

## Research Article

# Antimicrobial activity of a combination of three natural plant extracts and development of a herbal soap

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## ABSTRACT

*Azadirachta indica*, *Cassia fistula* and *Nelumbo nucifera* are medicinal plants that are frequently found in Sri Lanka. Although the antimicrobial activity of these plants has been investigated individually, the effectiveness of a combined extract has not been determined in previous studies. This study aimed to evaluate the antimicrobial activity of *A. indica*, *C. fistula*, *N. nucifera* and their combined extract to formulate an antimicrobial herbal soap. Different concentrations of aqueous and ethanol extracts of powdered leaves of *A. indica*, *C. fistula* and flowers of *N. nucifera* were prepared separately. Each extract was tested against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* using the agar well diffusion method. Combined extract was prepared using selected extracts of each plant with the highest antimicrobial activity. Combined extract formulated from ethanol extract of *C. fistula* and aqueous extracts of *A. indica* and *N. nucifera* was incorporated into a herbal soap. The antimicrobial activity of the combined extract and the herbal soap was determined using the agar well diffusion method. Combined extract and formulated soap exhibited antimicrobial activity against tested organisms with the highest activity against *S. aureus*. The physical and chemical parameters of the formulated herbal soap were determined. The pH of the formulated soap at 28°C was 9.11, the percentage of alcohol insoluble matter and free alkali were 24.6% and 1.6% respectively, which were within the accepted range. In conclusion, compared to the results of individual extracts, the soap has demonstrated enhanced antimicrobial activity against tested organisms. Further development of herbal soap as a value added product would be beneficial.

### Keywords:

*Azadirachta indica*, Antimicrobial activity, *Cassia fistula*, Combined extract, Herbal soap, *Nelumbo nucifera*

## 1. INTRODUCTION

Medicinal plants have been used in traditional medicine predominantly in Asian countries and they are considered as the root of traditional medicine. Many recent research studies showed that most of the medicinal plants possess diverse biological activities such as antibacterial, antifungal, antiviral, antioxidant and anti-inflammatory effects, etc<sup>1</sup>. As one of the best sources of obtaining antimicrobial agents, medicinal plants are gaining attention in the present world today. That is mainly due to increased resistance to synthetic compounds, inexpensive, fewer side effects compared to

synthetic compounds and availability. Recent studies have revealed that natural constituents of higher plants include new sources of antimicrobial agents<sup>2</sup>. Plants possess antimicrobial properties due to their secondary metabolites. Tannins, flavonoids, phenolic compounds and alkaloids have demonstrated *in vitro* antimicrobial activity<sup>3</sup>.

Sri Lanka has a diverse medicinal plant system. Medicinal herbs used in Ayurveda medicine are reported to possess antimicrobial activity. According to Hewage et al.,<sup>4</sup> in Sri Lanka, there are about 3300 flowering plants in which 830 species (25%) are endemic to Sri Lanka.

There are a vast number of conditions that can

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affect skin diseases. Skin infections are of several types that differ from the type of pathogen which causing infection such as fungal infections and bacterial infections<sup>5</sup>. Recently there is a surge of using various antiseptics, including commercially available synthetic soaps and alcohol based sanitizers. Even though these formulations reduce the transmission of contagious diseases, these are produced on a large scale with harsh chemicals, synthetic ingredients such as parabens, phthalates, petrochemicals, cheap fragrance oils and artificial colors all of which may lead to skin irritation and resistance among pathogens. Hence there is an urgent need to develop herbal soaps and other disinfectants and evaluate their efficacy.

*Cassia fistula* belongs to the family of *Fabaceae* and is commonly known as a golden shower plant. It has greenish-grey bark and has compound leaves. The conspicuous feature of this plant is golden yellow color hanging flower bunches about 40 cm in length<sup>6</sup>. Recent research findings have demonstrated that *C. fistula* possesses antioxidant, anti-mutagenic, antitumor, anti-microbial and anti-inflammatory activities<sup>7</sup>. *C. fistula* consists of various constituents such as flavonoids, phenols and proanthocyanidins. The bark, leaves and flowers of the plant were evaluated for potential constituents with antimicrobial activity<sup>7</sup>. *C. fistula* is considered as one of the abundantly used medicinal plants in Unani and Ayurvedic medical systems. Fruit pulp, bark, flowers, pods, leaves and roots of the plant are used<sup>8</sup>. Preparations like infusions, decoctions or powders which are either alone or in combination with other medicinal plants are used in traditional medicine<sup>9</sup>. Mainly the tender leaves are used as a purgative while the older ones together with the bark are ground into a paste and applied during ringworm infections, insect bites, rheumatism, facial paralysis, leprosy, chronic eczema and psoriasis<sup>10</sup>. In traditional system of medicine, the plant is used against skin diseases, diabetes, haematemesis, worm and microbial infections, abdominal diseases, abdominal tumours, heart diseases, during retention of urine, abdominal colic, haemostatic, due to its antipyretic and analgesic nature and to reduce oedema and prevent burning sensation<sup>8,11</sup>. Chakramardha tailamu, is a compound ayurvedic oil prepared using *C. fistula* which is beneficial in eczema, ringworm and other skin diseases. The plant is used in preparations such as Maha-marichayadi taila, aragvadhadi-kwatha etc<sup>9</sup>.

*Azadirachta indica*, which is often known as Neem, belongs to the family of *Meliaceae*. Leaves of the plant are compound and each carry 5-15 leaflets. According to several studies conducted, it has been demonstrated that different plant parts of *A. indica* possess diverse medicinal properties such as antibacterial, antifungal, antiviral, anti-oxidant, anti-malarial, etc<sup>12</sup>. Azadirachtin, nimbin, nimbidol and nimbolinin are important compounds that have been isolated from

the plant<sup>13</sup>. Mahmoodin, margolone, mergolonone and isomargolonone which have been isolated from *A. indica* show antibacterial activity whereas cyclic trisulphide and cyclic tetrasulphides show antifungal activity<sup>14</sup>. The plant is used in Ayurveda, Unani and homeopathy for the treatment of many infectious, metabolic or cancer diseases<sup>9</sup>. The leaves are bitter, astringent, acrid, depurative, demulcent and refrigerant<sup>15</sup>. A strong decoction of the fresh leaves has antiseptic properties and is used for cleaning wounds, ulcers and leprosy<sup>16</sup>. Leaves poultice is used for boils and sores treatment whereas the infusion is reported to be used in the treatment of malarial and intermittent fevers. Leaf juice is given in worms, jaundice and skin diseases<sup>11,16,17</sup>. In Sri Lanka, the juice of the fresh leaves is given with rock salt for intestinal worms and with honey for jaundice and skin diseases<sup>10</sup>. The leaf paste has been used externally for small pox treatments whereas the tincture is applied locally to treat bruises and sprains. Leaf ash mixed with ghee is useful in psoriasis<sup>16</sup>. Moreover, *A. indica* is useful in leucoderma, pruritus, ophthalmopathy, dyspepsia, tuberculosis, burning sensation and eczema and exhibits wound healing properties<sup>11,15</sup>.

*Nelumbo nucifera* is an aquatic plant, belonging to the family of *Nelumbonaceae*. This is a rhizomatous plant consisting of a creeping, elongated stem with nodal roots. It is having cup-shaped aerial leaves and flat-shaped floating leaves. The colour of the flower varies from white to reddish-pink and has a pleasant fragrance. The flower of *N. nucifera* demonstrated hypoglycemic, antipyretic, antioxidant, antimicrobial and antihypertensive abilities. Furthermore, it also acts as anti acne due to its antibacterial properties<sup>17</sup>. A variety of chemical constituents with different therapeutic activities were isolated from the flowers of *N. nucifera*<sup>18</sup>. The flower is used in Ayurveda, Siddha, Unani and other medicinal systems<sup>9</sup> and reported to be astringent refrigerant and cardiogenic<sup>14</sup>. The stamens of the flowers are used for bleeding piles and debility and weakness in children<sup>10</sup>. Flowers with stamens and juice of the flower stalk are used for diarrhea, cholera, fever, liver complaints and gastric ulcers in Ayurveda<sup>10</sup>. The dose of the flower extract used in decoctions is 12-24 g and has been claimed in the treatment of skin diseases<sup>9,11</sup>. Syrup made from flower is recommended for coughs, dysentery and haemorrhages and the ground petals are assumed to be effective in rectifying syphilis in Malaya<sup>10</sup>. A scanty literature was available on formulation of herbal soaps and evaluating their antimicrobial activity.

Moreover, the available literature did not support evidences on any herbal formulations based on combined extracts of *C. fistula*, *A. indica* and *N. nucifera*. Therefore, the purpose of this study was to formulate a herbal soap using the extracts of *C. fistula*, *A. indica* and *N. nucifera* and to investigate the antimicrobial activity

of the combined extract and the herbal soap against common microorganisms. Furthermore, this study was aimed to evaluate the physicochemical parameters of the prepared herbal formulation.

## 2. MATERIALS AND METHODS

### 2.1. Study setting

The study was conducted at the Department of Pharmacy and Pharmaceutical Sciences and Department of Medical Laboratory Sciences, Faculty of Allied Health Sciences, University of Sri Jayewardenepura, Sri Lanka and Industrial Technology Institute, Colombo 07, Sri Lanka.

### 2.2. Collection of plant materials and authentication

Fresh leaves of *A. indica*, white flowers of *N. nucifera*, and fresh leaves of *C. fistula* were collected from Southern Province and Western Province, Sri Lanka. Plants were authenticated at the National Herbarium, Botanical Gardens, Peradeniya, Sri Lanka and the voucher specimens were deposited under reference numbers, A Z Pharm .01 (*A. indica*), N N Pharm .01 (*N. nucifera*) and C F Pharm .01 (*C. fistula*).

### 2.3. Preparation of extracts

The collected plant parts (leaves and flowers) were washed, air dried for three days and dried in a hot air oven at 40°C until constant weight was obtained and powdered to a coarse powder, and stored in air tight bottles for the studies. A sample (50 g) of each plant was added to a round bottom flask containing 150 ml of ethanol or distilled water and boiled for four hours. Then the extract was filtered and the filtrate was concentrated using a rotary evaporator. Water extract was freeze-dried. The extracts were stored at 4°C until used<sup>19</sup>.

### 2.4. Determination of antimicrobial activity of selected plant extracts

The antimicrobial activities of the selected plant extracts were determined by the agar well diffusion method<sup>20</sup>. Isolates of *Candida albicans*, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were obtained from the Department of Microbiology, Faculty of Medical Sciences, University of Sri Jayewardenepura. Subcultured organisms were used to prepare microbial suspensions. The turbidity of the suspensions was adjusted to 0.5 McFarland standard ( $1 \times 10^8$  CFU/ml inoculum). The prepared inoculum was streaked by using a sterile cotton swab on the surface of Mueller Hinton Agar (MHA) using the spread plate method. Then the plates were allowed to dry for 5 min.

Wells were cut by using a sterile dropper with a 6 mm diameter. A drop of molten agar was added to each well to seal the bottom of the well. Stock solutions were prepared for each extract with a concentration of 1000 mg/ml in 1% dimethyl sulphoxide (DMSO). Each stock solution was diluted using 1% DMSO and a concentration series ranging from 31.25-1000 mg/ml was prepared. From each concentration, 50 µl were added to the wells.

Bacterial cultures and fungal cultures were incubated at 37°C for 24 hrs. and 48 hrs. respectively. 0.03% of gentamycin and 1% of clotrimazole were used as a positive control for bacteria and fungi respectively, and 1% DMSO was used as the negative control. Each concentration includes triplicates. Antimicrobial activity was determined by measuring the diameter of the zone of inhibition around the well against each microorganism<sup>20,21</sup>.

### 2.5. Determination of the effective extract of each plant and effective concentration of selected plant extracts against tested microorganisms to formulate a combined extract

According to the results of the antimicrobial activity of each extract of the three plants, dose-response curves were graphed separately. The suitable types of extracts were selected from each dose-response curve by considering the IC<sub>50</sub> value of each plant extract to incorporate into the combined extract. Effective concentrations of extracts selected were determined. Dose-response curves of selected plant extracts were graphed separately against each microorganism tested. Effective concentrations were selected at the highest point of the linear range. A common effective concentration for all tested organisms was selected from the linear range of the graph.

### 2.6. Formulation of herbal soap using the combined extract

Glycerin soap base was weighed and melted in a water bath. Previously determined concentrations of plant extracts were prepared by dissolving the relevant solvents (Assuming that the active ingredient should be about 5% of the total weight of soap). The plant extracts were added to the melted soap base. Stearic acid was dissolved in a small amount of hot water and added to the mixture. As the fragrance enhancer, the volatile oil of *N. nucifera* was added. The ultimate mixture was stirred using a magnetic stirrer. The melted mixture was poured into separate moulds and left until solidified<sup>22</sup>.

### 2.7. Determination of the antimicrobial activity of the combined plant extract and formulated herbal soap

The antimicrobial activity of the combined

extract was determined by the agar well diffusion method<sup>20</sup>. According to the proportions determined, the selected concentration of each plant extract was introduced into the wells of the agar plates that were previously streaked with *S. aureus*, *P. aeruginosa* and *C. albicans*. A percentage of 0.03% gentamycin and 1% clotrimazole were used as the positive control for bacteria and fungi respectively. 1% DMSO was used as the negative control. The plates were incubated at 37°C for 24 hrs. (48 hrs. for fungi) and the diameters of the zone of inhibition were measured. Six concentrations of formulated soap were prepared by dissolving it in 1% DMSO. Antimicrobial activity of each solution was tested by agar well diffusion method<sup>22</sup>.

## 2.8. Evaluation of physicochemical parameters of the formulated herbal soap

### 2.8.1. Physical characteristics

Odour, colour and appearance of the formulated soap were observed<sup>22</sup>.

### 2.8.2. Determination of pH

Formulated soap was dissolved in 100 ml of distilled water and stored for 2 hours. Then pH was measured by a calibrated pH meter<sup>22</sup>.

### 2.8.3. Determination of percentage free alkali

Formulated soap (5 g) was dissolved in 50 ml of neutralized alcohol. The mixture was refluxed for 30 minutes. The resulted mixture was cooled and titrated with 0.1 N HCl using phenolphthalein as the indicator<sup>22</sup>.

### 2.8.4. Foam height

Formulated soap (0.5 g) was dissolved in 25 ml of distilled water and it was transferred into a 100 ml measuring cylinder. Volume of soap mixture was made up to 50 ml with water. Then the cylinder was shaken 25 times and allowed to stand until the aqueous volume

makes up to 50 ml. Finally, the height of the foam was measured above the 50 ml mark of the measuring cylinder<sup>22</sup>.

### 2.8.5. Foam retention

Formulated soap solution (1%) was prepared by dissolving the soap in distilled water. A volume of 25 ml of soap mixture was placed in a 100 ml measuring cylinder and shaken it for 10 times. The volume of foam was recorded for 10 min at 1-min intervals<sup>22</sup>.

### 2.8.6. Alcohol insoluble matter

Formulated soap (5 g) was dissolved in 50 ml of warm ethanol vigorously. The resulting solution was filtered through a tarred filter paper with 20 ml of additional warm ethanol. Then the filter paper was kept in the oven for 1 hour at 105°C. Finally, the weight of the residue was weighed<sup>22</sup>.

### 2.8.7. Moisture / Volatile matter

Formulated soap (5 g) was placed in an oven for 2 hrs at a temperature of 105°C and repeated until a constant weight was obtained and the % moisture was calculated<sup>22</sup>.

## 2.9. Statistical analysis

The data were presented as mean±SD (Standard Deviation) of three replicates. One-way analysis of variance (ANOVA) was performed by using IBM SPSS Statistics 25.0 software to determine significant group differences and means were considered as statistically significant if  $p < 0.05$ .

## 3. RESULTS

### 3.1. Determination of the zones of inhibition of different concentrations of *C. fistula* leaf extracts against selected microorganisms

**Table 1.** Diameter of the zones of inhibition of different concentrations of leaf extracts of *C. fistula* against selected microorganisms.

Concentration of <i>C. fistula</i> leaf extract (mg/ml)	The diameter of zone of inhibition (mm) of leaf extracts of <i>C. fistula</i>					
	Ethanol extract			Aqueous extract		
	<i>S.aureus</i>	<i>P.aeruginosa</i>	<i>C. albicans</i>	<i>S. aureus</i>	<i>P.aeruginosa</i>	<i>C. albicans</i>
1000	21.0±0.7*	10.0±0.5*	0.0±0.0	18.0±0.7*	12.0±0.5*	0.0±0.0
500	18.0±0.7*	8.0±0.5*	0.0±0.0	16.0±0.7*	7.0±0.5*	0.0±0.0
250	15.0±0.7*	0.0±0.0	0.0±0.0	11.0±0.7*	0.0±0.0	0.0±0.0
125	13.0±0.7*	0.0±0.0	0.0±0.0	9.0±0.7*	0.0±0.0	0.0±0.0
62.5	9.0±0.7*	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
31.25	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Positive control	37.0±1.7*	32.0±1.7*	25.0±1.7*	33.0±1.7*	31.0±1.7*	31.0±1.7*
Negative control	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Data presented as Mean±SD

\*Significant when compared with the negative control;  $p < 0.05$

**Table 2.** Diameters of the zone of inhibition of different concentrations of white flower extracts of *N. nucifera* against selected microorganisms.

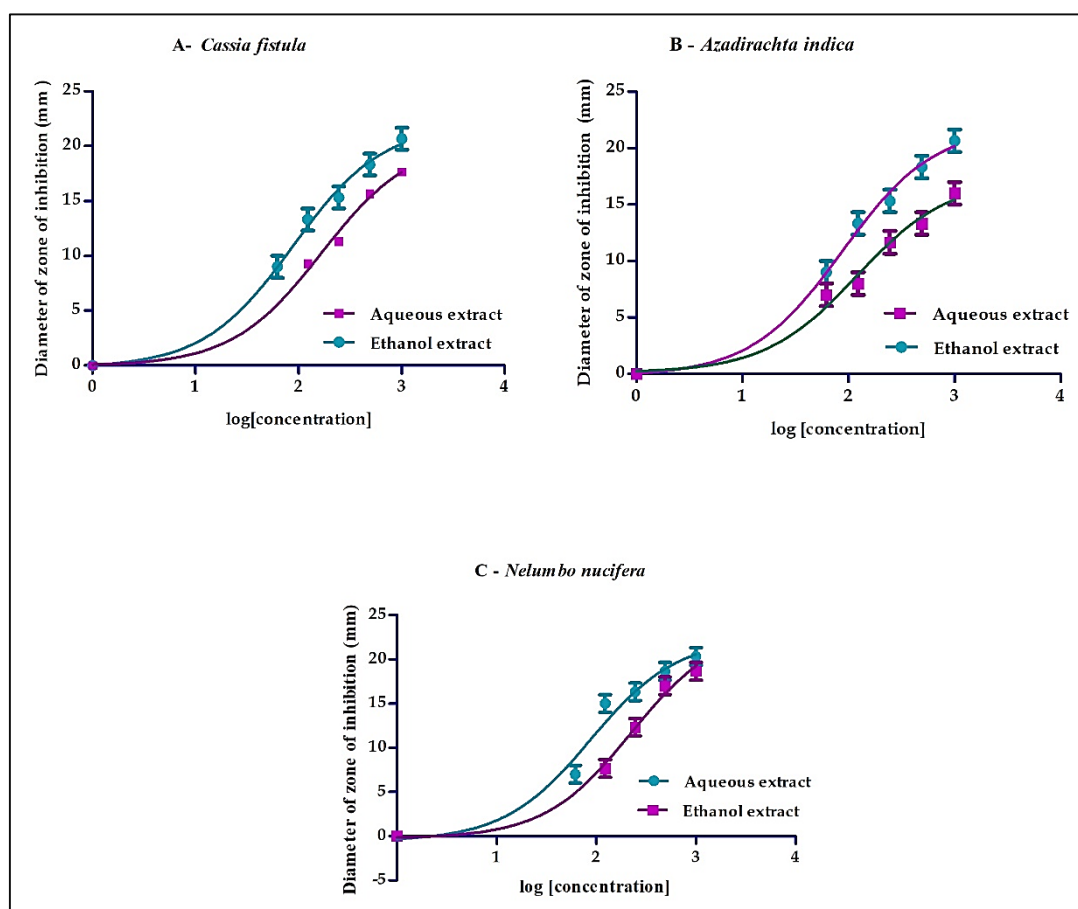
Concentration (mg/ml) of <i>N. nucifera</i> flower extract	The diameter of zone of inhibition (mm) of white flower extracts of <i>N. nucifera</i>					
	Ethanol extract			Aqueous extract		
	<i>S.aureus</i>	<i>P.aeruginosa</i>	<i>C. albicans</i>	<i>S. aureus</i>	<i>P.aeruginosa</i>	<i>C. albicans</i>
1000	19.0±0.5*	10.0±0.3*	0.0±0.0	20.0±0.5*	10.0±0.3*	10.0±0.3*
500	8.0±0.5*	9.0±0.3*	0.0±0.0	10.0±0.5*	7.0±0.3*	8.0±0.3*
250	12.0±0.5*	7.0±0.3*	0.0±0.0	16.0±0.5*	0.0±0.0	7.0±0.3*
125	8.0±0.5*	0.0±0.0	0.0±0.0	15.0±0.5*	0.0±0.0	0.0±0.0
62.5	0.0±0.0	0.0±0.0	0.0±0.0	7.0±0.5*	0.0±0.0	0.0±0.0
31.25	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Positive control	31.0±0.5*	31.0±0.3*	25.0±0.3*	30.0±0.5*	30.0±0.3*	31.0±0.3*
Negative control	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

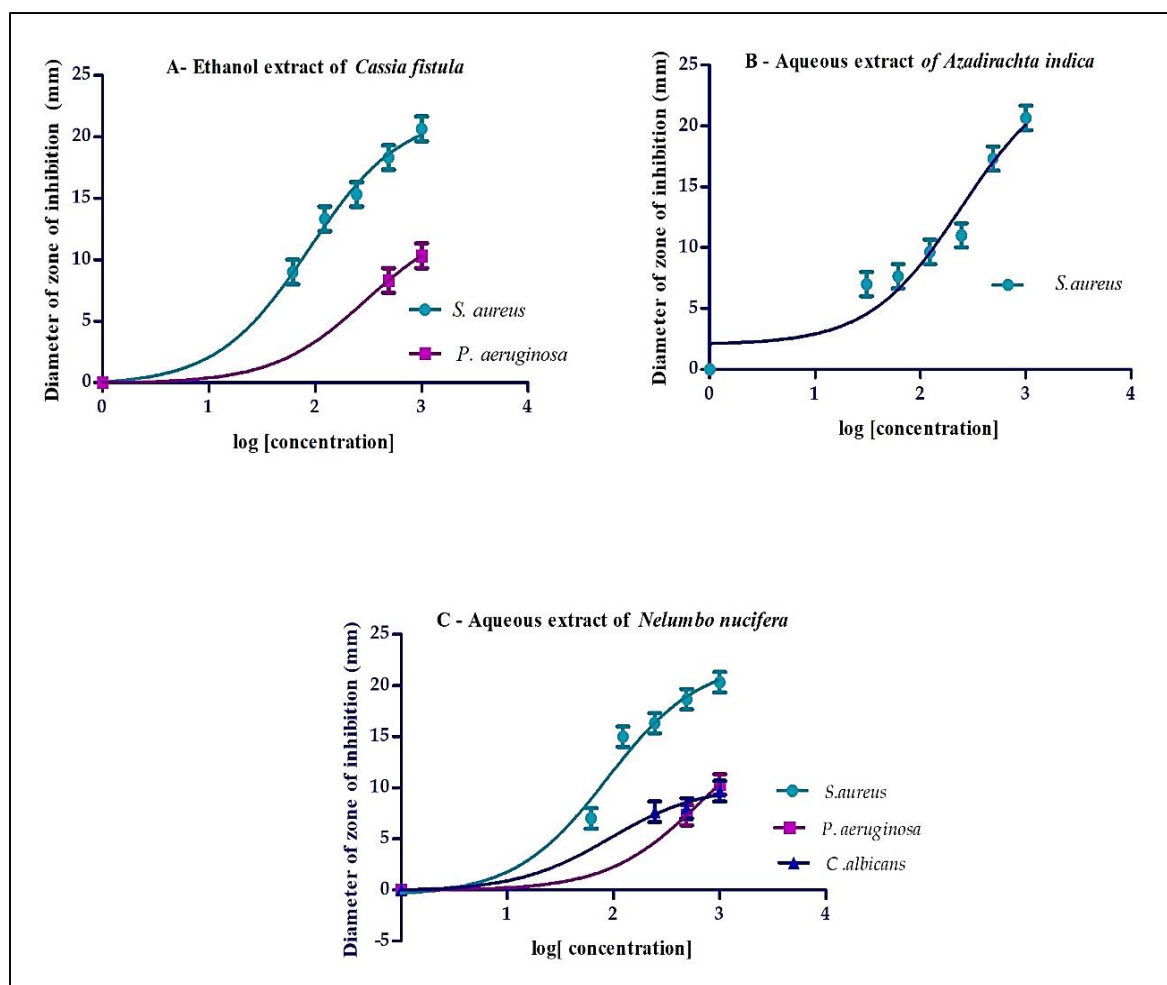
Data presented as Mean±SD

\*Significant when compared with the negative control;  $p < 0.05$ **Table 3.** Diameters of the zone of inhibition of different concentrations of leaf extracts of *A. indica* against selected microorganisms.

Concentration (mg/ml) of <i>A. indica</i> leaf extracts	The diameter of zone of inhibition (mm) of leaf extracts of <i>A. indica</i>					
	Ethanol extract			Aqueous extract		
	<i>S.aureus</i>	<i>P.aeruginosa</i>	<i>C. albicans</i>	<i>S. aureus</i>	<i>P.aeruginosa</i>	<i>C. albicans</i>
1000	16.0±0.5*	0.0±0.0	0.0±0.0	21.0±0.5*	0.0±0.0	0.0±0.0
500	13.0±0.5*	0.0±0.0	0.0±0.0	18.0±0.5*	0.0±0.0	0.0±0.0
250	12.0±0.5*	0.0±0.0	0.0±0.0	15.0±0.5*	0.0±0.0	0.0±0.0
125	8.0±0.5*	0.0±0.0	0.0±0.0	13.0±0.5*	0.0±0.0	0.0±0.0
62.5	7.0±0.5*	0.0±0.0	0.0±0.0	9.0±0.5*	0.0±0.0	0.0±0.0
31.25	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Positive control	34.0±0.5*	32.0±0.3*	25.0±0.3*	31.0±0.5*	30.0±0.3*	25.0±0.3
Negative control	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Data presented as Mean±SD

\*Significant when compared with the negative control;  $p < 0.05$ **Figure 1.** Dose-response curves of ethanol and aqueous extracts of *C. fistula*, *A. indica* and *N. nucifera* against *S. aureus*. A: *C. fistula*, B: *A. indica* and C: *N. nucifera*.



**Figure 2.** Dose-response curves of ethanol extract of *C. fistula*, aqueous extract of *A. indica* and aqueous extract of *N. nucifera* against tested organisms.

A: Ethanol extract of *C. fistula* against *S. aureus* and *P. aeruginosa*

B: Aqueous extract of *A. indica* against *S. aureus*

C: Aqueous extract of *N. nucifera* against *S. aureus*, *P. aeruginosa* and *C. albicans*

The antibacterial activity of different concentrations of ethanol and aqueous extracts of *C. fistula* is represented in Table 1. The results indicated that the plant extracts showed antibacterial activity at variable degrees against *S. aureus* and *P. aeruginosa*. The highest antibacterial activity was exerted at 1000 mg/ml of both extracts against *S. aureus* and *P. aeruginosa*. However, both extracts did not show antifungal activity against *C. albicans*. Both extracts showed moderate antibacterial activity against *P. aeruginosa* at the concentrations of 500 and 1000 mg/ml. When consider the both extracts, the ethanol extract of leaves of *C. fistula* at 1000 mg/ml showed the highest activity against *S. aureus*.

### 3.2. Determination of the zones of inhibition of different concentrations of white flower extracts of *N. nucifera* against selected microorganisms

The antibacterial activity of different concentrations of ethanol and aqueous extracts of *N. nucifera* flowers is represented in Table 2. The results indicated

that the different concentrations of aqueous extract of white flower of *N. nucifera* showed antimicrobial activity at variable degrees against all three selected organisms. The extract showed the highest antimicrobial activity against *S. aureus*. However, different concentrations of ethanol extract of white flowers of *N. nucifera* showed antimicrobial activity against *S. aureus* and *P. aeruginosa* except for *C. albicans*. When consider the both extracts, the aqueous extract of the white flower of *N. nucifera* showed the highest activity against *S. aureus*.

### 3.3. Determination of the zones of inhibition of different concentrations of leaf extracts of *A. indica* against selected microorganisms

The antimicrobial activity of different concentrations of ethanol and aqueous extracts of *A. indica* leaves is represented in Table 3. The results indicated that the ethanol and aqueous extracts of *A. indica* leaves showed antibacterial activity against *S. aureus* only. All



the tested concentrations of both extracts were exhibited antibacterial activity against *S. aureus* and the aqueous extract showed the highest activity against *S. aureus*. Furthermore, both extracts did not show antifungal activity against *C. albicans*.

### 3.4. Determination of the effective extract of each plant and effective concentration of selected plant extracts against tested microorganisms to formulate a combined extract

Ethanol and aqueous extracts of all three plants of *C. fistula*, *A. indica* and *N. nucifera* at concentrations ranging from 62.5-1000 mg/ml exhibited maximum antimicrobial activity against *S. aureus* with zones of inhibition ranging from 7.0 to 21.0 mm. Hence, the half-maximal inhibitory concentration (IC<sub>50</sub>) values of ethanol and aqueous extracts of all three plants of *C. fistula*, *A. indica* and *N. nucifera* were determined against *S. aureus*.

Figure 1 shows the IC<sub>50</sub> values of ethanol and aqueous extracts of *C. fistula*, *A. indica* and *N. nucifera* against *S. aureus*. The values of IC<sub>50</sub> of ethanol extracts of *C. fistula*, *A. indica* and *N. nucifera* were 87 mg/ml, 119.9 mg/ml and 221 mg/ml respectively. The value of IC<sub>50</sub> of aqueous extract of *C. fistula* was 166 mg/ml. The values of IC<sub>50</sub> of aqueous extracts of both plants of *A. indica* and *N. nucifera* were determined as 87 mg/ml. Since the ethanol extract of *C. fistula* and the aqueous extracts of *A. indica* and *N. nucifera* were demonstrated the maximum inhibition effect, these extracts were selected as the suitable extracts to incorporate into the combined extract.

Effective concentrations of ethanol extract of *C. fistula*, aqueous extract of *A. indica* and aqueous extract of *N. nucifera* were determined against the tested microorganisms. Figure 2 represents the dose-response curves of the ethanol extract of *C. fistula*, aqueous extract of *A. indica* and aqueous extract of *N. nucifera* against tested microorganisms.

According to the dose-response curve of the ethanol extract of *C. fistula*, the effective concentration ranges for *S. aureus* and *P. aeruginosa* were 30-257 mg/ml and 173-933 mg/ml respectively. Hence, the final effective concentration of ethanol extract of *C. fistula* was selected as 250 mg/ml for both microorganisms. Effective concentration range of aqueous extract of *A. indica* for *S. aureus* was range from 47.44 mg/ml to 1233.00 mg/ml. Hence 1000 mg/ml was selected as the effective concentration of aqueous extract of *A. indica* for *S. aureus*. By considering the dose-response curves of aqueous extract of *N. nucifera*, the effective concentration ranges for *S. aureus*, *P. aeruginosa* and *C. albicans* were 35-281 mg/ml, 36-309 mg/ml and 170-776 mg/ml. Hence, 250 mg/ml was selected as the effective concentration of aqueous extract of *N. nucifera* for *S.*

*aureus*, *P. aeruginosa* and *C. albicans*.

According to the effective concentrations selected from each extract, the proportions of the extract in the combination that incorporated into herbal soap were determined as follows.

<i>C. fistula</i>	: <i>N. nucifera</i>	: <i>A. indica</i>
250 mg/ml	: 250 mg/ml	: 1000 mg/ml

### 3.5. Determination of the antimicrobial activity of the combined plant extract and formulated herbal soap

Table 4 represents the antimicrobial activity of the combined plant extract. Combined extract showed the highest activity against *S. aureus*. Combined extract showed enhanced activity compared to individual extracts against the tested microorganisms.

The antimicrobial activity of the formulated herbal soap was investigated using the agar well diffusion method. Figure 3 and Table 5 represents the diameter of the zone of inhibition for *S. aureus*, *P. aeruginosa* and *C. albicans* at different concentrations of herbal soap. The diameter of the zone of inhibition exhibited by gentamycin for *S. aureus* and *P. aeruginosa* were 31.0±0.5 mm and 30±0.4 mm respectively and of clotrimazole for *C. albicans* was 26.0±0.4 mm.

The extracts that exhibited maximal activity were ethanol extract of *C. fistula* and the aqueous extracts of *A. indica* and *N. nucifera*. Ethanol extract of *C. fistula* exhibited zones of inhibition ranging from 9.0 to 21.0 mm and 8.0 to 10.0 mm against *S. aureus* and *P. aeruginosa* respectively. Aqueous extract of *A. indica* exhibited zones of inhibition ranging from 9.0 to 21.0 mm against *S. aureus* whereas aqueous extract of *N. nucifera* exhibited zones of inhibition ranging from 7.0 to 20.0 mm, 7.0 to 10.0 mm and 7.0 to 10.0 mm against *S. aureus*, *P. aeruginosa* and *C. albicans* respectively. According to the Table 5, formulated herbal soap exhibited zones of inhibition ranging from 15.0 to 28.0 mm, 15.0 to 25.0 mm and 15.0 to 25.0 against *S. aureus*, *P. aeruginosa* and *C. albicans* respectively at a similar range of concentrations. Hence, the formulated herbal soap showed enhanced antimicrobial activity compared to individual extracts.

### 3.6. Evaluation of physical and chemical parameters of formulated soap

The physical and chemical parameters of the formulated herbal soap were determined. Parameters such as color, odor, appearance, pH, etc. were tested. Table 6 represents the physical and chemical parameters of the formulated soap. The formulation exhibited good appearance characteristics as well as the pH was 9.1. Other parameters such as percentage free alkali, foam height, foam retention, alcohol insoluble matter and percentage of moisture were determined.

**Table 4.** Diameters of zone of inhibition of combined extract for selected microorganisms.

Microorganism	The diameter of the zone of inhibition of combined extract (mm)	The diameter of the zone of inhibition for the positive control (mm)	The diameter of the zone of inhibition for negative control (mm)
<i>S. aureus</i>	30.0±1.0	37.0±1.0	00.0±0.0
<i>P. aeruginosa</i>	26.0±1.0	32.0±1.0	0.0±0.00
<i>C. albicans</i>	16.0±1.0	25.0±1.0	0.0±0.00

Data presented as Mean±SD

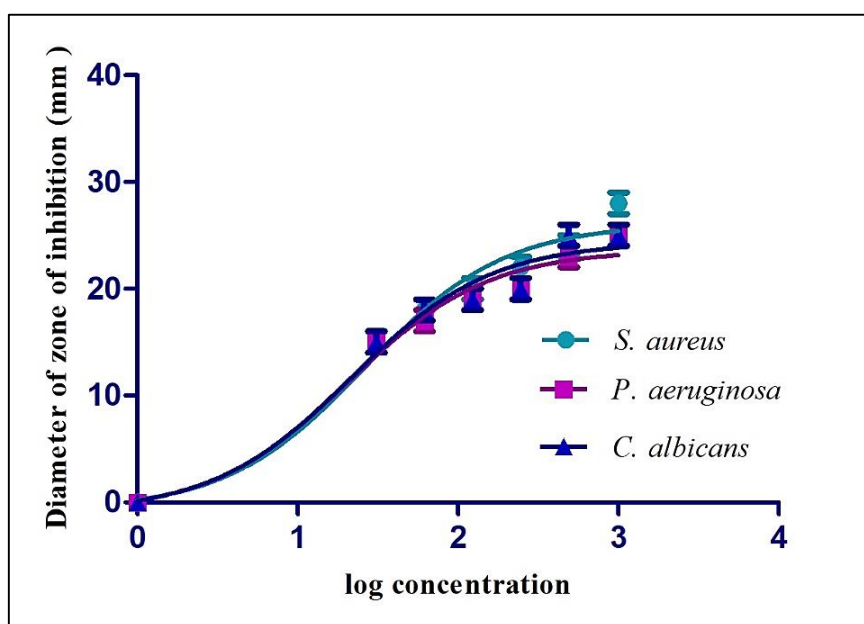
**Table 5.** Diameters of zone of inhibition of formulated soap for selected microorganisms.

Concentration (mg/ml)	The diameter of zone of inhibition (mm) of formulated herbal soap		
	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>
1000	28.0±0.5*	25.0±0.4*	25.0±0.4*
500	24.0±0.5*	23.0±0.4*	25.0±0.4*
250	22.0±0.5*	20.0±0.4*	20.0±0.4*
125	20.0±0.5*	19.0±0.4*	19.0±0.4*
62.5	18.0±0.5*	17.0±0.4*	18.0±0.4*
31.25	15.0±0.5*	15.0±0.4*	15.0±0.4*
Positive control	31.0±0.5*	30.0±0.4*	26.0±0.4*
Negative control	0.0±0.0	0.0±0.0	0.0±0.0

Data presented as Mean±SD

\*Significant when compared with the negative control;  $p < 0.05$ **Table 6.** Physical and chemical parameters of the formulated herbal soap.

Color	Odour	Appearance	pH	% free alkali	Foam height (cm)	Foam retention (min)	Alcohol insoluble matter (%)	Moisture/Volatile Matter (%)
Dark brown	Fragrant	Good	9.11	1.6	9.5	7	24.6	15.85

**Figure 3.** Dose-response curve for formulated soap against *S. aureus*, *P. aeruginosa* and *C. albicans*.

#### 4. DISCUSSION

Herbal soaps are products which are incorporated various plant extracts into the soap base. Nowadays, herbal soaps are gaining much attention due to the various advantages over synthetic soaps. Synthetic ingredients and artificial colors that are incorporated in soaps may lead to skin irritations.

Vast arrays of plants have the potential for antimicrobial activity. Mahato and Sharma<sup>23</sup> have reviewed and summarized the antimicrobial properties of some medicinal plants.

The present study has formulated a herbal soap using the extracts of *C. fistula*, *A. indica* and *N. nucifera* and the antimicrobial activity of the individual extracts, the combined extract and the herbal soap was evaluated



against the common microorganisms. Furthermore, the physicochemical parameters of the herbal soap were evaluated. Local availability and feasibility were considered in selecting plants possessing antimicrobial activity.

According to the literature, aqueous extract of leaves of *A. Indica* has shown antimicrobial activity against human pathogenic bacteria (*S. aureus*, *Enterococcus faecalis*, *Proteus mirabilis* and *P. aeruginosa*) and fungi (*Aspergillus fumigatus* and *C. albicans*)<sup>20</sup>. Hydro-alcohol extract of white flowers *N. nucifera* has shown antimicrobial activity against *S. aureus*, *P. aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia*, *Bacillus subtilis* and *A. niger*<sup>21</sup>. Hydro-alcohol extracts of the leaves of *C. fistula* possessed antimicrobial activity against human pathogenic bacteria (*S. aureus*, *Streptococcus pyogenes*, *E. coli*, *P. aeruginosa*) and fungi (*A. niger*, *A. clavatus* and *C. albicans*)<sup>24</sup>. *C. fistula*, *A. indica* and *N. nucifera* are considered as important herbs used in various traditional systems of medicine and all parts of the plants are used in traditional medicine. The selected plants are used in the treatment of skin diseases, worm and microbial infections, treatment of wounds, sores and ulcers etc<sup>8,10,11,18</sup>.

Selected plant extracts in the present study possessed antibacterial activity against both Gram-positive and Gram-negative bacterial isolates and antifungal activity against fungal isolates.

To establish the previous findings on Sri Lankan plants, we have evaluated the antimicrobial activity of aqueous and ethanolic extracts of leaves of *A. indica*, white flowers of *N. nucifera* and leaves of *C. fistula* against *S. aureus* (Gram-positive), *P. aeruginosa* (Gram-negative) and *C. albicans* (fungi). Further the findings of the study supported traditional claims in the literature.

Very few studies were reported about the formulation of herbal soaps and evaluating their antimicrobial activity. Polyherbal soap formulation prepared using coconut oil, castor oil, neem oil, mentha oil and rose petal extracts has exhibited antimicrobial activity against *E. coli*<sup>25</sup>. Afsar and Khanam, has formulated a herbal soap incorporating extracts of *C. fistula*, *Milletia pinnata* and *Ficus religiosa* and the antimicrobial activity was evaluated against *E. coli* (MTCC-1698), *S. aureus* (MTCC-1143) and *P. aeruginosa* (MTCC-2453)<sup>22</sup>. However, there are no any formulations based on combined extracts of *C. fistula*, *A. indica* and *N. nucifera* found in literature.

Authenticated plants were collected and basic operations such as pre-washing, drying and grinding were done to obtain a homogenous sample to facilitate extraction. Aqueous and ethanol extracts of selected medicinal plants were evaluated for antimicrobial activity by agar well diffusion method. According to the findings of the present study, aqueous and ethanol extracts of leaves of *C. fistula* have exhibited growth

inhibition against *S. aureus* and *P. aeruginosa*. A similar type of finding was observed in the study conducted by Bhalodia and Shukla<sup>24</sup>. However, there was a growth inhibition against *C. albicans* by hydroalcoholic extract of leaves of *C. fistula* in the study conducted by Bhalodia and Shukla<sup>24</sup> whereas no antifungal activity was detected in the present study against *C. albicans* by both aqueous and ethanol extracts of leaves of *C. fistula*.

Even though Reddy et al<sup>20</sup>, have confirmed the antimicrobial activity of aqueous extract of leaves of *A. indica* against *S. aureus*, *P. aeruginosa* and *C. albicans*, the present study did not show any growth inhibition against *P. aeruginosa* and *C. albicans*.

A limited literature was available on the antimicrobial activity of the white flower of *N. nucifera*. According to the literature hydroethanolic extract of *N. nucifera* white flowers showed antimicrobial activity against *S. aureus* and *P. aeruginosa*<sup>21</sup>. In this study, the aqueous extract of *N. nucifera* white flower has exhibited antimicrobial activity against *S. aureus*, *P. aeruginosa* and *C. albicans*. However, Brindha and Arthi<sup>21</sup> have not evaluated the antifungal activity against *C. albicans*.

When considering the antimicrobial activity of individual plant extracts of this study, the highest activity was detected against *S. aureus*, mild and poor activities were detected against *P. aeruginosa* and *C. albicans* respectively.

Antimicrobial activity was characterized by the IC<sub>50</sub> value. The IC<sub>50</sub> is used to assess and compare the effectiveness of inhibitory compounds<sup>26</sup>. Ethanol or aqueous extract of each plant was selected to formulate the combined extract. The most effective extract of each plant was selected by comparing the IC<sub>50</sub> values obtained from the log dose vs the response curve of each extract against *S. aureus*. Bhat et al.,<sup>27</sup> have used IC<sub>50</sub> values to determine the antimicrobial effectiveness of different extracts of Acacia powder. Ethanol extract of *C. fistula*, aqueous extracts of *A. indica* and *N. nucifera* were selected based on IC<sub>50</sub> values against *S. aureus* to incorporate into the soap formulation.

From all three types of plant extracts selected, a combination of extract was formulated. Proportions of each extract in the combination were determined by using the log dose vs. response curve for each extract considering all three organisms. (Concentration that lies within the range for all organisms was selected). Combined extract was included with all three extracts at a ratio of 1:1:4 (*C. fistula*: *N. nucifera*: *A. indica*).

In this study, the antimicrobial activity of the combined extract was determined. Results gained for the growth inhibition (diameter of the zone of inhibition) were comparatively higher than the results of individual extracts against the *S. aureus*, *C. albicans* and *P. aeruginosa*. Enhanced antimicrobial activity of the combined plant extract in the present study can be attributed to the synergistic effect produced after the combination of

individual extracts. A similar phenomenon has been discovered by other scientists by combining different plant extracts<sup>28,29</sup>. However, there was no reported literature on the combination of selected extracts of *C. fistula*, *A. indica* and *N. nucifera* and evaluation of its antimicrobial activity.

Antimicrobial herbal soap was formulated considering its microbiological, physical and chemical properties. The selected herbal extracts as active ingredients and several additives have been incorporated into the glycerin soap base which has been used as the vehicle for the formulation. Herbal soaps in 50 g in weight were formed by adding plant extracts, distilled water, stearic acid and natural volatile oil into the glycerin soap base. However, the active ingredients and added excipients should be compatible with the vehicle<sup>30</sup>.

Physicochemical characteristics of soap include moisture content, total fat matter, pH, free caustic alkalinity and percentage chloride. These characteristics depend mainly on the strength and purity of alkali, the kind of oil used and the completeness of saponification<sup>31</sup>. Appearance, color and odour were observed as physical properties of the final product. The dark brown color of the soap arises due to the plant extractions. Most of the herbal soaps exhibit a brown color due to the dominant color coming from various plant extracts<sup>33</sup>. Fragrant odor was the result of adding the natural volatile oil of *N. nucifera*.

Healthy skin has a pH range of 4 to 6<sup>32</sup>. The pH of the skin products is expected to maintain closer to this range as much as possible to reduce irritation<sup>33</sup>. As soap is alkaline (pH~10) in an aqueous solution because the soap base is the salt of a weak acid (fatty acid) and strong base (NaOH). The alkalinity favours detergency<sup>34</sup>. The pH of the formulated herbal soap at 28°C was 9.11. According to the SLS 1220 standard (Sri Lanka accreditation board for conformity assessment), the pH of the soap should be in the range of 4-10. The pH of the formulated soap was in an acceptable range and safe to use. The pH of a soap prepared from *A. indica* seed oil was 10.4<sup>34</sup> and the pH of a herbal soap formulated incorporating extracts of *C. fistula*, *Milletia pinnata* and *Ficus religiosa* was reported as 7.0<sup>27</sup>. The increased pH of the soap produces a significant increase in microbial growth<sup>35</sup>. Korting *et al.* examined the effect of different skin cleansing methods in healthy individuals aiming on the density of bacterial flora and the pH of the skin surface. After soap washing, the count of propionibacteria elevated strongly and dropped after application of syndet which is more acidic in nature<sup>36</sup>.

The total alkali means the presence of total alkaline components in the finished soap. The value of free caustic alkali measures the abrasiveness of any given soap. Free caustic alkali results from improper or incomplete saponification. High free alkali amount makes the soap more abrasive and it may cause harm to the skin<sup>37</sup>.

The herbal soap incorporated seed oil of *A. indica* reported 0.06% of free alkali<sup>38</sup>, whereas the said value of a herbal soap formulated incorporating extracts of *C. fistula* was 0.27%<sup>17</sup>. The free caustic alkali amount of the formulated herbal soap under this study was 1.6% which is much higher than the reported values. However, according to SLS 1220, the percentage of free alkali should be higher than 0.006%. 0.05% is the maximum amount declared by ISO standards<sup>39</sup>. Therefore, the soap does not comply with the ISO standards regarding the percentage of free alkali.

One of the parameters that use to detect the purity of the soap is matter insoluble in alcohol (MIA). This parameter is used to determine the non-soap ingredients known as builders or fillers such as sodium carbonate, sodium silicate and minor compounds such as whitening agents, bleachers in the final product<sup>40</sup>. The additives and foreign matter are known as ethanol-insoluble matter in soap. Additive/s is not a part of soap and includes colour additives, fragrance, herbs, silicon dioxide, etc. Inorganic matter such as carbonates, borates, perborates, chlorides and organic matter such as starches, dextrans, caseins, sugars, cellulose derivatives consider as foreign matter in soap. Main principle based for MIA is that separation of builders from active detergent and that is achieved by the use of alcohol. The standard procedure for analyzing commercial synthetic detergents consists in the separation of alcohol-soluble and insoluble fractions by extraction with ethanol<sup>37</sup>. The higher MIA value indicates that it contains a high level of impurities which may cause the level of impurities of alkali used for the soap<sup>31</sup>. The standard percentage of the MIA range is between 36-77%<sup>41</sup>. The value of MIA for formulated herbal soap was 24% and it was in an acceptable range. However, the herbal soap formulated by Afsar and Khanam<sup>22</sup>, incorporating extracts of *C. fistula* was 18%.

Moisture content is the major factor affecting the deterioration of formulated soap. It is a parameter that measures the shelf life. High moisture content leads to the hydrolysis of soap on storage and facilitates microbial growth<sup>33</sup>. The standard range for the moisture content is between 10-20%<sup>41</sup>. Moisture content of the formulated soap was 15.85% which falls in the acceptable range. Therefore, it has the potential to prevent deterioration that causes due to high moisture content. The herbal soap incorporating seed oil of *A. indica* reported 12.6% of moisture content<sup>34</sup>, which is lower than the product formulated under this study.

Another important physicochemical characteristic is the percentage chloride levels. Excess chloride levels may cause soaps to crack<sup>34</sup>. However, the percentage of chloride levels was not determined under this study.

Glycerin is the most effective humectant available to increase stratum corneum hydration<sup>42</sup>. Soap base used in the present study was composed of glycerin which promotes the moisturizing ability of the skin. This is a

potential benefit when using the soap product.

Varying time was observed for foam retention or foam stability. Herbal soap prepared with *C. fistula* reported 6 minutes of foam retention<sup>22</sup>, whereas the time observed under this study was 7 minutes. Even though both formulations were used glycerin soap base, the observed time for foam retention was different. This variation of foam retention time depends on the different concentrations of ingredients used in the soap base formulations<sup>33</sup>. Polyherbal soap formulation prepared using coconut oil, castor oil, neem oil, mentha oil and rose petals extract demonstrated 10 minutes of foam retention time<sup>25</sup>. The lather producing ability of the herbal soap prepared by Sindhu et al.<sup>25</sup> was more stable than the said ability of the soap prepared in this study.

## 5. CONCLUSIONS

Antimicrobial activity of aqueous and ethanol extracts of *A. indica*, *N. nucifera*, and *C. fistula* was assessed against *S. aureus*, *P. aeruginosa* and *C. albicans*. Enhanced antimicrobial activity was observed by the combined extract formulated from ethanol extract of *C. fistula* and aqueous extracts of *A. indica*, and *N. nucifera* and formulated herbal soap when compared to the individual plant extracts. The physical and chemical parameters of the formulated herbal soap were within the accepted range. In conclusion, combined extract has the potential in formulating herbal antimicrobial soaps. Further standardization and assessment of safety of formulated herbal soap would be beneficial in the commercialization of this product.

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