In Vitro Evaluation and Comparison of Antibacterial Activity of the Novel Herbal Aqueous Creams Containing Aqueous Extracts of the Leaves of *Ficus religiosa* L.

Randima T.R.[1*], Gajaman P.P.M.[1], Wimalasiri Y.S.G.[2], Perera P.K.[3], Navaratne V.[4] and Sanjeewani N.A.[1]

Abstract

In the current healthcare system, antibiotic resistance has become one of the crucial health issues. Discovering a novel antibiotic is a very complex and time-consuming process. Hence there is an increasing trend of conducting researches using herbal sources to treat infections which are resistant to currently available antibiotics. The objective of this study was to in vitro evaluation and comparison of the antibacterial property of herbal creams containing aqueous extracts of leaves of *Ficus religiosa* L. against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *Ficus religiosa* L. is a plant which has been using for many decades in Ayurvedic medicines to treat various types of illnesses including dermatological diseases. In this study, the antibacterial effect of the leaf extract was evaluated using agar well diffusion method. Using a dose-response curve, different concentrations of the extract were selected to formulate herbal creams. According to British Pharmacopoeia 2015, the aqueous cream samples were formulated without adding the preservative. Antibacterial effects of creams were evaluated against *S.aureus* and *P. aeruginosa.* The plant extract has shown a promising inhibitory action against the tested organisms. Some higher concentrations of plant extracts have shown a higher Zone of Inhibition when compared with the positive control (Gentamicin 50 µg/mL). All the cream samples have shown more inhibitory action against *S. aureus* than *P. aeruginosa.* The stability of the cream samples was evaluated. No change was observed in appearance, color and odor of cream samples in the period of 3 months. The overall results of this study indicate that the herbal creams have promising antibacterial properties and these results could be utilized in the pharmaceuticals industry to carry out more testing procedures to develop as an efficient antibacterial cream.

Keywords: Antibacterial property, Antibiotic resistance, *Ficus religiosa* L., Skin diseases, Formulations, Herbal creams

Introduction

The skin is considered as the largest organ in the human body. It is persistently exposed to potentially hazardous microbial and non-microbial agents of the environment. It acts as a physical barrier and provides the first lines of defense against these agents[1]. When treating dermatological diseases such as skin inflammations, microbial and fungal skin infections and skin cancer, the skin is considered as the main route of choice[2,3].

[1]*Department of Pharmacy, Faculty of Allied Health Sciences, General sir John Kotelawala Defence University, Sri Lanka.*
[2]*Unit of Swasthavritta, Department of Study in Ayurveda, Institute of Indigenous Medicine, University of Colombo, Sri Lanka.*
[3]*Unit of Dravyaguna vignana, Department of Study in Ayurveda, Institute of Indigenous Medicine, University of Colombo, Sri Lanka.*
[4]*Faculty of medicine, General sir John Kotelawala Defense University, Sri Lanka.*

**Correspondence: Randima T.R., Department of Pharmacy, Faculty of Allied Health Sciences, General sir John Kotelawala Defence University, Sri Lanka. Email: rushanirandima@gmail.com*

Figure 1: Tree of *Ficus religiosa* L

Ficus religiosa (Figure 1) belongs to the family "Moraceae". It *is* a medicinal plant which is commonly used in Ayurvedic and other traditional medical systems. It is known as the "Peepal" tree or "sacred fig" in English whereas in Sinhala it is known as the "Bo" tree. A wide range of diseases such as bacterial infections, viral infections, diabetes, helminthic diseases, dyslipidemia and cancers are treated using different parts of *F. religiosa* tree[4,5,6]. Various studies have been conducted to evaluate the antibacterial, antioxidant, antidiabetic, antiulcer, antiarthritic and anti-inflammatory effects of the different parts of the *F. religiosa* tree[6,7,8]. It is also used to treat various types of diseases of the gastro intestinal tract (vomiting, stomatitis, constipation, liver diseases), central nervous system (migraine, epilepsy), respiratory system (asthma, cough), infectious diseases (leprosy, tuberculosis, gonorrhea) and reproductive system. As well as the leaves and barks of *F. religiosa* are used to treat skin diseases[14,15,16].

Antibiotic treatment kills or suppresses the growth of a significant part of the microbial population by various mechanisms of action[11]. The effectiveness of presently available antibiotics is reducing significantly due to the emergence of resistance. According to World Health Organization (WHO), antimicrobial resistance is among one of the millennium developments goals at risk and endangers the achievement of sustainable development goals[12]. It is one of the greatest necessities to discover or develop novel antibiotics from natural/herbal sources to treat infections which are resistant to currently available antibiotics[13].

F. religiosa trees can be commonly found in the sub-Himalayan forests in Bengal and central India as well as in countries which are located in the Indian sub-continent like Pakistan, Bangladesh and Sri Lanka[17,14]. In Ayurveda, "*Panchavalkala*" is known as a combination of five barks of trees namely; *Nyagrodha* (*F. benghalensis* L.), *Udumbara* (*Ficus racemosa* Linn.), *Ashwatha* (*F. religiosa* L.), *Parisha* (*Thespesia populnea*) and *Plaksha* (*Ficus lacor*). This combination is widely used in Ayurveda as anthelmintic, antimicrobial, wound healing, anti-inflammatory agent and it is also used for cleansing purposes[18,19].

Many studies have been conducted to evaluate the antibacterial properties of *F. religiosa*, especially in India. An only a limited number of antibacterial preparations were formulated using this plant. The object of the present study was to *in vitro* evaluate, formulate and compare the antibacterial activity of herbal creams containing aqueous extract of *F. religiosa* leaves against *S. aureus* and *P. aeruginosa.*

Materials and Methods

Collection and preparation of plant material

The leaves were washed from distilled water to remove dust, soil and other materials on the surface of leaves and air-dried under shade for three weeks. Dried leaves were ground into powder by using an electric grinder. Powdered materials were stored in an airtight container for further use at room temperature (25 ± 5°C).

Preparation of aqueous extracts

Powdered leaves (50g) was added to a beaker containing 250 mL of distilled water. Each powder sample was kept on a mechanical shaker (Fisherbrand™ Seastar™ Digital Orbital Shaker) at 200 rpm and macerated for 48 hours, at room temperature. Then the macerates were filtered through two layers of clean muslin cloth in order to remove debris. The remaining aqueous portion was

removed using the freeze dryer (LABCONCO FreeZone 2.5). The extracts were labelled and stored in a refrigerator at 4-8°C until further use[20].

Preparation of concentration series for the microbial assay

A serial dilution was prepared using the final filtrate which started from 2000 µg/mL to 125 µg/mL.

Preparation of stock solution of Gentamicin as positive control

The stock solution was prepared using a commercially available Gentamicin (40 mg/mL) IV injection vial. 125 µL of Gentamicin solution was measured using a micropipette and transferred into a 100 mL volumetric flask. Then the flask was topped up with autoclaved distilled water until it reached the final volume of 100 mL.

Screening antibacterial effect of extracts of the leaves

Pure cultures of *P. aeruginosa* (ATCC 27853) and *S. aureus* (ATCC 25923) were obtained and sub-cultured in nutrient agar and stored in the refrigerator (4-8°C). The 0.5 McFarland standard was prepared by mixing 0.5 mL of 1.175% W/V $BaCl_2$ and 99.5 mL of 1% V/V H_2SO_4 with constant stirring to maintain a suspension.

Muller Hinton Agar (MHA) was prepared and after measuring the pH of the prepared agar solutions, they were placed in the autoclave at 121°C at 15 pounds per square inch for 15 minutes.

According to the direct colony suspension method, bacterial colonies of each organism (*S. aureus* and *P. aeruginosa*) were added separately to two sterile test tubes filled with 5 mL of saline solutions using a sterile inoculating loop. A single colony was added to saline solutions until turbidity is equivalent to the previously prepared 0.5 McFarland solution. These bacterial suspensions were used within 15 minutes of preparation.

Using separate micropipette tips per each bacteria, 1 mL of previously prepared bacterial suspensions were added into each MHA containing flask at about 40-45°C. Bacteria containing MHA solutions were added into autoclaved petri dishes using the pour plate method and six wells with equal diameter were prepared for each petri dish. Each well was filled by 200 µL of serial dilutions of the plant extract, Gentamicin 50 µg/mL (positive control) and distilled water (negative control). The plates were incubated at 37°C for 24 hours. After the 24 hours incubation period, the zones of inhibition around the wells were measured to the nearest millimeter and recorded.

Formulation of antibacterial herbal aqueous creams

According to the results of the antimicrobial assay of plant extracts, dose response curves were plotted using GraphPad Prism 6 software (version 6.01). Using the dose response curves, four concentrations of aqueous extract of *F. religiosa* were selected to formulate cream preparations.

As per BP 2015, emulsifying wax and Emulsifying ointment were prepared. The powdered extract was incorporated together with Emulsifying ointment, without adding the preservative (Phenoxyethanol) to prepare aqueous cream as described in BP.

Antibacterial assay of prepared herbal aqueous creams

Antibacterial assay of prepared herbal aqueous creams was carried out according to BP method of Gentamicin cream assay.

Microbial assay of cream samples

MHA medium was prepared and added test organisms were into MHA solutions. The mixtures were added into petri dishes using the pour plate method. In each petri dish, wells were prepared with equal diameter. Each well was filled by 200 µL of a solution obtained from the aqueous layer (top layer) of the separatory funnel containing herbal aqueous cream, Gentamicin (positive control) and the solution obtained from the aqueous layer (top layer) of the separatory.

Results

The results of the antimicrobial effects of leaves extract against *P. aeruginosa* are shown in Table 1.

Table 1: Zone of Inhibition shown by *Pseudomonas aeruginosa* with crude extracts of leaves

Concentration (µg/mL)	ZOI (mm)
2000	18.67±0.33
1750	18.50±0.40
1500	18.33±0.67
1250	18.00±0.00
1000	17.00±0.58
750	16.50±0.40
500	12.67±0.88
250	11.67±0.33
125	11.30±0.33
Gentamicin (50 µg/mL)	19±0.00
Distilled water	00.00

Data in the above table was expressed as mean Zone of Inhibition (mm) ± SEM.

According to the data expressed in Table 1, the positive control (Gentamicin 50 µg/mL) has shown a very clear ZOI compared to the ZOI which was shown by all plant extracts.

Antibacterial effect of leaves extracts against Staphylococcus aureus

The results of the antimicrobial effects of leaves extract against *S. aureus* are shown in Table 2.

According to the results expressed in Table 2, higher ZOI has exhibited against *S. aureus* by the aqueous extract of *F. religiosa* 1500 µg/mL, 1750 µg/mL and 2000 µg/mL concentrations when compared with the ZOI shown by Gentamicin 50 µg/mL against *S. aureus.*

Table 2: Zone of Inhibition shown by *Staphylococcus aureus* with crude extracts of leaves

Concentration (µg/mL)	ZOI (mm)
2000	18.33±0.67
1750	17.33±0.88
1500	17.00±0.00
1250	15.67±0.67
1000	12.67±0.88
750	12.33±0.88
500	11.50±0.40
250	11.30±0.67
125	11.00±0.00
Gentamicin (50 µg/mL)	15±0.00
Distilled water	0.00

Data in the above table was expressed as mean Zone of Inhibition (mm) ± SEM.

Dose-response study

Dose-response curves of aqueous leaves extract of *F. religiosa* against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Figure 2).

According to the dose-response curve in Figure 2, four concentrations of aqueous leaves extracts of *F. religiosa* were selected to formulate the herbal creams and they were labelled as follows (Table 3 and Figure 3 and Figure 4).

Table 3: Selected concentrations of aqueous leaves extracts of *Ficus religiosa* to formulate herbal aqueous creams

Concentration of crude extract (µg/mL)	Labelled as
500	FR aq 1
800	FR aq 2
1000	FR aq 3
1800	FR aq 4

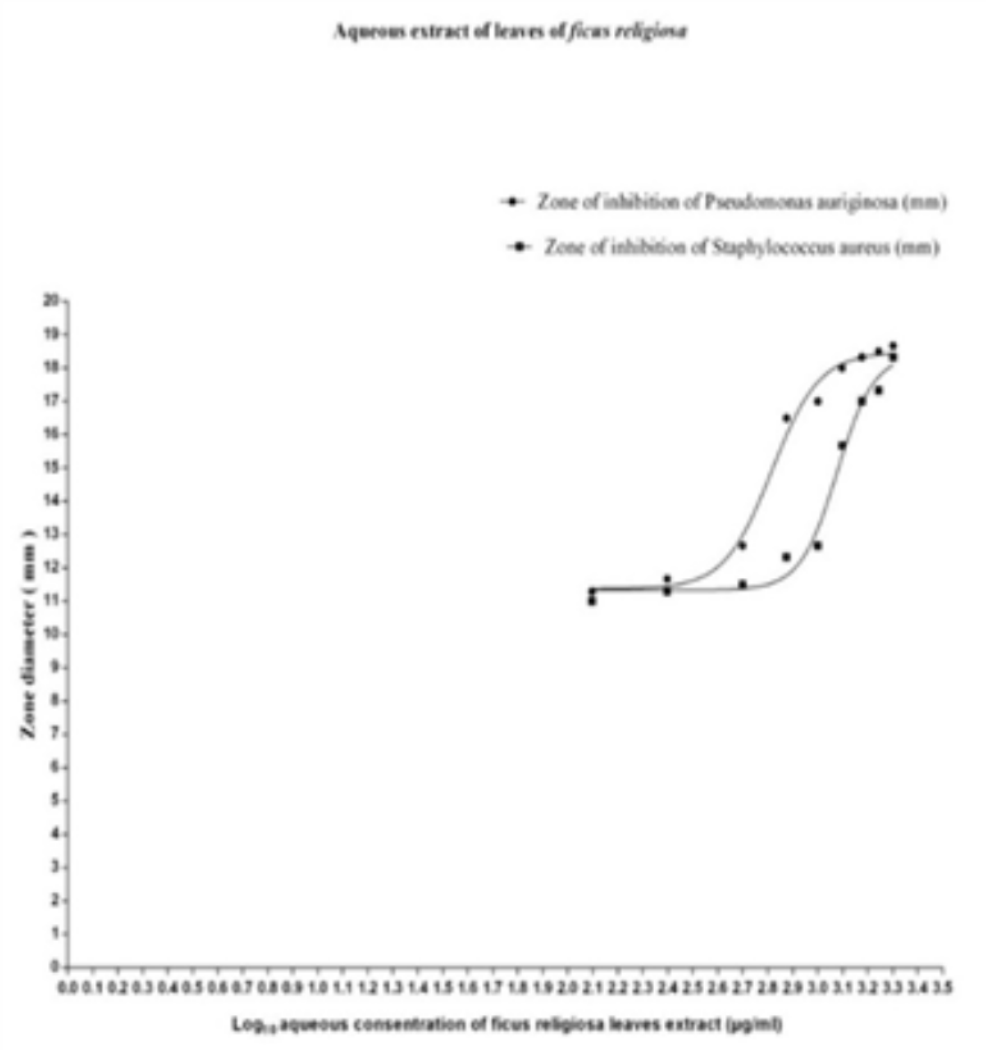

Figure 2: Dose response curve of aqueous leaves extracts of *Ficus religiosa* against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

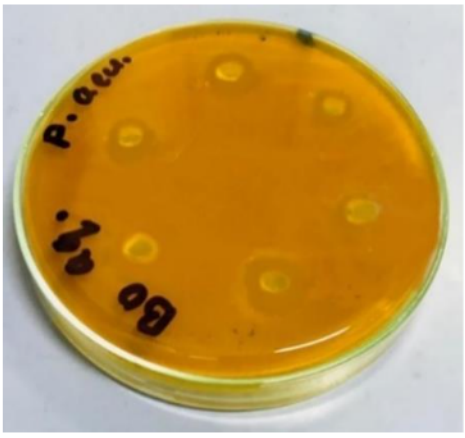

Figure 3: Antibacterial effect of aqueous extract of *Ficus religiosa*, against *Pseudomonas aeruginosa*

Figure 4: Antibacterial effect of aqueous extract of *Ficus religiosa*, against *Staphylococcus aureus*

Table 4 and Figure 5 and Figure 6 shows antibacterial effect of herbal aqueous cream containing aqueous extract of *Ficus religiosa* leaves against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

Table 4: Antibacterial effect of herbal aqueous cream containing aqueous extract of *Ficus religiosa* leaves against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

Sample	Zone of Inhibition (mm)	
	Staphylococcus aureus	***Pseudomonas aeruginosa***
FR aq 1	11.00±0.00	10.67±0.33
FR aq 2	12.00±0.00	10.67±0.33
FR aq 3	12.33±0.33	12.00±0.00
FR aq 4	14.00±0.58	12.00±0.58
Gentamicin (50 µg/mL)	19.00±0.00	15.00±0.00
Distilled water	0.00	0.00

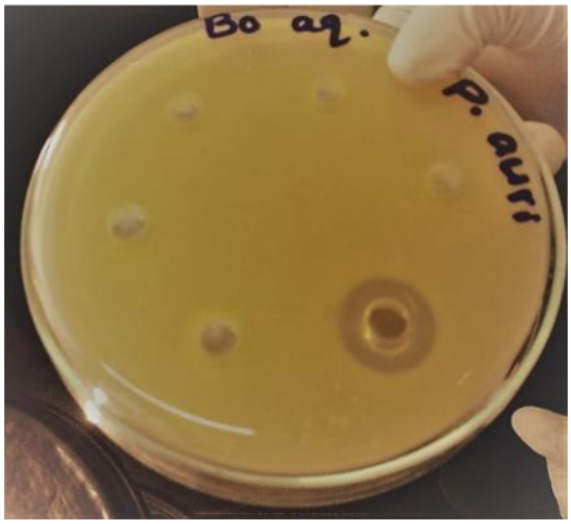

Figure 5: Antibacterial effect of herbal aqueous cream containing aqueous extract of *Ficus religiosa*, against *Pseudomonas aeruginosa*

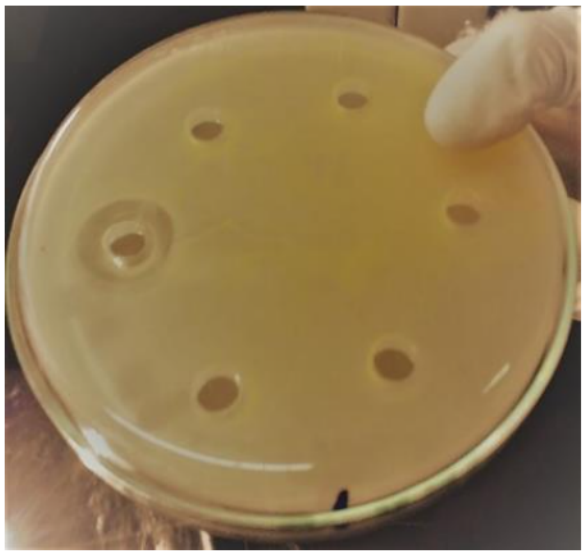

Figure 6: Antibacterial effect of herbal aqueous cream containing aqueous extract of *Ficus religiosa,* against *Staphylococcus aureus*

Stability evaluation

Evaluation of short-term stability

According to figure 7, four cream samples containing aqueous extracts *F. religiosa* were stable at room temperature throughout the study period of 16 days. Creaming, phase separation or coalescence couldn't be observed throughout the study period of 16 days.

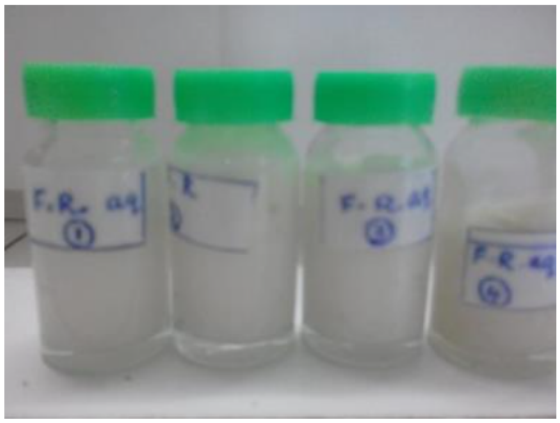

Figure 7: Aqueous herbal creams containing aqueous extracts of *Ficus religiosa* on the 16th day from the date of formulation

Figure 8 shows the aqueous herbal creams containing aqueous extracts of *Ficus religiosa* on the 90th day from the date of formulation

Figure 8: Aqueous herbal creams containing aqueous extracts of Ficus religiosa on the 90th day from the date of formulation

Evaluation of accelerated stability

Upon centrifugation of prepared herbal aqueous creams, the entire cream samples were stable throughout the period of 14 days (Figure 9).

Figure 9: On the 14th day after centrifugation aqueous herbal creams containing aqueous extracts of *Ficus religiosa*

Characterization of creams

pH evaluation

The pH values of all the cream preparations were within the range of 5.21-5.32 (Table 5) which are compatible with the pH range that should be included in a dermatological preparation (pH 4.5-6.0)22. No significant changes in pH were observed during the period of testing.

Viscosity

Viscosity of the cream samples at 28.2 ºC shown in Table 6.

Microscopic analysis

According to figure 10, herbal aqueous creams contain O/W emulsion.

Table 5: pH values of herbal cream samples containing aqueous extracts of *Ficus religiosa*

Type of cream	pH value					
	1st	15th	30th	60th	75th	90th
FR aq 1	5.21	5.23	5.19	5.20	5.22	5.22
FR aq 2	5.23	5.25	5.24	5.24	5.23	5.24
FR aq 3	5.22	5.24	5.23	5.23	5.25	5.23
FR aq 4	5.26	5.24	5.25	5.26	5.24	5.25

Table 6: Viscosity of the cream samples at 28.2 °C

Sample	Viscosity (cps) at rpm					
	0.3	0.6	1.5	3	6	12
FR aq 1	360,000	194,000	97570	65786	43590	31443
FR aq 2	359,700	192,580	97345	63580	42670	31170
FR aq 3	361,245	193,585	97438	63650	42680	31357
FR aq 4	359,860	193,000	97447	63570	42785	31379

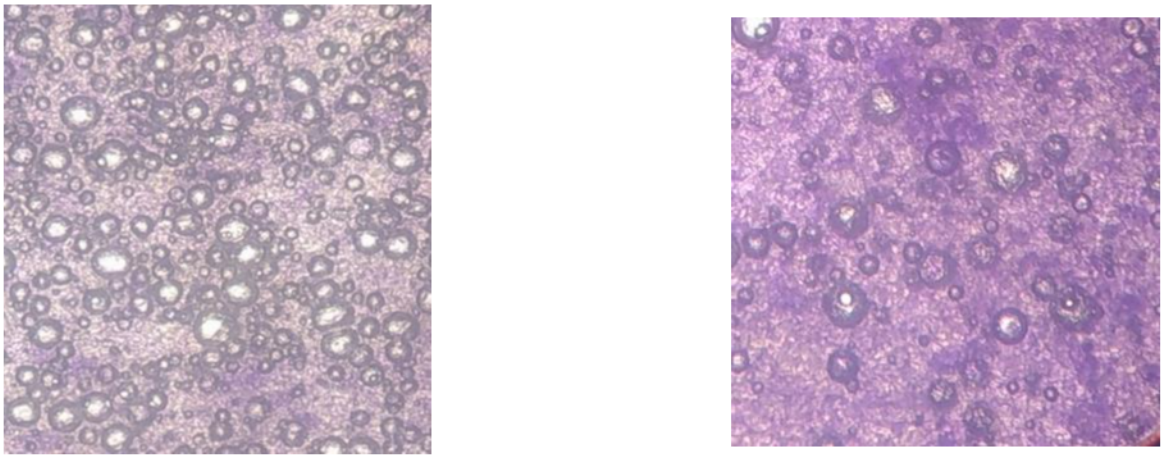

Figure 10: Microscope analysis of aqueous herbal creams containing aqueous extracts of *Ficus religiosa* under 100X magnification

Discussion

Creams have been used for many centuries to improve the wound healing process, to treat dermatological diseases, to protect the skin, as well as to improve natural beauty. Creams are one of the most popular dermatological preparations available, because of its semisolid properties facilitate ease of application, ease of removal and better penetration of drug/s into the skin layers[23,24].

Aqueous cream BP is a widely prescribed product as an emollient and soap substitute in the treatment of dry skin conditions. It relieves skin dryness by providing moisture to the skin. The Medicines and Healthcare products Regulatory Agency (MHRA) in the United Kingdom indicates that Sodium Lauryl Sulphate contained in aqueous cream is mainly responsible for stabilizing the O/W emulsion rather than its detergent property.

Dermatological diseases are one of the major health conditions in developing countries. The majority of cutaneous bacterial infections are caused by either *Staphylococcus aureus* or *Streptococci*[25,9].

In the current healthcare sector, antibiotic resistance has become one of the most critical health issues facing patients and healthcare workers[12]. Discovering a new therapeutic agent is a complex process and it consumes more than 10 years to reach a drug to the market[26]. However, many researches are being conducted to discover or develop novel antibiotics from herbal sources to treat infections which are resistant to currently available antibiotics[13].

Since ancient times, plants have been used to treat a vast range of diseases. At the present, there is an increasing trend to utilize herbal medicines and in most of the developing countries, people tend to use herbal medicines because of better compatibility with the body, lesser side effects and better cultural acceptability[26,27,28].

In the current study, *in vitro* antibacterial properties of aqueous extracts of *F. religiosa* were evaluated against *Staphylococcus aureus* and *Pseudomonas aeruginosa* separately. Moreover, using selected concentrations of this crude substance, aqueous herbal creams were formulated and evaluated for their antibacterial properties. This plant was selected to conduct this study because it is still used in Ayurvedic medicine in Sri Lanka. As well as a collection of plant materials is unchallenging because they are highly abundant in the area.

Before conducting the research, plant materials were authenticated by the National Herbarium, Peradeniya, Sri Lanka. It is one of the crucial steps to identify the plant materials prior to the study because many plants that belong to the same family are similar in appearance and it may cause misleading the results of the study.

Even though in Ayurveda medicine, barks of the plant are used to obtain the antimicrobial property, according to literature leaves of *F. religiosa* L. have been proven to possess more antibacterial activity than that of the barks of the tree[29,16]. Hence leaves were used in the current study to evaluate the antibacterial property.

In this study, the maceration process was used to obtain the aqueous plant extract. In order to increase contact between plant materials and the solvent, dried leaves were ground using a grinder. A mechanical shaker was used during the maceration process to increase the speed of the maceration.

In this research, the antibacterial effect of the crude plant extract and the herbal aqueous creams containing the plant extract was evaluated against two organisms. *Pseudomonas aeruginosa* is a Gram-negative bacteria while *Staphylococcus aureus* is a Gram-positive bacteria. These two kinds of bacteria were used in this study to identify and compare the inhibitory activity of *F. religiosa* against both kinds of bacteria.

According to the literature, *F. religiosa* leaves extracts have shown antibacterial action against both Gram-positive and Gram-negative bacteria[16,20,6]. Considering table 1 and 2, it has also been proven in this study. It was noted that aqueous extract has shown more inhibitory action against *P. aeruginosa* than *S. aureus.* The same result was obtained in the study carried by Shrijana *et al*., 2017.

Gentamicin (50 µg/mL) was used as the positive control in this study and it has shown 19 mm and 15

mm of ZOI against *P. aeruginosa* than *S. aureus* respectively.

According to table 2, 1250 µg/mL, 1500 µg/mL, 1750 µg/mL and 2000 µg/mL of aqueous extracts of *F. religiosa*, have shown the same or greater ZOI against *S. aureus* when compared to Gentamicin (50 µg/mL).

According to the literature, the majority of cutaneous bacterial infections are caused by *S. aureus* than *P. aeruginosa*[25,9]. Hence according to table 2, aqueous extract of *F. religiosa* has promising activity against skin infections caused by *S. aureus* when compared with Gentamicin (50 µg/mL).

Using the linear range of the dose-response curve of crude plant extract, suitable concentrations were selected to formulate the cream samples. The concentrations of the plant extract which were selected from the linear range of the dose-response curve were incorporated into the aqueous phase of the cream samples.

The herbal creams were formulated using the fusion method (Pharmalabs.unc.edu, 2017). The literature reveals that constituents in the leaves of the plant can be heated around 100°C without destroying the properties of the crude substances[26,16]. It is recommended to heat the aqueous phase a few degrees higher than the oil phase when both phases are being mixed to make a stable ointment because the aqueous phase tends to cool faster than the oil phase. This may lead to solidification of the active ingredient or excipients and finally, phase separation may occur[30].

In this study, the preservative of aqueous cream BP (Phenoxyethanol) was not included when preparing the herbal cream samples assuming it may have an impact on the result of the antibacterial effect of formulated herbal cream samples[31].

Prior to conduct the microbial assay of the prepared cream samples, plant extracts were separated according to the BP 2015 method of Gentamicin cream assay. In this study, only the aqueous layer inside the separatory funnel was used to evaluate the inhibitory action of the cream samples with the assumption of crude extracts may retain in the aqueous phase of the herbal creams without diffusing them to the oil phase.

According to tables 3, all tested concentrations of all the prepared herbal cream samples containing leaves extracts have shown less ZOI against both organisms when compared to the positive control (Gentamicin (50 µg/mL). The concentrations of each cream sample have diluted than the selected concentrations from the dose-response curves because the calculations were made to formulate the creams as per the selected concentrations were included in the aqueous phase of the creams. As well as during the separation procedure which was done prior to the assay of the creams, some amount of the crude extracts in the aqueous phase might be diffused to the oil phase. Considering the results in table 4, almost all the herbal creams have shown more inhibitory action against *S. aureus* than that of *P. aeruginosa*.

Stability testing of a drug product is crucial, in order to maintain the quality and safety and to determine the shelf life of the product. A formulation may undergo changes like its pH, consistency and appearance. Mainly there are two types of stability testing based on the time duration of stability evaluation namely; accelerated stability testing and real-time stability testing (Bhagwat *et al*., 2017). In this research, the samples were undergone stress conditions to evaluate the accelerated stability such as high temperature (37°C), low temperature (2-8°C) for four weeks and centrifugation.

No significant changes in color or odor were observed in each cream sample which was stored at room temperature, at 37-38°C and in the refrigerator (2-8°C) for 90 days.

The pH of the freshly prepared creams was measured in this study. The pH values of all the cream preparations were within the range of 5.21-5.32 (Tables 5) which are compatible with the pH range that should be included in a dermatological preparation (pH 4.5-6.0)[22].

The main purpose of this study was to formulate antibacterial creams using aqueous extracts of the leaves of *F. religiosa* L. compare the antibacterial activity of these formulations against tested

organisms and determine the stability of prepared herbal cream samples. The overall results of this study indicate that the herbal aqueous creams containing aqueous extract of the leaves of *F. religiosa* have promising antibacterial properties and these results could be utilized in the pharmaceuticals industry to carry out more testing procedures to develop as an efficient antibacterial cream.

Conclusion

The results of the present study suggest that selected plants can be used to treat various types of bacterial infections. According to the results obtained from the evaluation of the antibacterial effect of cream samples, it can be concluded that all cream samples have promising antibacterial activity against skin infections caused by *S. aureus* when compared with the skin infections caused by *P. aeruginosa.*

Recommendations

The present study indicates that the herbal aqueous creams containing aqueous extracts of the leaves of *F. religiosa* L. when further developed, can be used as effective dermal preparation for bacterial skin infections. The most active molecules can be identified and isolated by conducting a further pharmacological evaluation. As well as the safety and the activity of these cream samples can be further evaluated by conducting clinical trials.

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