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Evaluation of *in vitro* and *in vivo* antimicrobial efficacy of non-alcohol-based herbal hand scrubs developed against selected pathogens

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Abstract: Handwashing with water and soap limits the transmission of respiratory diseases, but considered as time spending method. The aim of this study was to formulate non-alcohol-based herbal hand scrubs (NABHHS) and to evaluate the antimicrobial efficacy against selected pathogens. Six types of hand scrubs (NAF_1-NAF_6) were formulated by incorporating essential oils and the physical stability was studied for three months at room temperature. Agar well diffusion method was performed to evaluate *in vitro* antimicrobial efficacy of the formulations developed against selected standard cultures and clinical isolates of pathogens compared with positive and negative controls. The most active and stable formulations (NAF_1 and NAF_3) were selected and subjected for the *in vivo* (phase II) efficacy test to evaluate the clinically proven safety, and skin compatibility. The formulations were found to be homogenous, liquid, and milky white colour with a pleasant odor. Among the mean values of zone inhibition for NAF_1, high values were obtained as 17.33±1.15, 25.33±0.58, 23.00±5.29, and 18.67±1.53 mm against *Proteus mirabilis, Candida albicans, Streptococcus pyogenes*, and *Staphylococcus aureus* respectively. Among the mean values of inhibition zones of NAF_3, high values were obtained as 23.33±1.15, 20.33±1.15, 34.67±1.15, and 21.00±0.00 mm against *Acinetobacter baumannii, Salmonella Typhi, Candida albicans*, and *Streptococcus pyogenes* respectively. Zero values of inhibition were obtained for distilled water (negative control) while the market product (positive control) showed the zones of inhibition of 8.00-12.15 mm against *Enterobacter cloacae, Salmonella Typhi, Candida albicans, Streptococcus pyogenes, Enterococcus faecium,* and *Staphylococcus aureus*. In conclusion, the NABHHSs developed in this study have promising *in vitro* antimicrobial activity against the tested pathogens and clinically proven safety, and skin compatibility against healthy human volunteers. Moreover, promising antifungal activity was showed by both formulations NAF_1 and NAF_3 against *Candida albicans.*

Keywords: Antimicrobial efficacy; essential oil; hand scrubs; pathogens

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INTRODUCTION

Healthcare-associated infections (HCAI) are a major problem for patient safety and its prevention must be the priority for settings and institutions committed to making health care safer (World Health Organization (WHO), 2009a). Since the explosion of viruses such as influenza A virus subtype H1N1, swine flu, coronavirus, and others, the health sector is reverted to emphasizing the importance of proper hand washing and maintaining hand hygiene as the most important step to protect human health against healthcare-associated infectious (HAI) diseases (World Health Organization (WHO), 2009b). Hand hygiene is the primary measure proven to be effective in preventing HCAI and the spread of antimicrobial resistance. However, it has been shown that healthcare workers (HCWs) encounter difficulties in complying with hand hygiene indications at different levels (World Health Organization (WHO), 2009a). Hand hygiene relates to the removal of visible soil and removal or killing of transient microorganisms from the hands (Provincial Infectious Diseases Advisory Committee, 2014).

The selection of a hand hygiene product has a positive impact on hand hygiene practice. It is a necessity to assess the criteria such as the efficacy of the product, the user-friendly of the product, and maintains skin tolerance when a hand hygiene product is being selected for a healthcare facility. Two types of hand hygiene products namely, alcohol-based and non-alcohol-based products are available at the market. They are alcohol-based (hand) rubs, antimicrobial (medicated) soaps, antiseptic agents, detergents (surfactant), waterless antiseptic agent (World Health Organization (WHO), 2007). Non-alcoholic products available at the market are known to have different active ingredients such as benzalkonium chloride, triclosan, chlorhexidine gluconate, chlorine derivatives, iodine, chloroxylenol (PCMX), quaternary ammonium compounds, (World Health Organization (WHO), 2007), didecyldimethylammonium chloride (Hygiene of Sweden, 2020), essential oils (Al-zahrani & Baghdadi, 2012; Acharya et al., 2018) with known side effects and less effectiveness against most microorganisms (Provincial Infectious Diseases Advisory Committee, 2014).

Nowadays, attention is being focused on the formulation and development of herbal products using natural biomaterials, which can overcome the apparent drawbacks of synthetic chemicals. Therefore, many research studies have been successfully carried out worldwide in order to investigate the potential antimicrobial agents from various medicinal plants and as well as to formulate novel herbal hand hygiene products. Among a large number of extracts obtained from natural products, it is reported that essential oils of clove, cinnamon, nutmeg, and casters from medicinal plants have proven antimicrobial activity against many microorganisms (Ramanoelina et al., 1987). Therefore, this study aimed to develop safer non-alcohol-based hand scrubs using plant extracts obtained from *Aloe vera*, clove, cinnamon, castor and nutmeg oils and to evaluate *in vitro and in vivo* antimicrobial efficacies against selected pathogens.

MATERIAL AND METHODS

Plant materials: The leaves of *Aloe vera* were collected from Galle District (geographical coordinates; latitude: 6.053519; longitude: 80.220978) in Southern Province of Sri Lanka and were authenticated by the Bandaranaike Memorial Ayurvedic Research Institute, Nawinna, Maharagama, Sri Lanka (14.02.2019.2030).

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Chemical materials: The chemicals performed in this study include glycerin, clove oil, and cinnamon oil, ethanol, polysorbate 20, and related products were commercially purchased (Clinipath Medical Services (Pvt) Ltd, Colombo, Sri Lanka). Muller Hinton Agar (Oxoid, UK) and Blood agar (Oxoid,, UK) were purchased from Hemsons International (Pvt) Ltd, Colombo, Sri Lanka.

Microorganisms: Namely, *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 23355), *Acinetobacter baumannii* (ATCC 19606), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 70603), *Salmonella Typhi* (clinical isolate), *Shigella sonnei* (clinical isolate), *Proteus mirabilis* (ATCC 12453), *Candida albicans* (ATCC 10231), *Streptococcus pyogenes* (ATCC 12384), *Enterococcus faecium* (ATCC 29212), and *Staphylococcus aureus* (ATCC 25923) were obtained from the Quality Control laboratory, Department of Bacteriology, Medical Research Institute, Colombo 08, Sri Lanka.

Sample preparation: The defective leaves were discarded and remains were used for the extract preparation. The extract of *A. vera* was prepared by blending (SISIL, Mixer, Grinder 550W, Greater Noida Dadri (ICD), Mumbai, India) flesh in small volume of distilled water.

Development of formulations of non-alcohol-based herbal hand scrubs: Different formulations of non-alcohol-based herbal hand scrubs (NAF_1-NAF_6) were prepared according to the compositions given in Table 1.

Table 1: Compositions of non-alcohol-based herbal hand scrubs (NABHHSs)

Ingredients	NAF 01/ mL	NAF 02/ mL	NAF 03/ mL	NAF 04/ mL	NAF 05/ mL	NAF 06/ mL
Glycerin	10	10	-	-	-	-
Aloe extract	20	20	30	30	30	30
Clove oil	60	-	60	-	-	-
Cinnamon oil	-	60	-	60	-	-
Castor oil	-	-	-	-	60	-
Nutmeg oil	-	-	-	-	-	60
Polysorbate 20	02	02	02	02	02	02
Distilled water	08	08	08	08	08	08
Total volume	100	100	100	100	100	100

NAF: Non-alcohol-based formula

Evaluation of physical stability, in vitro and in vivo antimicrobial efficacy of non-alcohol-based herbal hand scrubs developed: The physical stability parameters such as pH, odor, appearance, and color of the formulations developed were tested for 90 days [7, 30, and 90 d (s)] at room temperature. Agar well diffusion method (Kunicka & Kalemba, 2003; Wani et al., 2013) was performed to evaluate the *in vitro* antimicrobial efficacy of prepared NABHHSs against selected pathogens. The turbidity of the inoculum was prepared against McFarland 0.5. A 50 µL of each formulation, negative control (distilled water, N), and positive control (market product, P) were added separately to wells with 6 mm diameter. The seeded plates were incubated (Eyela incubator, SLI- 600D, Tokyo, Japan) overnight at 35±2°C, and the diameters of the zones of inhibition were calculated by using a calibrated vernier caliper. The most active formulations (NAF_1 and NAF_3) were selected by analyzing the results obtained for *in vitro* antimicrobial efficacy and subjected to phase II clinical trial by using the fingertip method on healthy human

volunteers (Jenkins & Belu, 2009; Karunanayaka & Parahitiyawa, 2013) to evaluate the *in vivo* antimicrobial efficacy and skin sensitivity of novel NABHHSs.

Ethical approval for the *in vivo* antimicrobial efficacy and post analysis on the skin sensitivity, safety, and compatibility was obtained from Medical Research Institute, Colombo 08, and Sri Lanka Clinical Trial Registry (SLCTR) of Sri Lanka Medical Association under registration number SLCTR/2019/016 (Sri Lanka Clinical Trial Registry, 2016) and the universal number U1111-1226-9390 to conduct this clinical trial. For one formulation, a group of healthy human volunteers (n=30) was randomly (Kac et al, 2005) recruited and each participant was requested to place both thumbs on two separate blood agar plates before application of any formulations. The formula to be tested was applied on one thumb and the control was applied on the other thumb simultaneously and placed both thumbs after 10 sec and 15 sec of application. After the incubation period at 35±2°C for 16-18 hours, the number of colony counts were recorded by using a bacterial colony counter (Stuart biocote colony counter, SC+6, Staffordshire, UK) and repeated on 15th and 30th day of preparation. Post-analysis on the skin sensitivity, safety, and compatibility against the tested hand scrubs was conducted for the most stable and active two formulations (NAF1 and NAF3) to identify the preference of participants on skin sensitivity, safety, and compatibility including itching, irritation, dryness, or rashes, and moisturizing effect, using a self-administered questionnaire given.

Statistical analysis: All experimental measurements were conducted in triplicate and results were expressed as mean (M) ± standard deviation (SD). Mean Log10 reduction value ± standard deviation (SD), reduction factor in percentage were analyzed and graphs were created using Microsoft Excel 2010 version. Significant levels ($p \leq 0.05$) in 95% confidence intervals were analyzed by multiple comparisons paired sample T-test using SPSS 16.0 software (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

The formulations developed namely. NAF_1-NAF_6 were physio-chemically liquid in the nature during the tested period of 90 days. All were pleasant in odor and milky white. The pH values of prepared formulations were around 7 with no remarkable changes. The results showed promising antimicrobial activity of NAF_1,-NAF_6 against the tested pathogens in comparison to the positive and negative control (Table 2).

Among the mean values of zone inhibition for NAF_1, high values were obtained as 17.33 ± 1.15, 25.33±0.58, 23.00±5.29, and 18.67±1.53 mm against *P. mirabilis, C. albicans, S. pyogenes*, and *S. aureus* respectively. Among the mean values of inhibition zones of NAF_3, high values were obtained as 23.33±1.15, 20.33±1.15, 34.67±1.15, and 21.00±0.00 mm against *A. baumannii,* S. Typhi*, C. albicans*, and *S. pyogenes* respectively. Moreover, promising antifungal activity was showed by both formulations NAF_1 and NAF_3 against *Candida albicans.* Zero values of inhibition were obtained for distilled water (negative control) while the market product (positive control) showed the zones of inhibition of 8.00-12.15 mm against *E. cloacae,* S. Typhi*, C. albicans, S. pyogenes, E. faecium,* and *S. aureus*. Significant reductions of colony forming units of tested formulation compared to negative and positive controls was revealed on 1st, 15th, and 30th day from the preparation (Table 3 & 4). Our finding is in agreement with a previous study in Canada, reported 100% reduction of colonies for fingertip colony count for alcohol-based hand rubs (Jenkins & Belu, 2009). A hand hygiene product which was formulated using a

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natural plant extract had shown 80.57% bacterial colony growth reduction compared to the controls in the study (Sharma *et al.*, 2016).

Table 2: Mean (M) ± standard deviation (SD) values of Zone of inhibition (mm) against selected pathogens

Formulation/ Control →	NAF_1	NAF_2	NAF_3	NAF_4	NAF_5	NAF_6	N	P
Pathogens ↓	M±SD	M±SD	M±SD	M±SD	M±SD	M±SD	M±SD	M±SD
Escherichia coli (ATCC 25922)	13.33 ±0.58	0.00	14.33 ±2.08	0.00	10.33 ±0.58	0.00	0	0.00
Enterobacter cloacae (ATCC 23355)	14.00 ±2.65	0.00	16.00 ±0.00	0.00	0.00	0.00	0	8.00 ±0.00
Acinetobacter baumannii (ATCC 19606)	20.00 ±1.00	10.00 ±0.00	23.33 ±1.15	10.00 ±0.00	10.33 ±0.58	0.00	0	0.00
Pseudomonas aeruginosa (ATCC 27853)	10.00 ±0.00	0.00	13.67 ±1.53	0.00	0.00	0.00	0	0.00
Klebsiella pneumoniae (ATCC 70603)	12.33 ±0.58	0.00	14.67 ±1.53	0.00	0.00	0.00	0	0.00
Salmonella Typhi (clinical isolates)	23.67 ±0.58	0.00	20.33 ±1.15	10.33 ±0.58	0.00	0.00	0	9.33 ±1.15
Shigella sonnei (clinical isolates)	16.00 ±1.00	0.00	19.00 ±1.73	10.67 ±0.58	10.00 ±0.00	0.00	0	0.00
Proteus mirabilis (ATCC 12453)	17.33 ±1.15	11.33 ±1.15	20.33 ±0.58	10.33 ±0.58	10.00 ±0.00	0.00	0	0.00
Candida albicans (ATCC 10231)	25.33 ±0.58	10.33 ±0.58	34.67 ±1.15	21.00 ±3.00	17.00 ±1.73	0.00	0.00	11.33 ±1.53
Streptococcus pyogenes (ATCC 12384)	23.00 ±5.29	14.67 ±4.04	21.00 ±0.00	12.33 ±0.58	18.33 ±0.58	15.67 ±1.53	0.00	10.00 ±0.00
Enterococcus faecium (ATCC 29212)	11.67 ±0.58	0.00	14.33 ±0.58	0.00	0.00	0.00	0.00	10.00 ±0.00
Staphylococcus aureus (ATCC 25923)	18.67 ±1.53	11.33 ±0.58	19.67 ±1.53	10.00 ±0.00	13.67 ±1.15	0.00	0.00	12.33 ±1.15

NAF: Non-alcohol-based formula; ATCC: American type culture collection; N: Negative control; M: Mean; P: Positive control SD: Standard deviation

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It is reported that natural herbs and essential oils can be utilized as an alternative to alcohol to formulate hand scrubs and currently, hundreds of herbal products had been formulated and launched in the market (Azelee *et al.*, 2020).

Table 3: Mean log $_{10}$ reduction values of colony-forming units (CFUs) and reduction factor (RF) for NABHHSs

	NAF_1		NAF_3	
	Mean Log $_{10}$ reduction of CFUs	Mean reduction Factor	Mean Log $_{10}$ reduction of CFUs	Mean reduction Factor
1st day				
Before vs 10 s after application	1.6902±0.51	100%	1.6655±0.58	100%
Before vs 15 s after application	1.6902±0.51	100%	1.6655±0.58	100%
15th day				
Before vs 10 s after application	1.2479±0.44	100%	1.1470±0.46	100%
Before vs 15 s after application	1.2479 ± 0.44	100%	1.1470±0.46	100%
30th day				
Before vs 10 s after application	1.1761±0.28	100%	1.2557±0.30	100%
Before vs 15 s after application	1.1761±0.28	100%	1.2557±0.30	100%

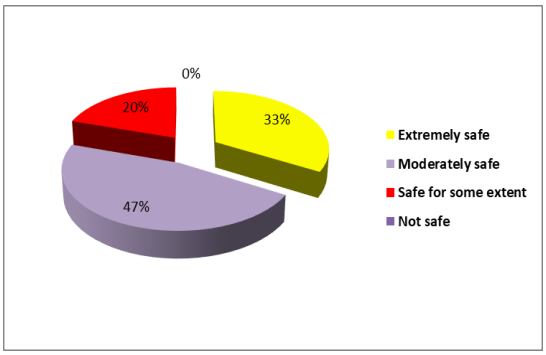
NAF: Non-alcohol-based formula; CFUs: Colony forming units; vs: versus; s: second

Table 4: Mean log $_{10}$ reduction values of colony-forming units (CFUs) and reduction factor (RF) for positive and negative control

	P		N	
	Mean Log $_{10}$ reduction of CFUs	Mean reduction Factor	Mean Log $_{10}$ reduction of CFUs	Mean reduction Factor
Before vs 10 s after application	0.9015±0.43	98.85%	0.0785±0.48	4.72%
Before vs 15 s after application	0.7768±0.43	94.69%	0.0406±0.48	- 3.06%

CFUs: Colony forming units; N: Negative control; P: Positive control; vs: versus; s: second

The results showed that 100% participants had no itching, irritation, rashes, or skin damages and drying, while more than 93% responded that there was no hesitation of using NAF_1, and NAF_3 (Figure 1 & 2).



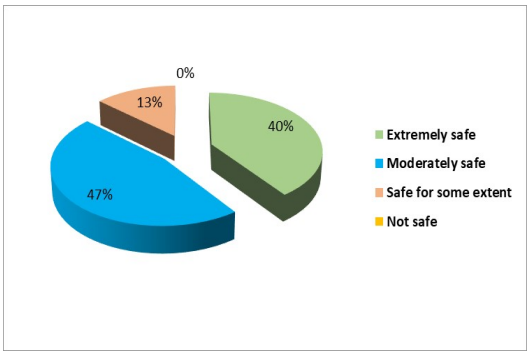


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Figure 1: Skin sensitivity of healthy human volunteers against novel NAF_1

Figure 2: Skin sensitivity of healthy human volunteers against novel NAF_3

More than 65% of participants mentioned that there was a moisturizing effect in all formulations developed. Majority (46%) of participants agreed that NAF_1 and NAF_3 were moderately safe (Figure 3, 4) to use in practice.

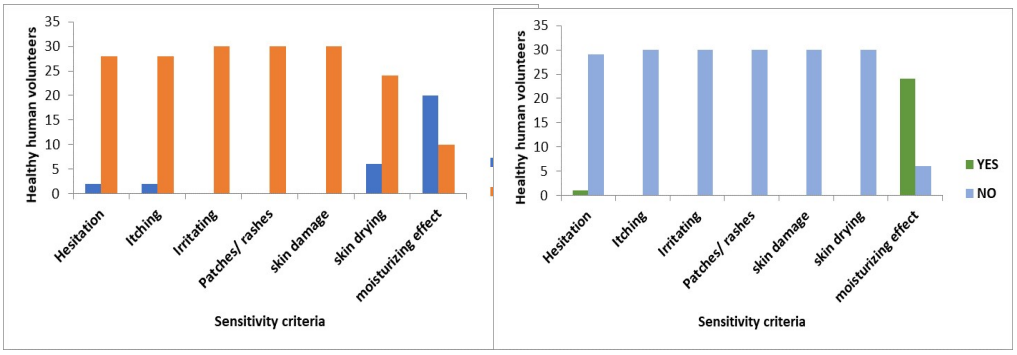

Figure 3: Preference for healthy human volunteers against novel NAF_1

Figure 4: Preference for healthy human volunteers against novel NAF_3

CONCLUSION

The tested formulations showed promising antimicrobial activity against the tested pathogens than the commercial product. NABHHSs with essential oils showed antimicrobial activity and the moisturizing effect given by medicinal *A. vera* plant. Further, the formulated NABHHSs have clinically proven low skin sensitivity, good safety, and compatibility against healthy human volunteers. Moreover, promising antifungal activity was shown by both formulations NAF_1 and NAF_3 against *Candida albicans.* Therefore, it is concluded that NAF_1 and NAF_3 can be further investigated as potent non-alcohol based herbal hand scrubs with remarkable antifungal activity.

ACKNOWLEDGMENTS

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DECLARATION OF CONFLICT OF INTEREST

We hereby declare that the study does not encompass any conflict of interest. The authors alone are responsible for the content and writing of this article.

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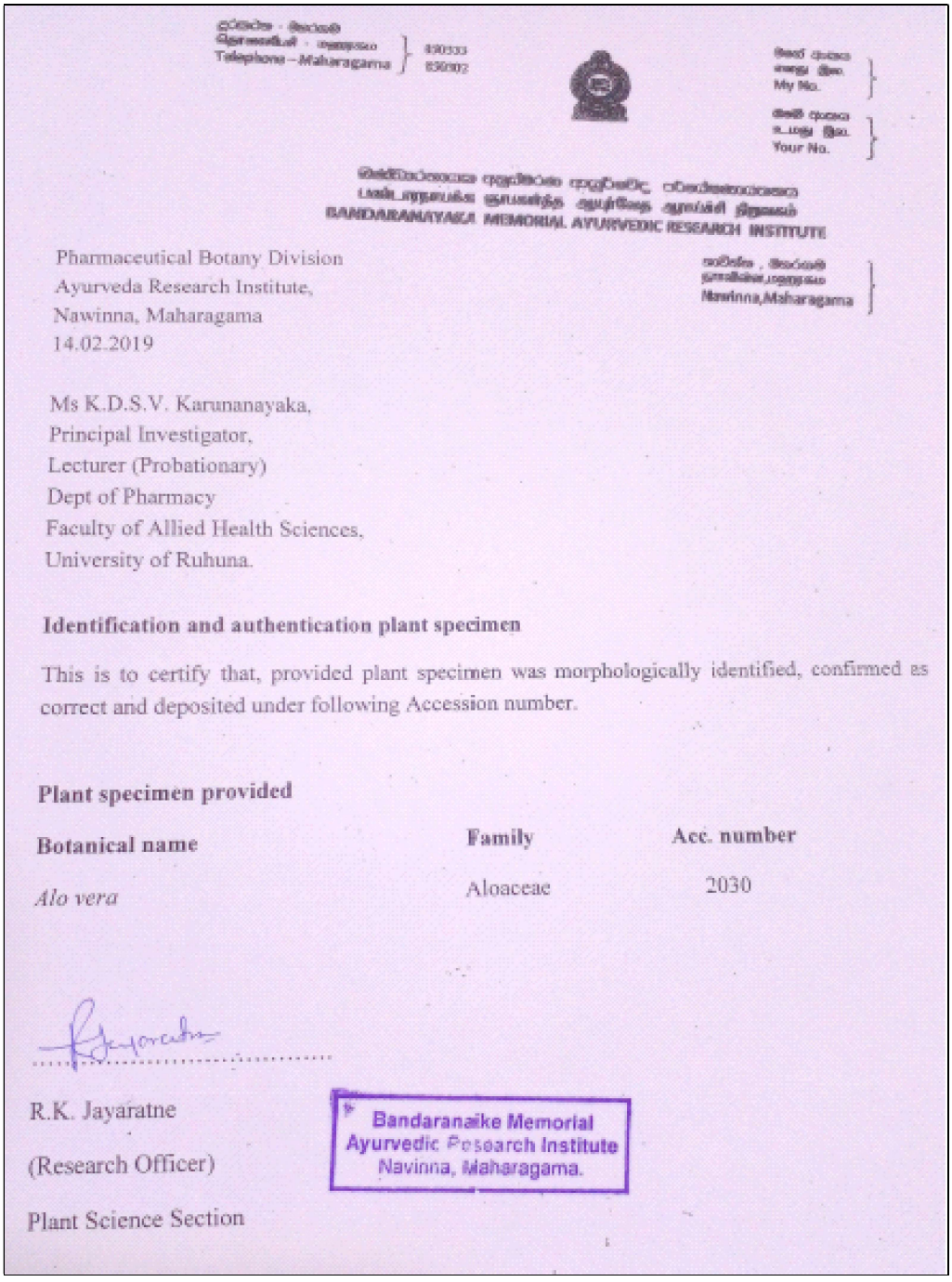
Annexure 1. : Authentication letter from Bandaranaike Memorial Ayurvedic Institute, Sri Lanka.



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BANDARANAYAKA MEMORIAL AYURVEDIC RESEARCH INSTITUTE

Pharmaceutical Botany Division
Ayurveda Research Institute,
Nawinna, Maharagama
14.02.2019

Nawinna,Maharagama

Ms K.D.S.V. Karunanayaka,
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University of Ruhuna.

Identification and authentication plant specimen

This is to certify that, provided plant specimen was morphologically identified, confirmed as correct and deposited under following Accession number.

Plant specimen provided

Botanical name	**Family**	**Acc. number**
Alo vera	Aloaceae	2030

R.K. Jayaratne
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Bandaranaike Memorial
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Annexure 02 : Degree & Designation of Authors

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