IJPSR (2020), Volume 11, Issue 11 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 22 November 2019; received in revised form, 17 February 2020; accepted, 14 March 2020; published 01 November 2020

FORMULATION OF EMULSIONS FROM PLANT ESSENTIAL OILS AGAINST MICROBIAL BIOFILMS

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Keywords:

Antibiofilm Inhibitors, Biofilm, Essential oil, Emulsion, Stability and ultrasonication

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ABSTRACT: A biofilms are sessile aggregate mode of growth in some bacteria, fungi, protozoa, and algae which makes the microorganism tolerant to adverse environmental conditions biofilm-forming microorganism may possibly create conditions including native valve endocarditis, osteomyelitis, dental caries, middle ear infections, medical device-related infections, ocular implant infections and chronic lung infections in cystic fibrosis patients. In an attempt to control biofilm formation on the wall, the emulsion was developed and evaluated against the bacteria isolated from biofilm layers of walls. In the current study, emulsion (O/W) was developed using oil extracted from the leaves of a *Cymbopogon citratus* (lemongrass), *Eucalyptus globules* and *Azadirachta indica* (neem) are used, which have antiseptic, antibacterial, antiviral, antioxidants and anti-diabetic activities. The emulsions were prepared in different ratios of oil and surfactant (Tween 80 and Tween 20) and evaluated for their mechanical and thermal stability. Transparent emulsion with a mean droplet diameter of 171 nm was obtained at a 6:4 ratio (v/v) of oil and surfactant and was found to be stable. This result was obtained with an emulsification time of 30 min. The emulsions were subjected to anti-biofilm assays against the bacterial cultures using a disc diffusion method and microtitration method. The results were compared to estimate the anti-biofilm efficacy of the emulsions. Out of 10, 4 emulsions showed higher biofilm activity than the control treatment. The study concluded that the essential oils being converted to emulsion has the potential to prevent microbial biofilms. Though, further investigation of the larvicidal activity of formulated nanoemulsion will be studied to prove the efficacy of nanoemulsion.

INTRODUCTION: Biofilm is a community of microorganisms attached to a solid substrate surface and submerged into the extracellular slimy matrix. Biofilm formation leads to the inefficiency of antibiotics due to their relative impermeability, variable physiological status of microorganisms, sub-populations of persistent strains, and variations of phenotypes present [1, 8].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.11(11).5725-32

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.11(11).5725-32

The formation of a biofilm begins with the attachment of free-floating microorganisms to a surface [24]. The growth of biofilm depends upon oxygen supply. Some harmful effects of biofilm include the deterioration of dental surfaces, contamination of surfaces in the food processing industry, and the deterioration of air quality in ventilation and air handling systems.

Nowadays, plant-derived substances are gaining more importance in the prevention of biofilms [8]. The essential oils are volatile compounds produced as secondary metabolites extracted from plant products *i.e.*, leaves, stems, roots, *etc*., are widely used in prevention and treatment of cancer, cardiovascular diseases including atherosclerosis

and thrombosis. Due to the presents of phytochemicals like phenols, alkaloids, steroids, flavonoids, glycosides, they act as antibacterial, antiviral, antitumor, antioxidants and antidiabetic activities [6, 9].

In this present study, essential oils are extracted from three plants, namely, *Cymbopogon citratus* (lemongrass), *Azadirachta indica* (neem), and *Eucalyptus globules* (Southern blue gum). Lemongrass oil possesses antibacterial, antifungal, antiseptic, antioxidant properties [2, 25]. Neem, a traditional plant abundant in India, is well known for its antibacterial, anticancer, antiviral, antifungal, insecticidal, and antidiabetic properties. In eucalyptus, a compound called 1,8-cineole plays a major role in its antibacterial activity. However, eucalyptus is widely used as a disinfectant, antiseptic, anesthetic, deodorant, insect repellent, fumigant, and in treatment for a variety of diseases, including diabetes, bronchitis, diarrhea, inflammation, leprosy, fever, flu and so on [12, 13].

The emulsion is kinetically stable that contains essential oil, water, and surfactants. Tween 20 and Tween 80, are non-ionic detergents in biochemical applications. Tween 20, due to its stability and relative nontoxicity [7]. It is used as an emulsifier in a number of domestic, scientific, and pharmacological applications. Tween 80, is also used as a surfactant in soaps and cosmetics (including eye drops), or a solubilizer such as in a mouthwash. Emulsions can be prepared by two methods, high energy and low energy methods. In this study, ultrasonication (high energy method) was carried out for nanoemulsion preparation [3, 5]. The advantages of using ultrasonication are that there is no radiation hazard in an ultrasonic examination, significant properties exhibited by the emulsions involves higher surface area per unit volume, robust stability, optical transparent appearance, *etc*. Emulsions have its applications as an overview of drug delivery, food, cosmetics, pharmaceuticals, and material synthesis [4].

It is observed that making up an emulsion to its nano-size under high energy inputs using ultrasonication would greatly lead to an innovative idea of the term 'BIO- PAINTS'. Bio-Paints has no Volatile Organic Compounds (VOC) and does not contribute to smog and pollution. It contains no lead, glycol derivates, aromatic hydrocarbons, and styrene compounds. It is based on natural clay, which helps to clean the air. Due to these properties, Bio-paints are considered to be eco-friendly, low allergy & inhalable. Nano-emulsion, when added with the base paint, would tremendously be efficient and resistant to the bacteria forming a biofilm.

In this present work, the emulsion that is stable against biofilm was identified by the particle size distribution method. In the future, the formulated nanoemulsion will be incorporated with the base paint, which acts as a 'BIO-PAINT'.

MATERIALS AND METHODS:

Collection of Plant Materials: The leaves of *Cymbopogon citratus* (lemongrass), *Azadirachta indica* (neem), and *Eucalyptus globules* (eucalyptus) were collected from in and around Sathyamangalam.

Extraction of Essential Oils: The leaves collected from the plant samples were washed with distilled water and allowed to shade dry. The dried leaves were crushed and made to a fine powder. 5 g of fine powder was mixed with 150 ml of solvent (ethanol) and subjected to steam distillation. Steam distillation is one of the conventional processes used for the extraction of oil from the plant extracts. It is a separation process in which temperature-sensitive materials which are insoluble in water decompose at their boiling point. The resulting distillate is exposed to liquid-liquid interaction in a separating funnel using Hexane as a solvent. The organic layer and the aqueous layer were separated in the funnel. The organic layer is present in the upper part due to its lower density, whereas the aqueous layer in the lower part due to its higher density. The organic layer is used for the preparation of emulsion [10].

Preparation of Emulsion: Emulsion was prepared by a mixture of three essential oils- lemongrass, eucalyptus and neem along with non-ionic surfactants (Tween 20 and Tween 80) and distilled water. The surfactant plays a role of blending the hydrophobic and hydrophilic groups. By using each emulsifier separately, five different emulsions were prepared by varying their concentration and emulsification time [7, 14].

Five mixtures were prepared by varying the concentration of three essential oils and surfactant mixture (emulsifier + hexane) as 2:8, 3:7, 4:6, 5:5, 6:4, respectively. Each mixture was made up to 25ml by adding 15 ml of water. These mixtures were subjected to different emulsification time as 5 min, 10 min, 15 min, 20 min, and 25 min under ultrasonication.

Determination of Stability: The obtained nanoemulsions from the ultrasonication process were subjected to mechanical and thermal stability.

Mechanical Stability: Mechanical stability is important in various manufacturing processes, and a number of empirical methods are used for testing. It is done by stirring a test portion at a moderate speed under load with shear stress. It can provide a more accurate indication of performance, stimulating the actual service conditions. Each emulsion mixtures were undergone centrifugation at five different rpm, *i.e.*, 2000, 3000, 4000, 5000, and 6000 for 15 min. The emulsion is left undisturbed for some time, and it is checked for stability [15].

Thermal Stability: The thermal stability is monitored by placing the samples under the five different temperatures of 4 ºC, 27 ºC, 35 ºC, 40 ºC and 45 ºC for a day. After 24 h, the emulsions were observed. The absence of phase separation indicates that prepared emulsions are highly stable.

Isolation of Biofilm Forming Bacteria: Samples were isolated from the contaminated wall in the laboratory. 500 mg of contaminated wall sample was dissolved in 100ml of distilled water and kept under shaker for 1 h. Using spread plate method, 100 µl of mixed solution is inoculated in nutrient agar media and incubated at 37 ºC for 1 day. Three individual bacterial colonies were isolated and further subcultured.

Biofilm Production Tube Assay: The three bacterial colonies were cultured in nutrient broth media. 5 ml of each bacterial culture is taken in a test tube and stored at 37 ºC for 3-5 days. The formation of biofilm is confirmed by adding 1% of crystal violet over the inner surface of the test tube. Crystal violet dye binds to negatively charged molecules such as polysaccharides present in the biofilm. After the addition of dye, the biofilm appears violet in color, which can be observed with the naked eye.

Microtiter Plate Biofilm Production Assay: Biofilm production assays of S1, S2, and S3 were performed by the microtiter plate reader method. A 50 µl of nutrient broth was added to the microtiter plate in addition to that inoculate 0.01ml of overnight cultures of S1, S2 & S3. Plates were made in duplicate, incubated, and sealed at 32 °C for 24 h. Each plate included three wells of nutrient broth as control wells. The cell turbidity was monitored using a microtiter plate reader at an OD 595 nm. A microtiter plate wells were washed five times with sterile distilled water, which is used to remove loosely associated bacteria.

Plates were allowed to air dry for 30 min, and each well was stained with 150 µl of 1% crystal violet solution in water for 45 min. After staining, plates were washed with sterile distilled water five times. Currently, it showed a purple ring formed on the side of each well. The concentration of biofilm was determined by adding 200 µl of 95% ethanol to destain the wells. 100 µl of well contents from each well was transferred to a new microtiter plate, and the absorbance was measured at 595 nm.

Antibiofilm Assay of Emulsion: The antimicrobial activity of the emulsions against biofilm-forming bacteria was determined using disc diffusion and microtiter plate methods. In Disc diffusion method, the bacterial cultures were spread over the nutrient agar medium. A filter paper was cut into round shaped pieces, soaked in the emulsion, and placed on the agar surface. The plates are incubated at 37 ºC overnight. The size of the zone of inhibition of growth indicates the antibiofilm activity of the emulsion. In 96 well microtiter plates, 50 µl of nutrient broth media is added to each well, and emulsions of different concentrations –1:10, 1:100, and 1:1000 were added. 1µl of bacterial culture is added to each well. After one day of incubation of the microtiter plate at 37 ºC, the absorbance value is measured using ELIZA reader. Results of disc diffusion method and ELIZA reader were compared [16, 17, 18].

Determination of Particle Size Distribution: The particle size distribution of prepared emulsion was determined using the scattering light intensity

method. This analysis is carried out to measure the homogeneity and stability of the droplet size in the emulsion system. Particle size was analyzed using particle size analyzer (Department of Nanoscience and Technology, SREC, Coimbatore). The droplet size was expressed in terms of nm and polydispersity index (PDI) was measured.

RESULTS AND DISCUSSION: The physicochemical properties of emulsions are used for practical applications because of their small droplet size and long-term stability. The emulsion formation with a stable droplet size is mainly based on the type of oil used during the emulsification process. Formulation of emulsion with essential oils resulted in stable emulsion with a droplet size of range 100 - 200 nm. Therefore the essential oils were used for emulsion preparation.

Isolation of Biofilm Producing Bacteria: Antibiotic resistance and biofilm formation play a crucial role in clinical infections. In conventional therapy, due to the increase in complexity of microbial infections and the resistance, a scientist has been incited to recognize alternatives for the microbial infections.

FIG. 1: ISOLATION OF BIOFILM MATRIX

Plant-based bioactive compounds have the ability to treat infections with less side effects while ancient times. From contaminated wall samples, 3 bacterial colonies were isolated after 24 h of incubation on a nutrient agar plate.

Screening of Biofilm Producing Bacteria: From the biofilm formation assay, three individual bacterial colonies were inoculated in the nutrient broth media and named as S1, S2, and S3. It was incubated under 37 ºC. The biofilm forming capability of the bacteria was confirmed by crystal violet assay. As a result, among the three bacterial cultures, S2 had shown the highest biofilm formation.

FIG. 2: BINDING OF CRYSTAL VIOLET WITH BIOFILM

Microtiter Plate Biofilm Production Assay: Biofilm production assay was used to evaluate biofilm formation of S1, S2 & S3 after 24 h of incubation at 32 °C. Biofilm production of S1, S2 & S3 ranging from an OD595 of 0.364 to 0.565. Our results indicated S2 strains have the highest biofilm formation than S1 & S3 strains. Similar results were obtained by crystal violet tube assay.

Emulsion Stability Studies: The stability analysis of formulated emulsion was studied by subjecting to centrifugation, heating-cooling cycle, and kinetic stability was investigated by determining droplet size in different time intervals. During the preparation of emulsion, an increase in the proportion of the emulsifier resulted in an increase in the turbidity of the emulsion. Emulsions were prepared by ultrasonication process. Four sets of emulsions were prepared and names from A to T.

TABLE 1: FORMULATION OF EMULSIONS

Variation of parameters	Tween 20	Tween 80
Concentration	SET 1- A, B, C, D and E	SET 3- K, L, M, N and O
Times	SET 2- F, G, H, I and J	SET 4- P, Q, R, S and T

Thermal Stability: The emulsions were subjected to different temperatures – 4 ºC, 25 ºC, 30 ºC, 35 ºC and 40 ºC and checked for phase separation. Inset 1, emulsions B, C, D, and E were stable in all the temperature ranges, whereas emulsion A was unstable. Inset 2, emulsion G is only stable. Inset 3 and set 4, all the emulsions (K to T) were stable. All the stable emulsions were carried for further tests.

TABLE 2: THERMAL STABILITY STUDIES OF EMULSIONS

T (ºC)	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
4 ºC	*	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25 ºC	*	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30 ºC	*	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35 ºC	*	-	-	-	-	*	-	*	*	*	-	-	-	-	-	-	-	-	-	-
40 ºC	*	-	-	-	-	*	-	*	*	*	-	-	-	-	-	-	-	-	-	-

(-) indicates no phase separation and (*) indicates phase separation

Mechanical Stability: The stable emulsions were subjected to centrifugation at five different rpm, *i.e.*, 2000, 3000, 4000, 5000, and 6000 for 15 min. Inset 1, emulsion C is mechanically stable. Inset 2, emulsion G is stable. Inset 3, emulsions M, N, and O are stable. Inset 4, emulsions P, Q, R, S, and T are stable.

TABLE 3: MECHANICAL STABILITY STUDIES OF EMULSIONS

Rpm	C	G	M	N	O	P	Q	R	S	T
2000	-	-	-	-	-	-	-	-	-	-
3000	------	-	-	-	-	-	-	-	-	-
4000	-	-	*	-	-	-	-	-	-	-
5000	-	-	*	-	-	-	-	-	-	-
6000	-	-	*	-	-	-	-	-	-	-

(-) indicates no phase separation and (*) indicates phase separation

Physical Stability: From the thermal and mechanical stability tests, among the stable emulsions, only C, G, O, and Q were selected for antibiofilm assay because of their physical appearance (milky white color).

FIG. 3: PHYSICAL APPEARANCE OF EMULSIONS C, G, O AND Q

Particle Size Distribution of Stable Emulsion: The emulsion was formulated by ultrasonication method, which yields a low polydispersity index with a minimized droplet size. Emulsification time has a direct correlation with the droplet diameter of the emulsion. When emulsification time increased from 05 min to 10 min using mixed oil emulsion (4:6) and Tween 20, the mean droplet size of the formulation C and G were reduced from 325.8 to 244.1 nm. Simultaneously, emulsification time significantly increase from 05 min to 10 min using mixed oil emulsion (6:4) and Tween 80, the mean droplet size of the formulation O and Q were reduced from 279.2 to 178.6 nm respectively.

The polydispersity index of the formulated emulsion was found to be minimum in the case of Q (0.016) and O (0.772) as compared to C (2.326) and G (0.228). The polydispersity index (PI) of the emulsions was below 0.2, which provides long-term stability to the emulsions. While the other formulations were not stable because of phase separation. It is used to measure the stability and homogeneity of formulated emulsion droplet size.

Leong *et al.*, (2009) and Tang *et al.*, (2013) observed a similar decreasing trend of droplet size and PDI with the increase in sonication time while formulating sunflower oil and aspirin nanoemulsion [19, 20]. The obtained results are parallel with a recent report by Ramisetty *et al.*, (2014), where they obtained reduced droplet diameter and PDI with increasing emulsification time and emulsifying agents (Span 80/Tween 80) [21].

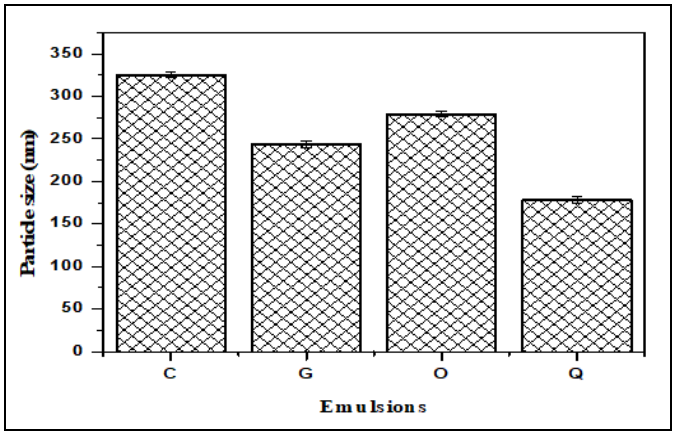

FIG. 4: EFFECT OF EMULSIFICATION TIME ON DROPLET SIZE OF EMULSIONS C, G, O & Q

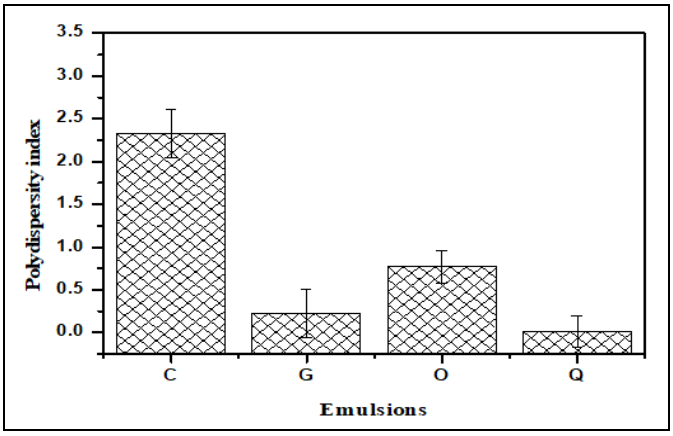

FIG. 5: EFFECT OF EMULSIFICATION TIME ON POLYDISPERSITY INDEX OF EMULSIONS C, G, O & Q

Antibiofilm Assay: The stable nanoemulsions were analyzed by the disc diffusion method and the microtiter plate method.

Disc Diffusion Method: Disc diffusion method was carried for the four stable emulsions C, G, O, and Q against biofilm bacteria such as S1, S2, and S3, respectively. Streptomycin (25 mca) was used as a positive control, and the negative control was the oil (dissolved in DMSO), and Tween 80. The surfactant solution did not exhibit antibacterial activity against all the tested strains. It can be observed that the emulsion demonstrated a higher zone of inhibition (mm) than oil for all the strains tested. The S1 was highly susceptible to zone size of 12 mm to mixed oil emulsion than S3 &S2, which showed a zone of 09 and 10 mm.

The reduction in particle size increases the surface area that may result in a greater interaction of emulsion with the bacterial membrane, thereby resulting in the enhanced antibacterial activity due to the activation of passive mechanisms of cell absorption. Our investigation based on the influence of the nanodroplet size on antimicrobial activity is similar to the previous reports [22, 23].

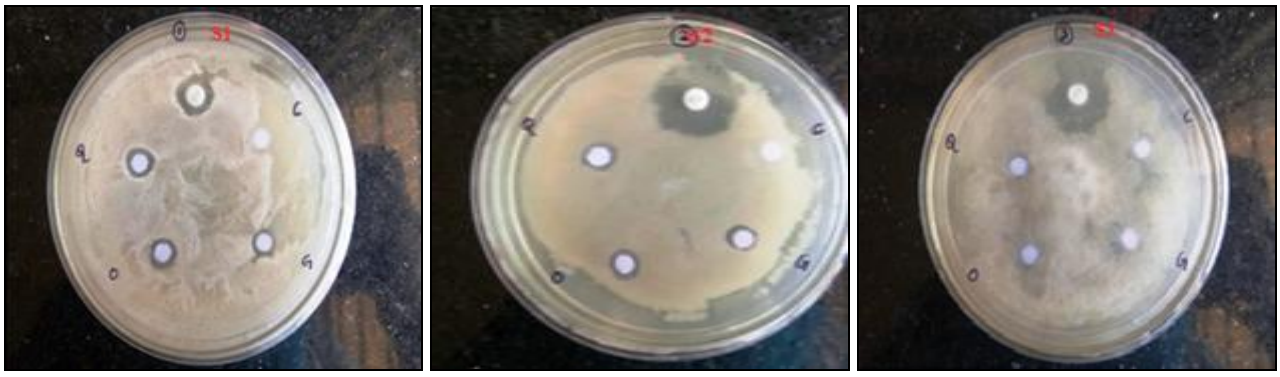

FIG. 6: ANTIBIOFILM ACTIVITY OF FORMULATED EMULSION C, G, O & Q

Antibiofilm Activity by Microtitre Plate Method: The study showed that the levels MIC observed range from 1:10 to 1:1000 ratios. Maximum Inhibition Concentration was observed with emulsion Q against S3 (1:10). Minimum Inhibition Concentration was shown in G against S3 (1:1000). Essential oils from plant extracts are a potentially useful source of antimicrobial compounds. In this study, Emulsion Q and C have significantly higher antibiofilm activity than G and O against the S1 strain.

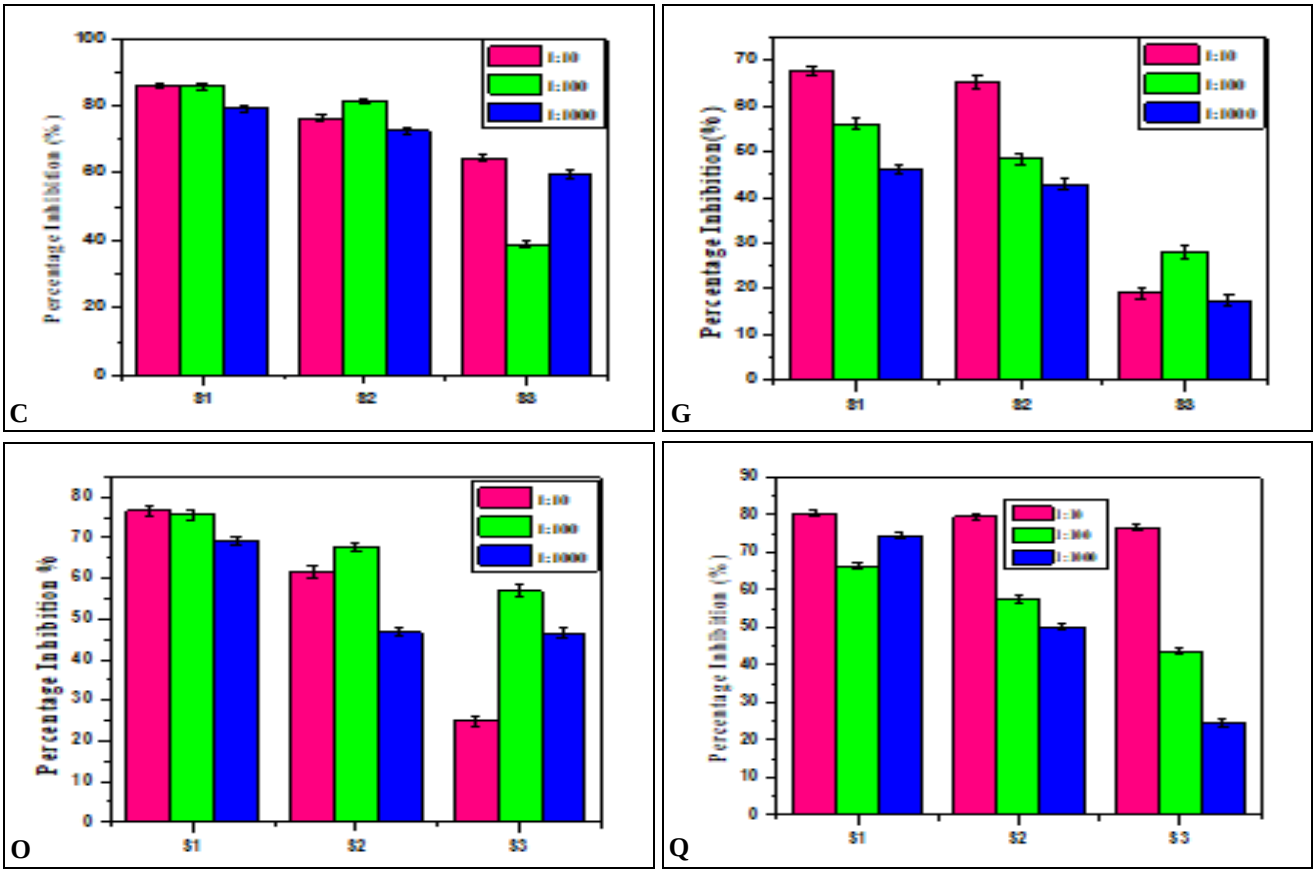

FIG. 7: INHIBITORY PERCENTAGE OF FORMULATED EMULSION C, G, O & Q

CONCLUSION: The plant samples were subjected to steam distillation for oil extraction. Emulsions were prepared by mixing extracted oils, surfactants, and water in different proportions. Thermal and mechanical stability of emulsions were studied, which confirmed that only emulsions C, G, N, O, P, Q, R, S, and T were stable. Simultaneously, three biofilm-forming bacterial species were isolated and checked for the effective formation of biofilm. Among the three cultures, S1, S2, and S3, S2 culture showed the highest biofilm formation. Among the stable emulsions, only C, G, O, and Q were selected for further antibiofilm assays because of their physical appearance. Results from disc diffusion method and microtitre plate method of antibiofilm assays proved that emulsions Q and C possess the highest antibiofilm capability than G and O against the S1 strain.

ACKNOWLEDGEMENT: I would like to acknowledge Department of Nanoscience and Technology, SREC, Coimbatore for their technical support.

CONFLICTS OF INTEREST: The authors declare that they have no conflicts of interest.

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How to cite this article:
Anandhi B, Subaharini M, Abinaya R and Ponnarmadha S: Formulation of emulsions from plant essential oils against microbial biofilms. Int J Pharm Sci & Res 2020; 11(11): 5725-32. doi: 10.13040/IJPSR.0975-8232.11(11).5725-32.