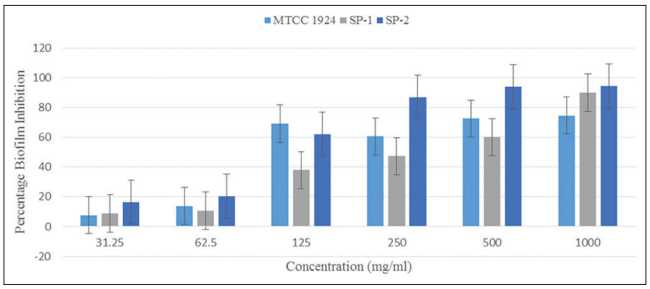

Fig. 3: Biofilm inhibition percentage of *Gymnema sylvestre* methanol extract at different concentrations against test isolates. Values are expressed as percentage inhibition

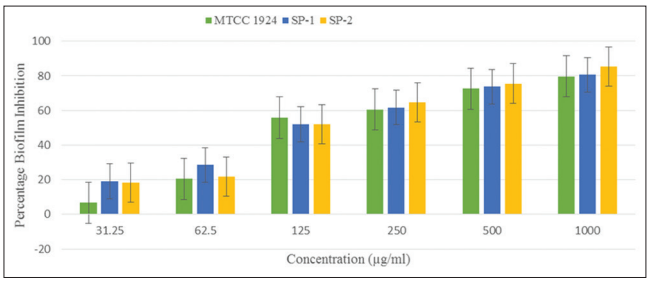

Fig. 4: Biofilm inhibition percentage of *Gymnema sylvestre* ethyl acetate extract at different concentrations against test isolates. Values are expressed as percentage inhibition

Growth curve analysis

The methanol and ethyl acetate extracts of *G. sylvestre* were analyzed for its effect on the growth of *S. pyogenes* at sub-MICs. The results showed no significant changes in the cell densities between treated and untreated cultures (Fig. 2). Prescott *et al.,* and Vlietinck *et al*. [25,26] noted that above MIC level (2.5 mg/ml) the plant extracts affected the bacterial growth. Thenmozhi *et al*. [27] also reported that at sub-MIC levels of CAB extracts (2 mg/ml) did not show any changes in growth curve analysis. Even after the addition of the extracts at biofilm inhibitory concentration (BIC) the growth of *S. pyogenes* was at the same level as that of control. This report correlated with our present study.

Biofilm biomass quantification assay

The inhibition of biofilm biomass by *G. sylvestre* solvent extracts against *S. pyogenes* isolates was determined (Figs. 3 and 4). *G. sylvestre* methanol extracts showed significant ($p \leq 0.05$) biofilm inhibition at a sub-MIC ranging from 1 to 0.0625 mg/ml. Methanol extracts of *G. sylvestre* exhibited higher inhibition compared to the ethyl acetate extracts. The percentage biofilm biomass inhibition of the methanol extract ranged from 53.0% to 96.8% and ethyl acetate extracts inhibition ranged from 50.0% to 90.0%. The results of the biofilm biomass inhibition by the *G. sylvestre* in this study correlates with our previous reports of *L. aspera* and *V. negundo* solvent extracts against the biofilm forming *S. pyogenes* [17].

Light microscopic observation

The *G. sylvestre* methanol and ethyl acetate extracts showed antibiofilm activity at a concentration of 1 mg/ml to 0.0625 mg/ml. It was also observed that the biofilm architecture was well established in control (untreated samples) as compared to test sample (Fig. 5).

The results of this study on the effect of *G. sylvestre* solvent extracts on the biofilm inhibition against *S. pyogenes* at sub-MIC (1 mg/ml) corroborate with our previous study on the antibiofilm property of

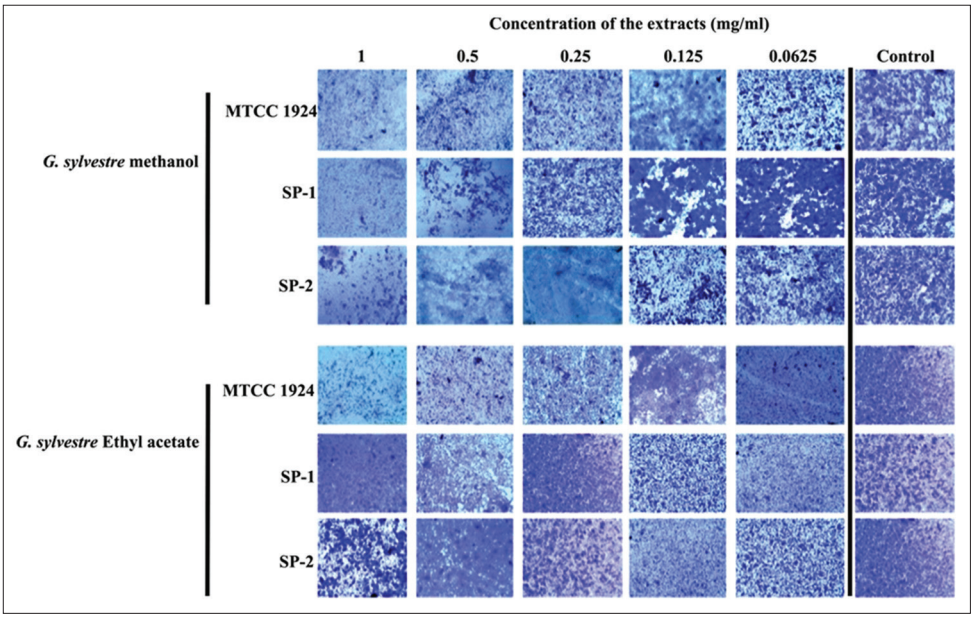

Fig. 5: Light microscopic images (×400; scale bar=20 µm) of *Streptococcus pyogenes* biofilms grown in the absence and/or presence of *Gymnema sylvestre* solvent extracts

L. aspera and *V. negundo* extracts against biofilm forming *S. pyogenes* in which *L. aspera* and *V. negundo* solvent extracts (2 mg/ml; sub-MIC) treated samples developed poor biofilm growth as compared control samples [17]. This study of biofilm inhibition concentration results were also in accordance with [28,29] in which the methanol and ethyl acetate extracts of *Piper longum* and *Piper nigrum* were reported to affect the cell-cell communication at sub-MIC of 2 mg/ml. About 50% biofilm inhibition and formation of microcolonies of *S. pyogenes* isolates at a minimal concentration of 0.5 mg/ml. According to Limsuwan and Voravuthikunchai, [30] the architecture of *S. pyogenes* microcolonies that were treated with CAB extracts was looser because the CAB extracts showed a reduction in hydrophobicity at BIC or sub-BICs. As the biofilm inhibition results in the present study as confirmed from the light microscopy analysis were similar to the previous reports it could be hypothesized that the solvent extracts of *G. sylvestre* were efficient in affecting the biofilm formation as compared to the untreated control.

CONCLUSION

G. sylvestre is a well establish medicinal plant owing to its rich phytochemicals and secondary metabolites. It is widely used as alternative medicine in treatment for human health. The present preliminary study has revealed the biofilm inhibition potential of *G. sylvestre* against the biofilm forming *S. pyogenes* at minimum concentrations. Further studies are needed to determine the mechanism involved in the antibiofilm property of the *G. sylvestre* extracts at the molecular level and evaluate their biological functions for their safety in developing an herbal product.

ACKNOWLEDGMENT

The authors thank Karpagam Academy of higher Education for providing the infrastructure facility to conduct the research work.

REFERENCES

1. Cunningham MW. Pathogenesis of group a streptococcal infections. Clin Microbiol Rev 2000;13(3):470-511.
2. Usha PT, Jose S, Nisha AU. Antimicrobial drug resistance - A global concern. Vet World 2010;3(3):138-9.
3. Aiyegoro OA, Afolayan AJ, Okoh AI. Synergistic interaction of *Helichrysum pedunculatum* leaf extracts with antibiotics against wound infection associated bacteria. Biol Res 2009;42(3):327-38.
4. O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. Annu Rev Microbiol 2000;54:49-79.
5. Hoyle BD, Costerton JW. Bacterial resistance to antibiotics: The role of biofilms. In: Progress in Drug Research/Fortschritte der Arzneimittelforschung/Progrès des Recherches Pharmaceutiques. Basel: Birkhäuser; 1991. p. 91-105.
6. Bisno AL, Gerber MA, Gwaltney JM Jr, Kaplan EL, Schwartz RH. Practice guidelines for the diagnosis and management of Group A streptococcal pharyngitis. Infectious Diseases Society of America. Clin Infect Dis 2002;35(2):113-25.
7. Baldassarri L, Creti R, Recchia S, Imperi M, Facinelli B, Giovanetti E, *et al.* Therapeutic failures of antibiotics used to treat macrolide-susceptible *Streptococcus pyogenes* infections may be due to biofilm formation. J Clin Microbiol 2006;44(8):2721-7.
8. Essawi T, Srour M. Screening of some Palestinian medicinal plants for antibacterial activity. J Ethnopharmacol 2000;70(3):343-9.
9. He J, Chen L, Heber D, Shi W, Lu QY. Antibacterial compounds from *Glycyrrhiza uralensis*. J Nat Prod 2006;69(1):121-4.
10. Islam B, Khan SN, Haque I, Alam M, Mushfiq M, Khan AU. Novel anti-adherence activity of mulberry leaves: Inhibition of *Streptococcus mutans* biofilm by 1-deoxynojirimycin isolated from *Morus alba.* J Antimicrob Chemother 2008;62(4):751-7.
11. Koo H, Duarte S, Murata RM, Scott-Anne K, Gregoire S, Watson GE, *et al.* Influence of cranberry proanthocyanidins on formation of biofilms by *Streptococcus mutans* on saliva-coated apatitic surface and on dental caries development *in vivo*. Caries Res 2010;44(2):116-26.
12. Stoecklin W. Chemistry and physiological properties of gymnemic acid, the antisaccharine principle of the leaves of *Gymnema sylvestre*. J Agric Food Chem 1969;17(4):704-8.
13. Shanmugasundaram KR, Panneerselvam C, Samudram P, Shanmugasundaram ER. Enzyme changes and glucose utilisation in diabetic rabbits: The effect of *Gymnema sylvestre*, R. Br. J Ethnopharmacol 1983;7(2):205-34.
14. Kurihara Y. Characteristics of antisweet substances, sweet proteins, and sweetness-inducing proteins. Crit Rev Food Sci Nutr 1992;32(3):231-52.
15. Bishayee A, Chatterjee M. Hypolipidaemic and antiatherosclerotic effects of oral *Gymnema sylvestre* R. Br. Leaf extract in albino rats fed on a high fat diet. Phytother Res 1994;8(2):118-20.
16. Kapoor LD. CRC Handbook of Ayurvedic Plants. Boca Raton, FL: CRC Press; 1990. p. 200-1.
17. Dineshbabu J, Srinivasan P, Manimekalai K, Guna GD, Darsini TP. Uses of traditional medicinal plants against the biofilm forming *Streptococcus pyogenes* isolated from upper respiratory tract. Int J Pharm Biol Sci 2015;6(2):(B)464-79.
18. 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.
19. Khattak KF, Simpson TJ. Effect of gamma irradiation on the antimicrobial and free radical scavenging activities of *Glycyrrhiza glabra* root. Radiat Phys Chem 2010;79(4):507-12.
20. Perez C, Pauli M, Bazerque P. An antibiotic assay by the agar well diffusion method. Acta Biol Med Exp 1990;15:113-5.
21. Ponnuraj S, Jaganathan D, Kanagarajan M, Castro J, Deivamarudachalam TP. Influence of *Limonia acidissima* L. Against the biofilm forming *Aeromonas hydrophila* isolated from fresh water fishes. J Biochem Technol 2015;6(1):910-21.
22. Prouty AM, Schwesinger WH, Gunn JS. Biofilm formation and interaction with the surfaces of gallstones by *Salmonella* spp. Infect Immun 2002;70(5):2640-9.
23. Naidu GK, Naidu KC, Sujatha B. *In vitro* antibacterial activity and phytochemical analysis of leaves of *Gymnema sylvestre* Retz. R. Br. Int J PharmTech Res 2013;5(3):1315-20.
24. Paul JP, Jayapriya K. Screening of antibacterial effects of *Gymnema sylvestre* (L.) R. Br. A medicinal plant. Pharmacol Online 2009;3:832-6.
25. Prescott LM, Harley JP, Klein DA. Microorganisms in aquatic environments. In: Microbiology. 5th ed. New York: McGram-Hill Companies Inc.; 2002. p. 632-63.
26. Vlietinck AJ, Van Hoof L, Totté J, Lasure A, Vanden Berghe D, Rwangabo PC, *et al.* Screening of hundred Rwandese medicinal plants for antimicrobial and antiviral properties. J Ethnopharmacol 1995;46(1):31-47.
27. Thenmozhi R, Nithyanand P, Rathna J, Pandian SK. Antibiofilm activity of coral-associated bacteria against different clinical M serotypes of *Streptococcus pyogenes*. FEMS Immunol Med Microbiol 2009;57(3):284-94.
28. Darsini DT, Srinivasan P, Guna G, Manimekalai K, Dineshbabu J. *In vitro* antibiofilm 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.
29. Chew AL, Jessica JJ, Sasidharan S. Antioxidant and antibacterial activity of different parts of *Leucas aspera*. Asian Pac J Trop Biomed 2012;2(3):176-80.
30. Limsuwan S, Voravuthikunchai SP. *Boesenbergia pandurata* (Roxb.) Schltr. *Eleutherine americana* Merr. and *Rhodomyrtus tomentosa* (Aiton) Hassk. As antibiofilm producing and antiquorum sensing in *Streptococcus pyogenes*. FEMS Immunol Med Microbiol 2008;53(3):429-36.