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Fruit Pulp Extracts of *Ficus racemosa* and *Aegle marmelos:* Ethnopharmacological Approach for curing the Diabetic Foot Ulcer

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ABSTRACT

Diabetic Foot Ulcer (DFU) are the most common complication represent a central cause of morbidity among *Diabetes mellitus* (DM) mostly type–II, also reported amputation of foot region the impact of this complication cause mortality until treated. Biofilms are the sole responsible for over 90% of all chronic wounds in case of DFU. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Proteus* sp, are prime pathogens causing biofilm, whereas higher prevalence occupied by *Pseudomonas aeruginosa* during chronic condition. The objective of the current investigation is to determine the value of methanolic fruit pulp extracts of *Ficus racemosa* and *Aegle marmelos* against isolated key biofilm former *Pseudomonas aeruginosa* and associated bacterial pathogens. The phytochemical constituents for fruit pulp extracts of *F. racemosa* and *A. marmelos* were determined using FT-IR analysis. Minimum Inhibitory Concentration (MIC), Biofilm Inhibitory Concentration (BIC) and antibacterial activity were performed to find out the efficiency of the extract. *F. racemosa* was shown the better antibiofilm activity than *A. marmelos*. Remarkably, Sub-MIC level showed increasing inhibitory activity as concentration increases (0.5 mg/mL). Microscopic analysis showed dose dependant reduction in the biofilm architecture as compared to control. The extracts of fruit pulp of *F. racemosa* and *A. marmelos* show the anti-biofilm activity and which might be used as a substitute medicine in DFU. But it remains for the further analysis to elucidate the active binding molecule against biofilm former *P. aeruginosa.*

Keywords: Diabetic Foot Ulcer, Biofilms, *Pseudomonas aeruginosa*, MIC, *Ficus racemosa*, Fruit pulp.

INTRODUCTION

Diabetic Foot Ulcer (DFU) is referred to a clinical complaint, but simply it is a merely wound on the foot of a patient who is diabetic (Green *et al.,* 2013). A 50%–70% of chance to formation of diabetic foot in persons with *diabetes mellitus* (Markakis *et al.,* 2016; Kirsner *et al.,* 2010; Boulton *et al.,* 2005). Foot lesions induces the high morbidity and mortality and represent the most common origin of hospitalization in patients with diabetes. It influences a person's quality of life, resulting to a decline to social, physical and psychological functions (Davis *et al.,* 2006) which have high frequency to amputation of the leg (Jeffcoate and Harding, 2003). Diabetics with foot ulcers commonly experience infection with *Gram-positive* (*Staphylococcus aureus* and *Enterococcus*) and *Gram-negative* (*Pseudomonas aeruginosa, Escherichia coli, Klebsiella sp, Proteus sp*, etc., and some anaerobes) organisms (Khan *et al.,* 2016) which lined up in the infection site in the form of "Biofilms".

Now-a-days, the formation of multidrug-resistant bacteria is of great concern due to wide-ranging use of antibiotics, GMO's and so on. These bacteria have built-in abilities to discovery new ways to be resistant and can pass along genetic materials that allow other bacteria to become drug-resistant as well (CDC, 2017). Multi-drug resistant gene can transfer through the conjugation process between populations or within population in the natural environment (Stickler *et al.,* 1998). Nowadays, plant-based products have found widespread application in the medical and food industries as alternatives to conventional therapy. Generally, essential oils of plants have been widely used to treat several ailments (Ríos and Recio, 2005) and the use of its extracts has also gained popularity as they contain various bioactive compounds whose antimicrobial properties yield wide spread application in the pharmaceutical industry (Shan *et al.,* 2011).

In traditional, *Ficus racemosa* L and *Aegle marmelos* L, are used as medicine for the treatment of numerous disorders. Root, fruits, latex, leaf and bark are used to treat the diseases such as carminative, leprosy, diarrhea, anti-dysentery, menorrhagia, vermifuge, astringent, circulatory and respiratory disorders (Sharma and Gupta, 2008). Gluanol acetate, tiglic acid, glucanol, taraxasterol, friedelin, and lupeol acetate are the major component of fruits extracts (Joseph and Raj, 2010) and also have various phenolics in the fruit as chlorogenic acid, ellagic acid, ferulic acid, gallic acid, protocatechuic acid and quercetin (Dhan *et al.,* 2011). Charoensiddhi and Anprung, (2008) reported the presence of Dehydro-p-cymene, hexadecanoic acid, α-Cubebene, β-phellandrene, humulene oxide, caryophyllene oxide, β-caryophyllene and dihydro-β-Ionone in the extract of *A. marmelos*.

The objective of present investigation is to explore the use of ethno-medicinally properties of *Ficus racemosa* and *Aegle marmelos* fruit pulp extracts for the possible remedy of the DFU biofilm former bacterial pathogens.

MATERIALS AND METHODS

Bacterial strains

Four bacterial pathogens (*Pseudomonas aeruginosa, Pseudomonas mendocina, Proteus mirabilis* and *Shewanella xiamenensis*) were found from wound swabs of diabetes foot ulcers patients from Karpagam Medical College Hospital, Coimbatore with proper authentication and directions. Todd Hewitt's Broth (THB) was employed for cultivation of bacterial pathogens and which was maintained at -20°C for future studies.

Fruit pulp extraction and FT-IR analysis

F. racemosa and *A. marmelos* were obtained from local market in Coimbatore, Tamil Nadu. 50 mL of methanol was used for preparation of extraction from 10 g of fruit pulps. The prepared extract was filtered using the filter paper. The process was carried out by rotary vacuum evaporation to remove the solvents from filtrate. The concentrated extract was dissolved in DMSO and store in -4°C for further analysis (Packiavathy *et al.,*2012). The functional groups of derived extracts were determined by the Fourier-transform infrared spectroscopy using the protocol of Durrell (1964).

Antibacterial activity assays

The different concentration of prepared extracts (25, 50 and 75 μg/mL) were prepared and used for Agar well diffusion assay. Streptomycin (0.03 mg/mL) was used as positive control and meanwhile DMSO was employed as negative control. The isolated bacteria

(*Pseudomonas aeruginosa, Pseudomonas mendocina, Proteus mirabilis* and *Shewanella xiamenensis*) were swabbed on Muller Hinton agar plates. After that well was created and different concentration of extracts was poured. Finally, the plates were incubated at 37°C for 24 hours. The zone of inhibition was measured after the incubation period. The growth curve analysis was done using the 2 mg/mL of *F. racemosa* and *A. marmelos* fruit pulp extracts for bacterial pathogens. The bacterial pathogens were grown in LB broth and supplemented with fruit pulp extracts. The cell density was determined according the procedure of Abraham *et al.,*(2011) and assessed using the UV-Visible spectrophotometer at 600nm. The Minimal Inhibitory Concentration (MIC) assay was performed using the *F. racemosa* and *A. marmelos* fruit pulp extracts for inhibition of isolated bacterial growth.

Antibiofilm assay (Microscopic view)

The inhibition of biofilm formation by bacterial pathogens was determined by the antibiofilm assay via protocol of Nithya *et al.,* (2011). Fruit pulp extracts of *Ficus racemosa* and *Aegle marmelos* (0.0625-1 mg/mL) were prepared and used for this analysis.

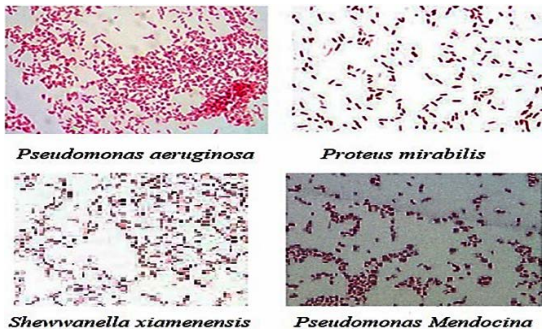

Fig. 1. Microscopic observation of isolates

RESULTS

FT-IR analysis of fruit pulp extracts

The functional group of *Ficus racemosa* possess the active components based on the peak value in the infra-red radiation. The FT-IR spectrum was identified 09 functional groups between 500 to 4000 frequency wavelengths. The presence of intense bands at 817.28 cm^{-1}, 948.52 cm^{-1}, 1049.28 cm^{-1}, 1311.59 cm^{-1}, 1419.61 cm^{-1}, 1674.21 cm^{-1}, 2908.65 cm^{-1}, 2993.52 cm^{-1} and 3641.60 cm^{-1} corresponding to C-H, C-H (S), H–C and C=C bond vibration indicate the presence of Alkyl groups, Aryl groups, Cyclohexane ring vibrations, Alcohol, Hydroxyl, Olefinic group, Alkene and Alcohol respectively (Fig. 2 & Table 1). In case of A. marmelos, the bands at 948.98 cm^{-1}, 1033.85 cm^{-1}, 1311.59 cm^{-1}, 1419.61 cm^{-1}, 1728.22 cm^{-1}, 1990.54 cm^{-1}, 2916.37 cm^{-1}, 3001.24 cm^{-1} and 3626.17 cm^{-1} corresponding to (P – O-C stretch), (Si–O-Si), C–H, C–H stretch, (-NCS), C-H asym/sym stretch and OH stretch bond vibration respectively, indicate the presence of Aromatic phosphates, Silicon-oxy compounds, Carbonyl compound, Olefinic (alkene) group, Aldehyde, Isothiocyanate, Methylene and Secondary alcohol respectively (Fig. 3 & Table 2).

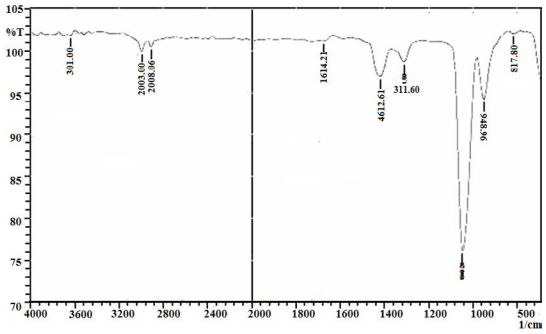

Fig. 2. FTIR analysis of Ficus racemosa fruit pulp methanol extract

Table 1: Phytoconstituents of *Ficus racemosa* by FTIR analysis

S. No	Frequency (500-4000 cm^{-1})	Bond and type of Vibrations*	Functional groups
1	817.28	C-H	Alkyl groups
2	948.98	C – H	Aryl groups
3	1049.28	C –H	Cyclohexane ring vibrations
4	1311.59	O – H	Alcohol and Hydroxyl compound
5	1419.61	C – H	Olefinic group
6	1674.21	C=C	Alkene
7	2908.65	-	-
8	2993.52	-	-
9	3641.60	O – H	Alcohol group

Table 2: Phytoconstituents of *A. marmelos* by FTIR analysis

S. No	Frequency (500-4000 cm^{-1})	Bond and type of Vibrations*	Functional groups
1	948.98	(P – O - C stretch)	Aromatic phosphates
2	1033.85	(Si – O - Si)	Silicon-oxy compounds
3	1311.59	C - H	carbonyl compound
4	1419.61	C - H	Olefinic (alkene) group
5	1728.22	C - H stretch.	Aldehyde
6	1990.54	(- NCS)	Isothiocyanate
7	2916.37	C- H asym. / sym. stretch	Methylene
8	3626.17	OH stretch	Secondary alcohol

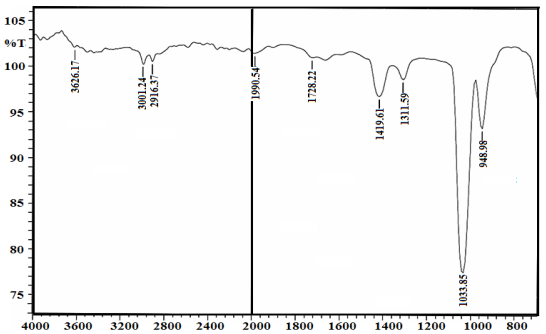

Fig. 3. FTIR analysis of *A. marmelos* fruit pulp methanol extract

Antibacterial activity assay

The fruit pulp of methanol extracts from *Ficus racemosa* showed maximum antibacterial activity against test pathogens of *Pseudomonas aeruginosa* (20mm) and *Proteus mirabilis* (17mm) at varying degree of inhibition than *A. marmelos*. The maximum inhibition was observed in high concentration at 75 μL against the test bacterial pathogen (Table 3).

Growth curve analysis

In growth curve analysis, extracts had good effect on the cell biomass inhibition against bacterial pathogens at 1 mg/mL concentration which was apparent from relatively high to low growth in the cell density. Results reveal that the cell mass inhibition was observed in *Ficus racemosa* extract possess high bactericidal effects at the frequent incubation periods than *A. marmelos* (Figure 4).

Minimal Inhibitory Concentration (MIC) Assay

Sub–MIC of 2 mg/mL were taken at vary concentrations (0.062-0.5 mg/mL) and inhibition of all pathogens was drastically observed as the concentration increases. *Pseudomonas aeruginosa* and Proteus mirabilis highly shown the sensitivity against the fruit pulp extract of *Ficus racemosa* compared to Aegle marmelos at 0.5 μL concentration (Figure 5).

Table 3: Antibacterial activities of methanolic fruit pulp extracts

S. No	Bacterial Pathogens	*Ficus racemosa* (μg/mL)			*A. marmelos* (μg/mL)		
		25	50	75	25	50	75
		Zone of Inhibition (mm)					
1	*Pseudomonas aeruginosa*	14	17	20	6	10	15
2	*Pseudomonas mendocina*	-	6	12	-	-	5
3	*Proteus mirabilis*	11	12	17	6	4	10
4	*Shewanella xiamenensis*	7	10	14	2	6	10
5	Streptomycin (0.03 mg/mL) (Positive control)	17	16	17	15	14	16
6	DMSO (Negative control)	-	-	-	-	-	-

Inhibition assay (Microscopic View)

In Light microscopic analysis, *Pseudomonas aeruginosa* and *Proteus mirabilis* were subjected to the methanol extracts of *Ficus racemosa* and *Aegle marmelos* due to the high sensitivity, biofilm susceptibility analysis was assessed using the inhibitory concentrations of 0.0625-1 mg/mL concentration (Fig. 6). Significant reduction in the biofilm formation was observed in the test concentration when compared to their respective controls. This elucidated that the fruit pulp extracts of Ficus aeruginosa inhibited the biofilm former than *Aegle marmelos*.

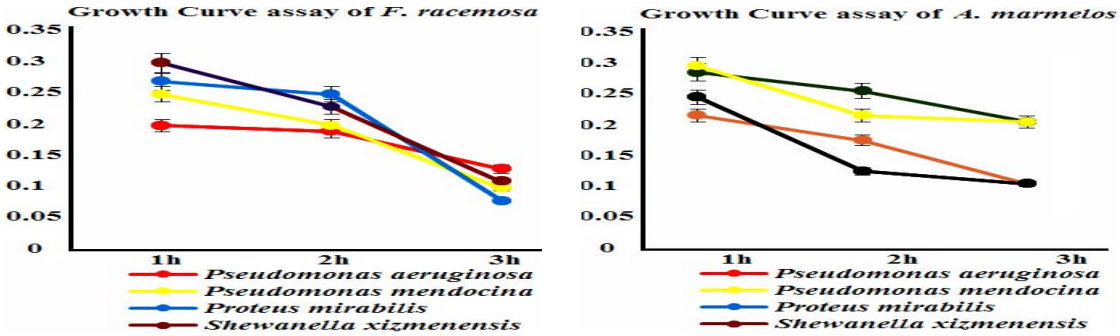

Fig. 4. Growth Curve analysis of fruit pulp extracts

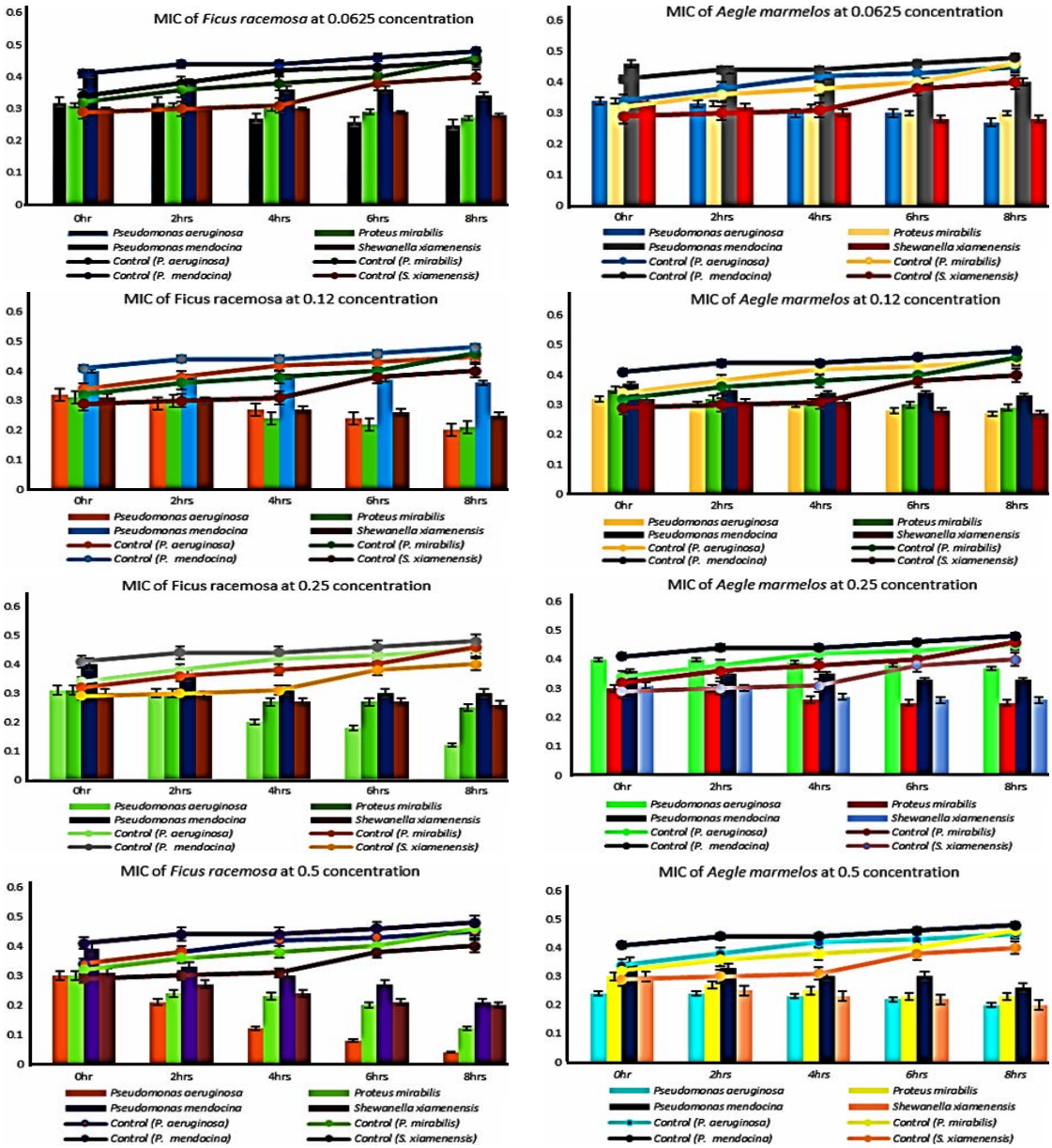

Fig. 5. Minimal Inhibitory Concentration of fruit pulp extracts

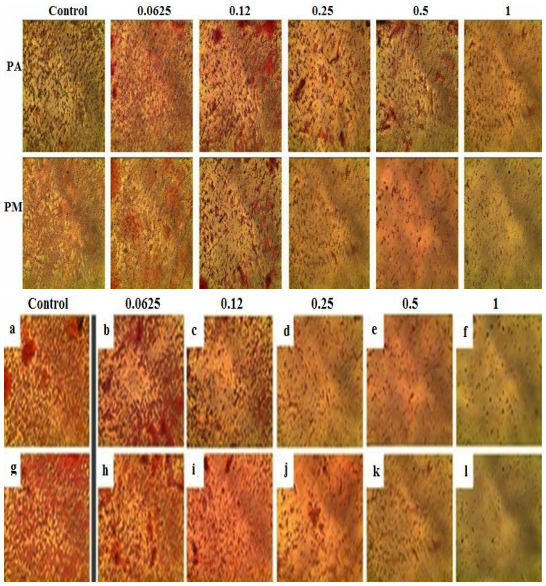

Fig 6. Light microscopic images of *Pseudomonas aeruginosa* and *Proteus mirabilis* biofilms

DISCUSSION

In Diabetic Foot Ulcers (DFU), deep tissues are unprotected to bacterial colonization, once the protective layer of skin is damaged (Vuorisalo *et al.,* 2009). Based on the DFU swabs 96% of *S. aureus*, 99% for β-haemolytic *Streptococcus* and 96% of *P. aeruginosa* pathogens were reported (Gardner *et al.,* 2014). Multicombinations of bacterial pathogens like *Staphylococcus aureus*, *Enterococcus, Pseudomonas aeruginosa, E. coli, Klebsiella sp* and *Proteus sp* form polymicrobial communities within a matrix of EPS (Extracellular Polymeric Substances) (Banu *et al.,* 2015) may possibly communicate via Quorum Sensing (QS) where the systems interact mainly with exopolysaccharide (EPS) production (De Kievit *et al.,* 2001). There are 2 QS systems in *Pseudomonas aeruginosa*-Las (LasR & LasI synthase proteins) and Rhl (RhlI and RhlR proteins). The Rhl system showed the prominent characteristics having a role in immune invasion and biofilm formation (Lequette and Greenberg, 2005). In this study, Ethnopharmacology approaches were designed to eradicate the biofilm former because these isolates highly resist in multi-drug combinations and also required high doses of antibiotics to eliminate this biofilm phase. Isolated pathogens were exposed to fruit pulp extracts of *F. racemosa* and *Aegle marmelos* which shown an efficient activity against the biofilm formers. FTIR analysis showed the presence of active compounds with varying medicinal properties includes alkyl groups, aryl groups, carbonyl compounds, cyclohexane ring vibrations, Hydroxyl, Isothiocyanate, Methylene, Olefinic group, Phosphate (Aromatic), Alkene and Alcohol group compound possess anti-cancer, anti-oxidant, anti-microbial, wound healing activity. Proteus mirabilis and *Pseudomonas aeruginosa* divide in one plane in chains of varying lengths in selection agar media and *Gram staining* (Todar, 2006). They are generally strong fermenters of carbohydrates, resulting in the production of lactic acid, a property used in the dairy industry and hold a catalase, Oxidase and IMViC negative (Kilian and Bulo, 1976). The present study results were show the correlate with the earlier report of *Gram staining* and Biochemical test.

Fruit pulp extracts of *Ficus racemosa* showed good antibacterial activity against test pathogens of *Pseudomonas aeruginosa* (20mm) and *Proteus mirabilis* (17mm) at the concentration of 75 μL respectively, whereas in *A. marmelos*, *Pseudomonas aeruginosa* (15mm) and Proteus mirabilis (10mm) showed its activity at the maximum concentration of 75 μl. Sequential inhibition rate were noted as the concentration increases. Comparatively, fruit extracts of *F. racemosa* showed maximum activity than *A. marmelos*.

In Growth Curve analysis, both the fruit extracts minimize the cell density of all pathogens especially *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Pseudomonas mendocina*. As compared with *A. marmelos*, *Ficus racemosa* exhibited the maximum inhibitory level, thus biofilm activity against the *Pseudomonas aeruginosa* and *Proteus mirabilis* of *Ficus racemosa* was proceeded for further analysis. Similar results were observed in Minimum Inhibitory Concentration (MIC), extracts of *Ficus racemosa* showed the inhibition range at 0.5 mg/mL in case of *Pseudomonas aeruginosa* and *Proteus mirabilis*, followed by *Pseudomonas mendocina* and *Shewanella xiamenensis* and the extracts of *Aegle marmelos* showed the meager response compared to *F. racemosa* respectively. The antibiofilm activity at vary concentrations (0.062-1 mg/mL) of fruit pulp extracts showed gradual inhibitory effects from 0.25 concentration, whereas in *A. marmelos* inhibitory range were steadily low

from the range of 0.25. *Pseudomonas aeruginosa* and *Proteus mirabilis* were steadily viewed under microscope which was exposed to fruit pulp extracts since these are chief biofilm formers.

CONCLUSION

The present investigation clearly indicated the fruit pulp of *Ficus racemosa* extracts contain the primary and secondary phytochemicals which are high effective against the prime biofilm former *Pseudomonas aeruginosa* and *Proteus mirabilis* in efficient way which was evident and confirmed by different *In vitro* studies. Studies has also ensured or suggested that this bioburden will be reduced or eradicated through substitute biomaterial in the form of dressing bandage or spray which coated with phytoconstituents from *Ficus racemosa* could be a possible remedy for DFU patients. Furthermore, studies should involve in understanding of action to exploit as potent of *in vivo* biofilm mechanism against pathogens.

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Conflict of interest

The authors declare no conflict of interest.

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