# DEVELOPMENT OF FOAMING HAND SANITIZER CONTAINING *ANDROGRAPHIS PANICULATA* EXTRACT.

SASID SUKSUMIT

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in Pharmaceutical Chemistry and Natural Products
2022

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By Sasid Suksumit

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Pharmaceutical Chemistry and Natural Products of Naresuan University

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| **Title** | DEVELOPMENT OF FOAMING HAND SANITIZER CONTAINING *ANDROGRAPHIS PANICULATA* EXTRACT. |
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## ABSTRACT

*Andrographis paniculata* (*A. paniculata*) is a herbal plant containing natural compounds commonly found in southern Asia, China, and Europe. The plant is traditionally used to treat fevers, common colds, and skin infections. *A. paniculata* has been reported to have numerous potential bioactivities, with emphasis particular emphasis on its antibacterial properties. This study aimed to formulate a completely developed foaming hand sanitizer product containing *A. paniculata* extract, which has good antibacterial activity and stability. The ethanolic characteristic of the *A. paniculata* extract (EAE) obtained in this current study was a dark green, sticky paste, with a yield percentage of 15.63% (w/w). The EAE was prepared to quantify andrographolide and evaluate antibacterial activity. The quantitative estimation of andrographolide content in EAE was performed using the reversed-phase HPLC method with a C18 column using a mixture of water and methanol (50:50) as the mobile phase. The quantity of andrographolide was 9.80 ± 0.43 µg/ml in the solution of amorphous residue. The andrographolide was found at 9.80% (w/w) in the EAE. In addition, the EAE was evaluated for antibacterial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* by zone of inhibition and determining the minimum inhibitory concentration (MIC). The study's results compared the zone of inhibition and MIC values with those of tetracycline (10 µg/disc). Results revealed that the EAE is potent in inhibiting both *S. aureus* and *S. epidermidis*, which showed

the clear zone of inhibition diameter was 11.2 ± 0.3 mm. and 10.17 ± 0.29 mm, respectively. Moreover, the MIC value of the EAE against both germs is 50 mg/ml. The result of this study shows that the EAE exerted inhibitory activities, indicating its potential use as an alternative ingredient for formulation. The foaming hand sanitizer was formulated using ethanolic extract of *A. paniculata*, disodium laureth sulfosuccinate, cocamide DEA, cocamidopropyl betaine, and PEG-7 Glyceryl cocoate in various proportions added to a 100% aqueous solution. The PEG-40 hydrogenated castor oil is used as a solubilizer with 5 %(w/w) EAE. A small amount of phenoxyethanol and o-Cymen-5-ol were added as preservatives, and the pH was adjusted with triethanolamine. Several tests, such as color, viscosity, foam volume, foam retention, pH value, etc., were performed to determine the physical properties of all formulations. The formulation Rx 6.2 showed low viscosity, soft foam, foam retention, clear solutions, good smells, and no drying effect after use. Moreover, the antibacterial activity of Rx 6.2 reduced more than 99.999% of the bacteria for tested bacterial strains *S. aureus* in 5 minutes at 34°C under conditions. This result indicated that the Rx 6.2 under test fulfilled the requirements of the EN 1276:2009 standard for the bactericidal activity of chemical disinfectants and antiseptic products. The stability evaluation of the foaming hand sanitizer formulation showed positive results, as the formulation was found to be stable for up to 30 days at 5 ± 2°C, 75%RH ± 5%. No significant changes in the test parameters were detected during this period. Further studies are needed to investigate the clinical effectiveness of the foaming hand sanitizer product in demonstrating antibacterial activities. Studies regarding safety are also necessary to prove that the product is safe for use.

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Sasid Suksumit

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**Rationale of study**

In 2019, an outbreak of the COVID-19 pandemic rapidly spread around the world. It is an infectious disease caused by a new strain of coronavirus that is spread primarily through respiratory droplets and contact routes (Murugan, Pandian, & Jeyakanthan, 2020).

Touching infected people and contaminated surfaces by hand is one of the main transmission routes. Therefore, washing your hands often and properly with soap can effectively prevent the transmission and spread of the virus. However, frequent washing and disinfection can cause the skin to become dry and can lead to skin inflammation in the form of redness, irritation, and itching. The hand sanitizer should be as mild as possible and suitable for frequent use to avoid these effects.

In 2019 global statistics showed that death from infectious diseases was reported to be 50–75% of all deaths in hospitals. The increasing bacterial resistance to common drugs is a significant public health concern, and therefore, the treatment of infectious diseases must be an utmost concern. Fortunately, there are various traditional herbal plants readily available that can overcome this problem. One of these herbal plants, *Andrographis paniculata* (AP), which contains natural compounds, has been reported to possess potential antimicrobial activity (Banerjee, Parai, Chattopadhyay, & Mukherjee, 2017).

Herbal products are frequently used to treat various diseases and have become more popular and widely used worldwide. The use of herbal products gains a rising high interest from customers. In industrialized countries, 10-50% of the population regularly uses herbal products because of their better tolerability compared to chemical-based products or synthetic drugs. Moreover, herbal products and medicines are widely available and are more affordable in developing countries.

AP, an annual herbaceous plant in the family *Acanthaceae,* is traditionally used to treat fever, common cold, diarrhea, and skin infections in southern Asia, China, and Europe (Jarukamjorn & Nemoto, 2008). This plant has been reported to possess numerous potential pharmacological activities, especially antibacterial properties (Banerjee et al., 2017). The extract from AP exhibits good inhibitory effects against most pathogenic and nonpathogenic (Abubacker & Vasantha, 2010). Therefore, it deserves further investigation as a potential source for a new class of antibiotics and natural ingredients in cosmetics. In this current study, we focus on the development of a foaming hand sanitizer containing AP extract that offers good antibacterial activity, efficiency, and stability.

# CHAPTER I
# INTRODUCTION

**Purposes of the study**

1. To develop foaming hand sanitizer products containing AP extract
2. To determine the physical and chemical stability of the developed product
3. To evaluate the antibacterial activity of the developed product

**Hypotheses of the study**

1. The ethanolic extract from AP can inhibit *Staphylococcus epidermidis* and *Staphylococcus aureus.*
2. The formulation of foaming hand sanitizer containing AP extract has good antibacterial efficacy and stability.

**Expected benefits from the research**

1. To know that ethanolic extract from AP can inhibit *S. epidermidis* and *S. aureus*
2. To get foaming hand sanitizer containing AP extract product with good efficacy and stability

**Scope of the study**

This study focuses on developing a foaming hand sanitizer product containing AP extract. The andrographolide present in the extract is qualified and quantified using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), respectively. The study also focuses on evaluating the physical stability of the product and assessing its antibacterial activity and efficacy in preventing bacterial growth.

# CHAPTER II

# LITERATURE REVIEW

## Description of plant

*Andrographis paniculata* (Burm.f.) Wall ex Nees, commonly known as “fa-ta-lai-jorn” or “Andrographis”, is an annual herbaceous plant in the family *Acanthaceae* that has been widely used as an essential medicinal plant around the world. It is normally found in Southeast Asia, especially in Thailand (Boopathi, 2000a).

The stem is acutely quadrangular. The leaves are simple, opposite, and lanceolate with 2 to 12 cm. long, 1 to 3 cm. wide, acute, glabrous, entire-slightly undulate, and upper ones often bractiform: petiole short.

The flower is calyx 5-partite, small, linear, corolla tube narrow, about 6 mm long, limb not shorter than the tube, upper lip oblong, bilabiate, white with a yellowish top, lower lip broadly cuneate, trifid, violet with white marking; stamens 2, ovary superior, 2-celled, inserted in the throat and far exerted, anther basally bearded; style far exerted.

The fruit is capsule, thinly glandular-hairy, compressed, erect, longitudinally furrowed on the broad faces. The seed is small and subquadrate (Hossain, Urbi, Sule, & Hafizur Rahman, 2014).

Figure 1 Morphology of AP

**Traditional use**

AP, also known as fa-talai-jorn, is an essential medicinal plant that has been used in Ayurvedic medicine for centuries in India. It is frequently used for several traditional medicines for the treatment of fever, upper respiratory infections, herpes, throat, sore, and other chronic and infectious diseases (Boopathi, 2000b). In traditional Asian medicine, the whole plant of AP is used, including its roots and aerial parts. AP is also popularly used to treat inflammation, stomachaches, intermittent fevers, and pyrexia (Chao & Lin, 2010).

**Chemical constituents**

The phytochemistry of AP is related to various chemical constituents. Notable among these, they are mostly terpenoids (ent-labdane diterpenoid lactones). Other chemical constituents can also be isolated, such as flavonoids, xanthones, noriridoides, and polyphenols.

Terpenoids

The major chemical constituents of the aerial parts of AP are diterpene lactones in both glycosidic and free forms including andrographolide (Figure 2A) (Levita, Nawawi, Mutalib, & Ibrahim, 2010), neoandrographolide (Figure 2B) (Koteswara Rao, Vimalamma, Venkata Rao, & Tzeng, 2004), 14-deoxyandrographolide (Figure 2C), and 14-deoxy-11,12-didehydroandrographolide (Figure 2D).

Andrographolide (Figure 2A) is colorless crystalline with bitter taste. It is labdane diterpenoid which has a broad range of therapeutic applications including anti-inflammatory (Abu-Ghefreh, Canatan, & Ezeamuzie, 2009), anti-cancer (Ye et al., 2011), and anti-bacterial activity (Banerjee et al., 2017; Tan, Oyong, Shen, & Ragasa, 2017). Andrographolide shows an anti-bacterial activity against both normal and drug-resistant strain in gram negative and positive bacteria in different minimum inhibitory concentration levels. Among these, *Staphylococcus aureus* was found to be the most sensitive with a minimal inhibitory concentration value of 100 µg/mL (Banerjee et al., 2017).

Besides, other chemical constituents show various biological activities. First, neoandrographolide (Figure 2B) is also a labdane diterpenoid that has been shown anti-viral activity, such as SARS-CoV-2 (or Covid-19). Recently, Natarajan et al. (1) reported that it could be used as a cost-effective drug-analog for treatment of covid-19 infection in developing countries.

Second, 14-deoxyandrographolide (Figure 2C), isolated from ethanolic crude extract, exhibited antimicrobial properties using disc-diffusion technique. The result showed mild antimicrobial activity against *Escherichia coli* with a zone of inhibition of 8 mm at a concentration of 50 µg/disc (Rashid, Ahmed, Rahaman, & Muhit, 2018).

Furthermore, 14-deoxy-11,12-didehydroandrographolide (Figure 2D) has been found to reduce synthesis of extracellular protease by *Pseudomonas aeruginosa* at around 90% (Majumdar, Misra, & Roy, 2020).

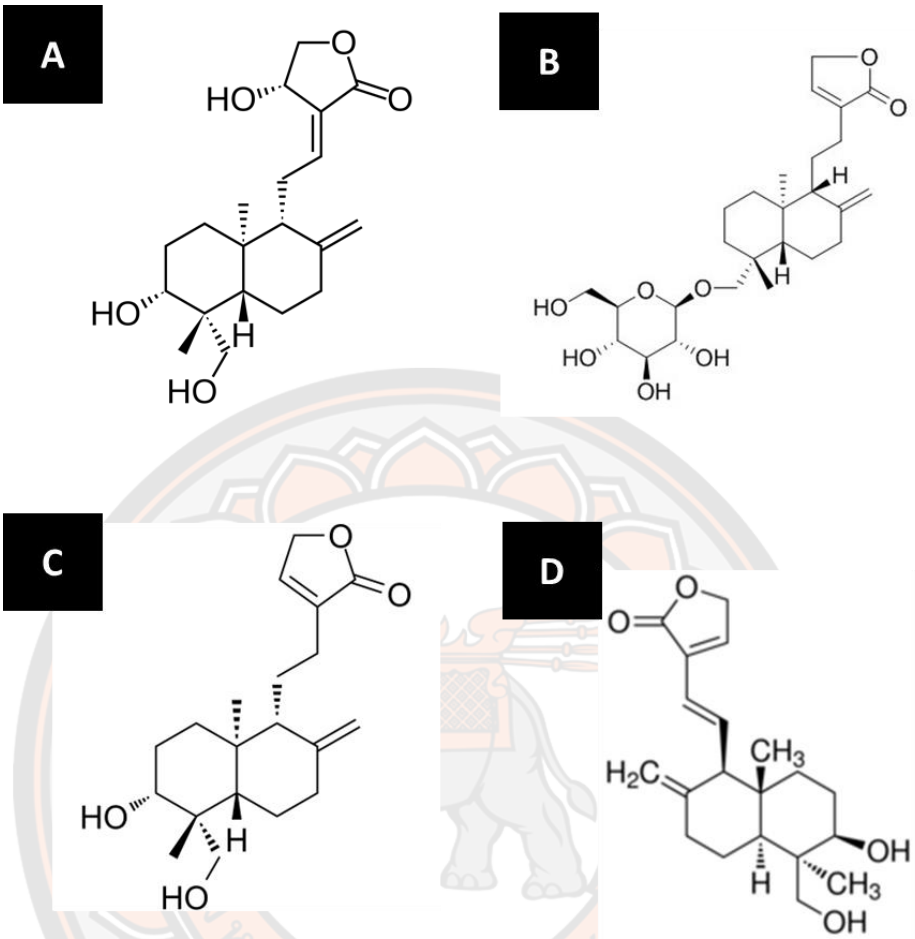



Figure 2 Chemical structure of andrographolide (A), neoandrographolide (B), 14-deoxyandrographolide (C), and 14-deoxy-11, 12-didehydroandrographolide (D)

Flavonoids (flavones)

Flavones are major flavonoids that have been isolated from the roots, aerial parts, and whole plant of *A. paniculata.* 5-Hydroxy-7,2',6'-trimethoxyflavone is flavones (Reddy et al., 2003).

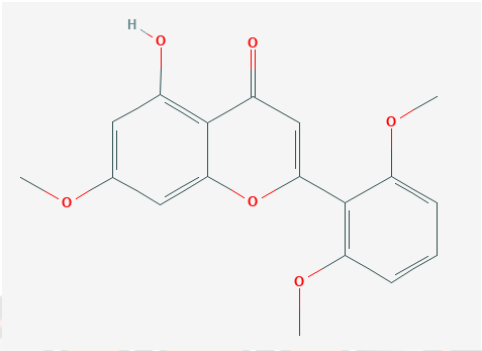


Figure 3 Chemical structure of 5-hydroxy-7,2',6'-trimethoxyflavone

Xanthones

The xanthones are a class of biologically active compounds. Four xanthones have been isolated from the root of AP. They were characterized as 1,8-di-hydroxy-3,7-dimethoxy-xanthone (Figure 4A), 4,8-dihydroxy-2,7-dimethoxy-xanthone (Figure 4B), 1,2-dihydroxy-6,8-dimethoxy-xanthone (Figure 4C), and 3,7,8-trimethoxy-1-hydroxy xanthone (Figure 4C). The one of xanthones that shown anti-malarial activity without cytotoxicity with $IC_{50}$ values > 16 g/mL is 1,2-dihydroxy-6,8-dimethoxy xanthone (Dua et al., 2004).

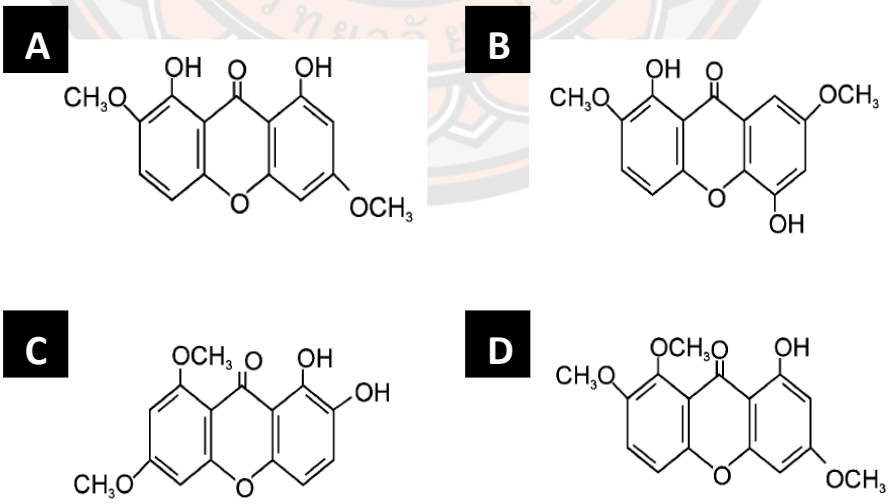



Figure 4 Chemical structure of 1,8-di-hydroxy-3,7-dimethoxy-xanthone (A), 4,8-dihydroxy-2,7-dimethoxy-xanthone (B), 1,2-dihydroxy-6,8-dimethoxy-xanthone (C), and (D) 3,7,8-trimethoxy-1-hydroxy xanthone.

**Pharmacological activities**

Antiviral activity

Antiviral activity of AP and other pharmacological activities have been reported over the past two decades. In 2015, an ethanolic extract from leaves of AP showed an inhibition of the SRV virus titer like the positive control lamivudine (Churiyah, Pongtuluran, Rofaani, & Tarwadi, 2015).

In 2020, phytochemicals isolated from AP plant were reported to have their potency against SARS-CoV-2 or Covid-19 by using in silico method computational approaches called molecular docking when compared with the commercial drug. The result showed the binding affinity of neoandrographolide in the active site of four target proteins including 3CLpro, PLpro, RdRp and spike protein that required for inhibiting in a therapeutic way for antiviral activity. Therefore, neoandrographolide could be suggested as a cost-effective drug for treatment of covid-19 infection (Murugan et al., 2020).

Antibacterial activity

The inhibition of bacterial growth of both gram-positive and gram-negative was found in an ethanolic extract shown as MIC values compared to ciprofloxacin that is standard in 100 and 200 µg/mL (Mishra, Mishra, Kumari, Pn, & Naik, 2009). In addition, the ethyl acetate extract of AP exhibits significant antibacterial activity against both susceptible and resistant strains of *Klebsiella Pneumoniae* and *E. coli* through the broth dilution method. The extract has also been found to be effective in controlling the growth of E. coli and K. pneumoniae, and inhibiting biofilm formation (Sah, Rasool, Ali, & Hemalatha, 2019; Sah, Rasool, & Hemalatha, 2019).

For aqueous methanol (50% v/v), crude extracts of the whole plant displayed antibacterial activity against *Bacillus subtilis* and *Proteus vulgaris* but not *E. coli* (Nakanishi et al., 1965). For aqueous methanol (70%v/v), the inhibition effect towards gram-positive bacteria was found. In contrast, methanol extract showed more potent inhibitory activity against both gram-positive and gram-negative bacteria such as *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* at the concentration of 50-100 mg/mL (Chuah, Mun, & Teo, 2017).

However, an antibacterial activity of aqueous extract to pathogens including *E. coli*, *Salmonella*, and *S. aureus*, was not demonstrable even in a solution containing 25 mg/mL of crude powder (Leelarasamee, Trakulsomboon, & Sittisomwong, 1990).

It can be concluded that AP extract has shown good inhibitory effects against most pathogenic and nonpathogenic. Therefore, it can be further investigated and applied as a potential source of a new class of antibiotics and new ingredients in cosmetic (Sule et al., 2012).

# CHAPTER III

# RESEARCH METHODOLOGY

The aim of the present study is to prepare foaming hand sanitizer containing AP extract and to investigate an antimicrobial activity of the extracts against common organisms such as *Staphylococcus aureus* and *Staphylococcus epidermidis*. Furthermore, the stability and phytochemical parameters of the prepared formulations were evaluated.

Our study was divided into two parts. The first part was an extraction of AP, a study of antibacterial activity of the whole plant, and qualitative and quantitative determination of phytochemicals from crude extract.

The second part involved formulation of foaming hand sanitizer containing AP extract along with antibacterial activity test and stability test (Figure 5).

**Experimental design in this study**

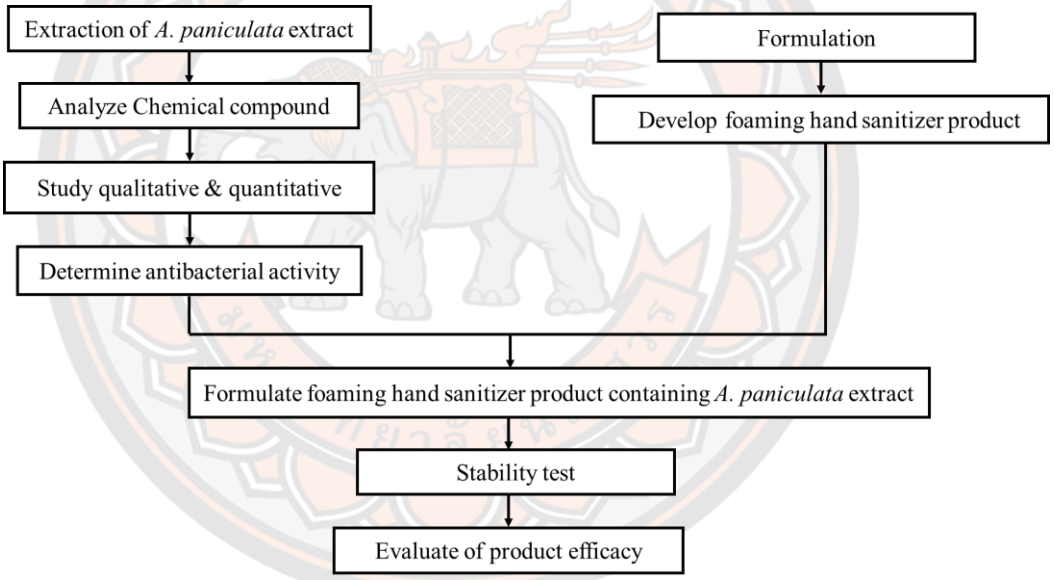



Figure 5 Experimental design in this study

**Chemicals and reagents**

1. Disodium EDTA (Cosmetic grade, Nouryon, China)
2. SulFoam, Disodium Laureth Sulfosuccinate (Cosmetic grade, BASF, Thailand)
3. Comperlan KD, Cocamide DEA (Cosmetic grade, BASF, Thailand)
4. N-alkyl betain, Cocamidopropyl betaine (Cosmetic grade, BASF, Thailand)
5. Coco Wash™, PEG-7 Glyceryl Cocoate (Cosmetic grade, NIKKOL, Janpan)
6. PEG-40 Hydeogenated Castor oil, PEG-40 Hydeogenated Castor oil (Cosmetic grade, NIKKOL, Janpan)
7. CH. ONLY BL M10, Fragrance, (Cosmetic grade, Hong huat, Thailand)
8. Thyme Kill™, Isopropyl methylphenol (Cosmetic grade, NIKKOL, Janpan)
9. Phenoxyethanol (Cosmetic grade, Chemipan, Thailand)
10. Soyabean Casein Digest Agar (Tryptone soya Agar) (Casein Soybean Digest Agar) (HIMEDIA, Mumbai, India)
11. Mueller Hinton Agar (HIMEDIA, Mumbai, India)
12. Dey-Engley Neutralizing Broth Base (HIMEDIA, Mumbai, India)
13. Trytone Broth (Tryptone Water) (HIMEDIA, Mumbai, India)
14. Phosphate buffered saline (Sigma-Aldrich, Chemie Gmbh, Munich, Germany)
15. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Chemie Gmbh, Munich, Germany)
16. Tween -20 (B LOBA CHEMIE PVT. LTD., Mumbai, India)
17. Tetracycline (Oxoid™, United Kingdom)
18. Methanol (HPLC grade, RCLabscan, Thailand)
19. 3,5-Dinitro-Benzoic acid (Analytical grade, Chemie Gmbh, Munich, Germany)
20. Ethanol (Analytical grade, RCLabscan, Thailand)
21. Andrographolide standard (purity≥97%) (Chemika, NSW, Australia)
22. Chloroform (Analytical grade, RCLabscan, Thailand)

**Instruments**

1. High Performance Liquid Chromatography (HPLC) (LC-20AT, Shimadzu, Kyoto, Japan)
2. Column C18 Jupiter 5u C18 300A (150 x 4.60mm.) (Part Number: 00G-4053-E0, Phenomenex)
3. SecurityGuard™ Guard Cartridge Kit (Part Number: KJ0-4282, Phenomenex)
4. Rotary evaporator (BUCHI, interface t-300, Postfach, Switzerland)
5. Incubator (VO400cool, MEMMERT, Schwabach, Germany)
6. Blender (HR2020, PHILIPS, Amsterdam, Netherlands)
7. pH meter (PL-700, Gondo, Nangang, Taiwan)

**Methodology**

Plant materials

The whole plant of AP was collected from Nakhon Phanom, Thailand. The plant identification was made by Assistant Professor Pranee Nangngam, Ph.D. from Department of Biology, Faculty of Sciences, Naresuan University. A voucher herbarium specimen of AP is made and stored at PNU Herbarium voucher number 005689 for future reference. The whole plant was dried at a temperature of approx. 30-40ºC. The dried plants were then homogenized into fine powder by blender.


Figure 6 Herbarium voucher of AP.

Preparation of ethanolic AP extract

The dried powder of AP (100 g.) was extracted with 1.2 L of 95% ethanol for 48 hr. The AP extract was filtered and concentrated in vacuum under reduced pressure (Figure 6).

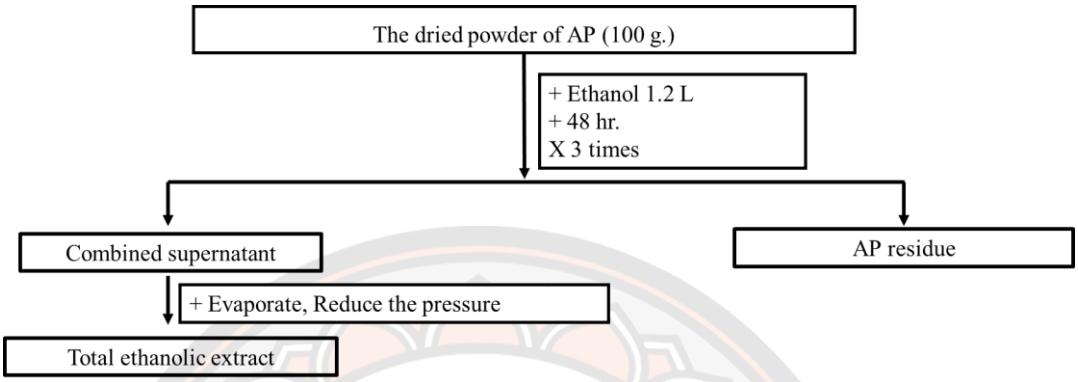



Figure 7 Schematic diagram of AP extract

Qualitative determination of phytochemicals from AP extract.

The AP plant is also known for its antibacterial and antiviral properties (Banerjee et al., 2017; Chuah et al., 2017; Dua et al., 2004). The major constituent of AP is andrographolide which has many pharmacological activities, especially antibacterial activity (Banerjee et al., 2017).

Thin layer chromatography analysis (TLC)

The determination of andrographolide in AP extract was confirmed by thin layer chromatography (TLC) using silica gel GF254 as the coating substance and a mixture of 85:15 volumes of chloroform and absolute ethanol as the mobile phase.

For the preparation of the sample, 1 g of AP extract was dissolved in 1 mL of ethanol. For the standard solution, 2 mg of standard commercial andrographolide was dissolved in 1 mL of ethanol.

The AP extract and the standard solution were applied separately on the TLC plate, with 5 μL of each solution. The TLC plate was removed and left to air dry before being viewed under ultraviolet light (254 nm). The TLC plate was sprayed with a 2 percent w/v 3,5-dinitrobenzoic acid in methanol and a 5.7 percent w/v potassium hydroxide solution in methanol, respectively, after examination. The chromatogram of the AP extract appeared to have a quenching spot (hRf; values 52–56) that corresponded to the andrographolide spots in standard solution (*Thai herbal pharmacopoeia*, 2009).

Quantitative determination of andrographolide from AP extract

High-performance liquid chromatography (HPLC) analysis

The determination of andrographolide was further confirmed using high-performance liquid chromatography (HPLC) analysis. The mobile phase was made up of a 1:1 combination of distilled water and methanol.

Standard preparation

An accurate quantity of andrographolide was dissolved in sufficient methanol and diluted with mobile phase to obtain a stock solution at a known concentration of 200 μg/mL. This solution was diluted quantitatively and sequentially with mobile phase to get six different solutions with concentrations of 2.5, 5, 10, 20, and 40 g/mL.

Sample preparation

One mg of AP extract was dissolved in 1 mL methanol, then diluted with 0.9 mL mobile phase and filtered over a 0.45 m nylon membrane.

Separation system

The separation was performed using a reverse phase HPLC column (150 x 4.60 mm.) filled with octadecylsilane chemically linked to porous silica or ceramic microparticles (5 m) at a flow rate of 1 mL per min with 224 nm detection.

Procedure and calculation

The 20 µL of both standard solution and AP extract solution were separately injected into the column. The signal was recorded on the chromatograms and determined for the major peak. By reference to the standard curve. (*Thai herbal pharmacopoeia*, 2009).

Determination of antibacterial activity

*Staphylococcus aureus* (B-2-1399) and *Staphylococcus epidermidis* (B-2-1407) applied in our research were provided by The National Institute of Health (NIH), a part of the Department of Medical Sciences, Ministry of Public Health Thailand. Naresuan University Institutional Biosafety Committee has accepted this protocol as a biosafety number of NUIBC MI 63-12-59. Before usage, the bacterial stock cultures were maintained on Mueller Hinton Agar (MHA). (A et al., 2013; Mishra et al., 2009; Rajalakshmi & Banu, 2016).

*Bacterial Culture and the AP extract solution*

Mueller Hinton Agar was used to cultivate *S. aureus* and *S. epidermidis* for 24 hours at 37°C before the experiment. After that, the bacteria stains on MHA agar were suspended in 0.85% normal saline. The bacteria suspension was then evaluated at 600 nm using the microplate reader. The absorbance should be between 0.080 and 0.100. Furthermore, to achieve a concentration of 100 g/disc, the AP extract was re-suspended in 5% DMSO and 5% Tween 20.

*Determination of antibacterial susceptibility test of AP extract*

The disc diffusion method was used to investigate the antibacterial susceptibility of AP extract. The 100 µL of bacteria stock solution was spread on their MHA. The discs were coated with 20 µL of AP extract solution (100 mg/disc) and incubated at 40°C for 30 minutes. The discs were placed on MHA and incubated for 24 hours at 37°C.

Antibacterial activity of AP extract was determined by measuring the diameter of the inhibitory zone (mm). Negative and positive controls were utilized, with 5% w/w Dimethyl sulfoxide (DMSO) and 5% w/w Tween 20 in water and 30 g of tetracycline, respectively. This test was performed three times and the results were presented as mean ± SD. A paired Student's t-test was used to assess the data. When the *p*-value was less than 0.05, the differences were significant.

Minimum inhibitory concentration (MIC) analysis

The microdilution assay was used to define MIC of AP extract. The Mueller Hinton broth (MHB) was used to prepare *S. aureus* and *S. epidermidis* in $1.5x10^5$ CFU/mL.

50 µL of AP extract stock solution in 5% w/w Tween 80 was used to prepare the final concentration of 6-70 mg/mL with the mixture of 50 µL bacteria suspension compared with 50 µL of MHB and 50 µL of bacteria suspension as a positive control and 50 µL MHB and 50 µL of 5% Tween 80 as a negative control.

Both strains were incubated at 37°C for 24h. After that, the microplate readers were used to measure AP extract at wavelength 600 nm. The measurement was triplicately carried out. Moreover, the MIC value was calculated by using percentage of inhibition (%I) as followed:

$$\%I = \frac{(TFGrowth - T0Growth) - (TFBlank - T0Blank)}{(TFAPE - T0\text{APE}) - (TFBlank - T0Blank)}$$

Where:

$T_0$APE = The absorbance of AP extract before incubation.

$T_F$APE = The absorbance of AP extract after incubation.

$T_0$Blank = The absorbance of blank before incubation.

$T_F$Blank = The absorbance of blank after incubation.

$T_0$Growth = The absorbance of positive control before incubation

$T_F$Growth = The absorbance of positive control after incubation

The MIC of the AP extract should be at least 80% to define as a potential of bacteria inhibitor according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

Minimum bactericidal concentration (MBC) analysis

All concentrations exhibited at least 80% of MIC were selected to test minimum bactericidal concentration (MBC). The selected concentrations were spread on MHA at 37°C for 24h. The MBC values were identified as no single colony on agar.

Development of formulation

This research focused on developing a foaming hand sanitizer that was both effective and safe. As shown in Table 1, the researcher varied the percentages of components and AP extract.

Table 1 Composition of formulations of hand sanitizer

| **Trade name** | **INCI name** | **Part** | **(In formula)** |
|---|---|---|---|
| **Water** | Water | A | Solvent |
| **Disodium EDTA** | Disodium EDTA | | Chelating agent |
| **Thyme Kill™** | Isopropyl Methyl Phenol | | Preservative |
| **DMDM Hydantoin** | 1,3-Bis(hydroxymethyl)-5,5-dimethylimidazolidine-2,4-dione | | Preservative |
| **Aloe Vera extract** | Aloe vera callus extract | | Humectant |
| **SulFoam** | Disodium laureth sulfosuccinate | B | Cleansing agent |
| **Cocamide DEA** | Cocamide DEA | | Foam booster |
| **Cocamidopropyl betaine** | Cocamidopropyl betaine | | Foam booster |
| **PEG-7 Glyceryl cocoate** | PEG-7 Glyceryl cocoate | | Foam booster |
| **PEG-40 Hydrogenated castor oil** | PEG-40 Hydrogenated castor oil | C | Solubilizer |
| **AP extract** | *Andrographis paniculata* extract | | Active ingredient |
| **CH. ONLY BL M10** | Fragrance | | Fragrance |
| **Triethanolamine** | Triethanolamine | D | pH adjuster |

Evaluation of foaming hand sanitizer.

T The developed formulation was evaluated for appearance, viscosity, foam ability, odor, pH value, and percentage of remaining andrographolide.

*Measurement of viscosity*

The viscosity of the sample was measured by using Brookfield viscometer at 25°C with spindle CP 40. Viscosity measurements were made at 10 - 120 rpm (AlQuadeib, Eltahir, Banafa, & Al-Hadhairi, 2018).

*Measurement of foam height and foam retention*

The foaming ability and foam stability were determined using a modified cylinder stirring method. In a 250 mL graduated measuring cylinder, 50 mL of the 1% sample solution was deposited and covered with parafilm. For one minute, the measurement cylinder was shaken. After 1 minute of shaking, the foam's total volume was measured. The method was repeated for another 5 minutes to ensure foam retention (AlQuadeib et al., 2018).

*Measurement of pH*

The developed formulation was diluted using distilled water to prepare a 10 % sample. The pH of the prepared sample was checked using a pH meter at room temperature (AlQuadeib et al., 2018).

*Measurement of physical appearance*

The appearance was checked by obscured visually on a black background (Tarun, Susan, Suria, Susan, & Criton, 2014).

*Measurement of percentage remaining andrographolide in formulation*

The percentage of remaining andrographolide in the formulation was determined by using high-performance liquid chromatography (HPLC) analysis. The standard preparation, sample preparation, and conditions in HPLC are used for quantitative determination of the AP extract.

Qualitative determination of antimicrobial activity in foaming hand sanitizer product

*Staphylococcus epidermidis* (B-2-1407) and *Staphylococcus aureus* (B-2-1399) bacterial strains were used to determine the antimicrobial activity of the foaming hand sanitizer as part of the protocol. Fresh cultures of the isolated bacteria were grown on Mueller Hinton Agar (MHA) (A et al., 2013; Mishra et al., 2009; Rajalakshmi & Banu, 2016).

The antibacterial activity was used by disc diffusion method for qualitative measuring the efficacy of foaming hand sanitizer product. Firstly, 100 µL of tested bacteria suspension was spread on MHA. Then, the 20 µL of formulation Rx 6.2 cooperated with AP extract was dropped into the discs (200 µg/disc), and the discs was incubated by at 40°C for 30 minutes. After that, put the discs into an agar plate of each stain and incubate at 37°C for 24 hr.

The formulation Rx 6.2 containing AP extract exhibits antimicrobial activity if a clear and distinct zone of inhibition surrounding the disc is observed. The antimicrobial activity of the extract was determined by measuring the diameter of the zone of inhibition in millimeters (mm). To compare the results, a control formulation Rx 6.2 without AP extract was used as the vehicle control, and a commercial product (Kirei Kirei ®, JAPAN) was used as the positive control. The experiment was performed in triplicate and expressed as mean ± SD.

Quantitative determination of antimicrobial activity in foaming hand sanitizer product

The European Hand Disinfectant Standards EN 1276:2009 Chemical disinfectants and antiseptics with some modifications following the Thai FDA recommendations were used to determine the hand sanitizer product's efficacy in reducing bacteria (Tienungoon, 2019).

This method specifies a test method and the minimum requirements for antibacterial activity of antiseptic products. It was a quantitative suspension test to evaluate of bactericidal activity of antiseptics and chemical disinfectants.

*Test Products*

The formulation Rx 6.2 containing AP extract was tested for bactericidal efficacy. The test results were then compared with the Rx 6.2 without AP extract as vehicle control and the commercial product (Kirei Kirei ®, JAPAN) as the positive control.

*Test organisms and inoculum preparation*

The Staphylococcus aureus ATCC 6538 was used as the standard bacterial strain. The second subculture was used as a working culture. The test suspension was prepared by a direct colony suspension method by which the bacterial colonies were suspended in 0.85% NaCl. The test suspensions were prepared and diluted to $1^{-5}$ x $10^9$ cfu/mL (N) and between 3.0 x $10^3$ and 3.0 x $10^4$ cfu/mL (Nv) for use in test experiments and control methods, respectively. The number of bacterial cells was validated by plating on the surfaces of tryptone soya agar (TSA) plates. The Dey-Engley Neutralizing Broth Base was used as neutralizer.

*Test procedures*

The test procedures consisted of one test experiment and three control methods: experimental condition control, neutralizer control, and method validation.

*Test experiment method*

The test experiment method involved mixing 0.1 mL of the test bacterial suspension (1-5 x $10^9$ cfu/mL) with 0.1 mL of 30% (w/v) albumin. After that, 9.8 mL of the test product was added to the mixture. After 1 min and 5 min contact time, 1 mL of the mixture was added to 9 mL of the neutralizer. Following a 5 min neutralization period, 1 mL was sampled out and serial diluted (if needed) before plating onto TSA plates to detect surviving bacteria. The tests were performed twice with two replicates at 34°C.

*Product efficacy calculation and Criteria*

The averages of the plate counts were converted to $\log_{10}$cfu/mL. The $\text{Log}_{10}$reduction ($\text{Log}_{10}$R) was calculated by the following equation:

$\text{Log}_{10}\text{R} = \log_{10}\text{initial count} - \log_{10}\text{bacterial count at 1 min contact time}$

To pass the EN1276 standard with some modifications as Thai FDA recommended, the product test must achieve a 5 $\log_{10}$reduction in test bacteria or the product must kill 99.999% of bacteria in 1 min at 34°C.

*The control methods*

To ensure that there was no biocidal effect from any experimental parameters, the same procedure as the test experiment method was performed. Validated bacterial suspension (Nv) between $3.0 \times 10^3$ and $3.0 \times 10^4$ cfu/mL and distilled purified water was used in place of the test bacterial suspension (1-5 x $10^9$ cfu/mL) and neutralizer, respectively. The number of surviving bacteria was counted on TSA plate.

*The neutralizer control:*

The neutralizer control was performed to make sure that the neutralizer can inhibit biocidal effect of products and the neutralizer has no effect against tested bacterial strains. The test mixtures of 9.8 mL neutralizer, 0.1 mL distilled purified water and 0.1 mL of the validated bacterial suspension (Nv) was prepared and left for 5 min at 34°C. Then, 1 mL of the mixtures was plated onto TSA plates to detect the number of surviving bacteria.

*The method validation:*

To validate the neutralization method, the test mixtures of 100 µL of bovine albumin, 100 µL of 0.1% Tryptone water, and 9.8 mL of the sample were mixed and placed at 34°C ± 1°C for 5 min (contact time), then 1.0 mL of the test mixture was transferred to 8.9 mL of neutralizer. After 5 min, 0.1 mL of the validated bacterial suspension (Nv) was added and left for 30 min before plating 1 mL of the mixtures onto with TSA plate to count the number of surviving bacterial cells. All three control methods were performed along with the test experiment method on the same day using the Nv that was prepared using test bacterial suspension dilation.

*Recovery ratio calculation and criteria*

The recovery ratios for all control methods were calculated by dividing the number of surviving bacterial cells by the number of validated bacterial suspension (Nv). To pass the EN: 1276 standard, the recovery ratios from all control methods must be in the acceptance criterion of ≥ 0.5 x number of bacteria in validated bacterial suspension.

Stability test

Stability studies were performed according to ICH guidelines for accelerated testing with required modifications. The formulation was evaluated for chemical stability and physical stability (viscosity, color, odor, pH, and foaming ability). The formulation was stored in packaging that protected it from light at 45 ± 2°C, 75% ± 5% RH for days 0, 10, and 30 after formulation. ("Appendix C - Additional ICH and FDA guidelines," 2016). The chemical stability tests were evaluated by High-performance liquid chromatography (HPLC) analysis to quantify andrographolide from formulation.

Statistical analysis

All data were expressed as mean ± SD calculated from at least three different experiments. The data were analyzed using paired Student’s t-test. Differences were considered statistically significant when p value was lower than 0.05.

# CHAPTER IV

# RESULTS AND DISCUSSION

## Extraction of AP

The extracted AP powder was soaked in 95% ethanol for 48 hours to obtain the AP extract. The resulting extract appeared as a sticky dark green paste, as shown in Figure 8. The yield of the extract was calculated as a percentage of the weight of the extract per 100 g of dried AP powder, which was found to be 15.63% (w/w), as illustrated in Table 2.

Figure 8 The appearance of AP extract

Table 2 The percent yield of AP extract

| Weight of AP powder | Weight of AP extract | % Yields |
|---|---|---|
| 100 g. | 15.63 g. | 15.63 |

**Determination of andrographolide from AP extract using TLC**

The sample solution was selected to perform the TLC analysis with a reference standard of andrographolide where a mixture of chloroform and ethanol (85:15) was used as the mobile phase. The Rf value of the AP extract (0.53) and standard andrographolide (0.55) matched completely, which indicated the presence of andrographolide in a sample as shown in Figure 9.

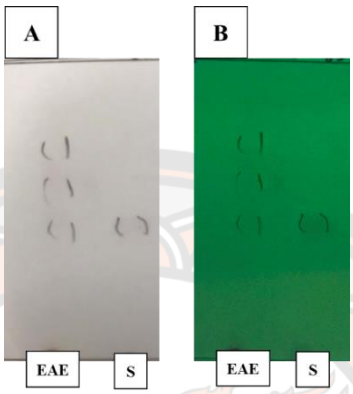



Figure 9 TLC analysis of AP extract (EAE) and standard andrographolide solution (S) under natural light [A] and under ultraviolet light (254 nm) [B]

**Quantification of andrographolide content in the extract using HPLC technique**

HPLC was used to determine the amount of andrographolide in the AP extract. As illustrated in Figure 10, a standard solution of reference andrographolide was injected, and the retention duration was found to be 4.3 minutes. When compared to reference andrographolide, the AP extract had the same retention time. This revealed that andrographolide was present in the AP extract.

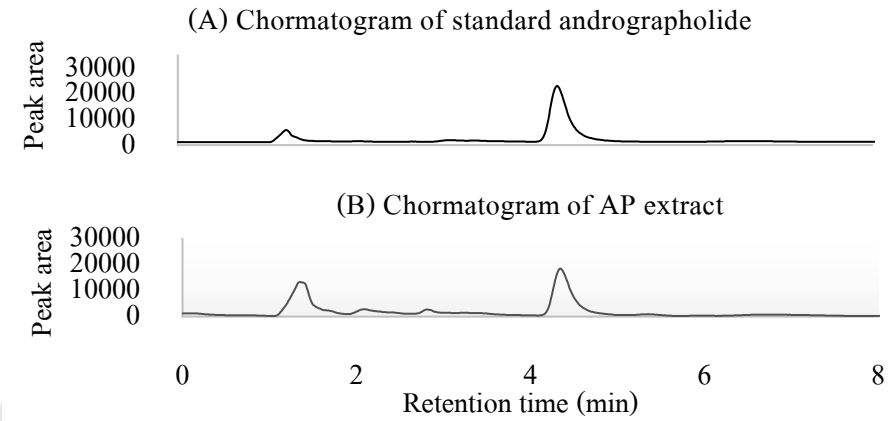



Figure 10 HPLC chromatograms of the standard andrographolide at 10.00 µg/ml [A] and AP extract [B]

A calibration curve was provided by peak areas and concentration as shown in Figure 10 of standard solutions, and the content of andrographolide content in the extract was quantified using a linear regression equation of the calibration curve. The linear regression equation of calibration curve was "y = 31207x + 16380", where X = concentration (µg/mL) and Y = peak area with coefficient of determination ($R^2$) is 0.9999.

Table 3 Peak area of HPLC chromatogram for various concentrations of andrographolide.

| **Standard curve of andrographolide** | | | | | | |
|---|---|---|---|---|---|---|
| **Concentration (µg/ml)** | | **Area** | **Retention time** | **Height** | **Average of area** | **STD of area** |
| **40** | N1 | 1239848 | 4.342 | 76134 | 1265301.67 | 24420.18 |
| | N2 | 1288537 | 4.346 | 80646 | | |
| | N3 | 1267520 | 4.338 | 80303 | | |
| **20** | N1 | 599038 | 4.352 | 37826 | 635971.67 | 32144.72 |
| | N2 | 651243 | 4.363 | 41886 | | |
| | N3 | 657634 | 4.343 | 42487 | | |
| **10** | N1 | 330611 | 4.364 | 21691 | 334378.00 | 12908.54 |
| | N2 | 323772 | 4.362 | 21221 | | |
| | N3 | 348751 | 4.36 | 23473 | | |
| **5** | N1 | 175593 | 4.365 | 12434 | 176564.00 | 1023.52 |
| | N2 | 176466 | 4.363 | 11703 | | |
| | N3 | 177633 | 4.375 | 12277 | | |
| **2.5** | N1 | 87950 | 4.392 | 5924 | 88199.67 | 823.40 |
| | N2 | 89119 | 4.381 | 5987 | | |
| | N3 | 87530 | 4.372 | 6136 | | |

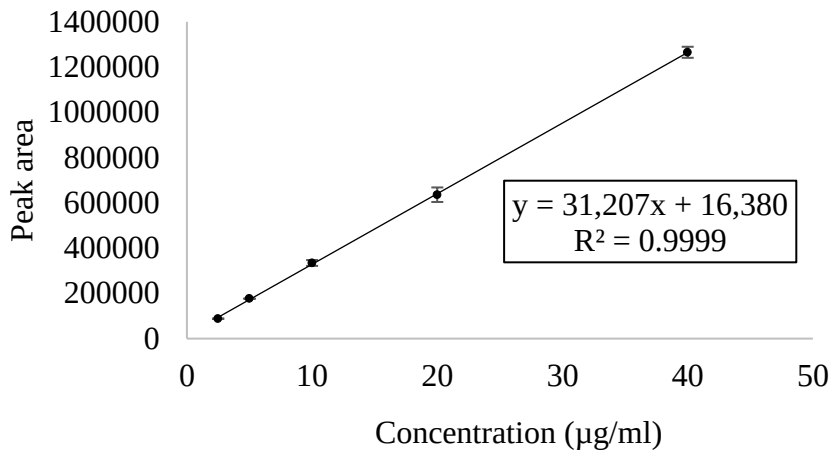



Figure 11 Calibration curve of standard andrographolide

The retention time for andrographolide was found to be 4.3 minutes after injecting a solution of AP extract (100 g/ml) under similar chromatographic conditions as the reference standard. The peak areas of the AP extract analyses were obtained after three replications with the prepared sample solutions. The concentration of andrographolide in the amorphous residue solution was 9.80 ± 0.43 µg/ml. The andrographolide was found at 9.80% w/w in the AP extract. The precision reached 4.35 % RSD for the sample studied. These findings are lower than those of a previous study, which discovered that AP extract was extracted using 95% ethanol. The andrographolide content in the AP extracts was 10.2 percent w/w. (Rafi et al., 2020).

Some research found a higher percentage of andrographolide concentration than this one (Masaenah, Elya, Setiawan, Fadhilah, & Arianti, 2021). Even though they used the same extraction, they discovered that andrographolide content in the AP extract was 14.5 percent. The percentage of andrographolide content widely differs in terms of the part used, season, geography, and harvesting time. That's the reason why in this study, AP extracts showed 9.80% w/w of andrographolide content, lower than other studies.

Table 4 Quantification of andrographolide in AP extract

| **Concentration of sample (y = 31207x+16380, $R^2$ 0.9999)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Sample (mg/ml)** | **Peak area** | | | **Concentration (µg/ml)** | | | **Average (µg/ml)** | **SD** | **%RSD** |
| | **N1** | **N2** | **N3** | **N1** | **N2** | **N3** | | | |
| **0.10** | 337,418.00 | 313,420.00 | 315,448.00 | 10.29 | 9.52 | 9.58 | 9.80 | 0.43 | 4.35 |

**Determination of antibacterial efficacy test of the AP extract**

Determination of antibacterial susceptibility test

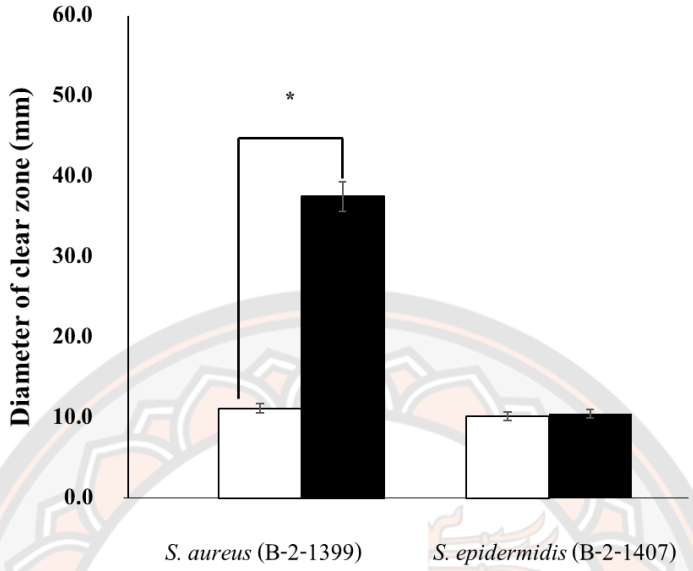



Figure 12 Antibacterial susceptibility determination of AP extract (light bar) 100 mg/disc and Tetracycline (dark bar) 10 µg/disc against *S. aureus* (B-2-1399) and *S. epidermidis* (B-2-1407). Each bar represents mean ± SD of triplicate study. *p<0.5, when compared between each group (n = 3, Student's *t*-test).

Antibacterial susceptibility determination was determined by the disc diffusion method. The diameter of clear zone (mm.) was expressed to evaluate AP extract against *S. aureus* (B-2-1399) and *S. epidermidis* (B-2-1407) as shown in Figure 13.

The results showed that the AP extract (100 mg/disc) and the positive control, Tetracycline (10 µg/disc), inhibited *S. aureus* by 11.2 ± 0.3 mm and 37.5 ± 1.0 mm, respectively. While AP extract presented the inhibitory activity against *S. epidermidis* (10.17 ± 0.29 mm) which nearly by Tetracycline (10 µg/disc) as the positive control significantly (*p*-value < 0.5) with the diameter of clear zone of 10.5 ± 0.4 mm.

The bacteriostatic effects of andrographolide, which consisted of AP extract, played an important role in inhibiting staphylococcal biofilm formation. Moreover, andrographolide can suppress the transcriptional regulator SarA which is an important transcriptional regulator of S. aureus. The transcriptional regulator SarA can directly regulate the expression of various virulence factors in humans, such as enterotoxin B and toxic shock syndrome (Zielinska et al., 2011).

**Determination of minimum inhibitory concentration (MIC)**

Table 5 MIC and MBC of AP extract against *S. aureus* and *S. epidermidis*

| Microorganism | MIC (mg/ml) | MBC (mg/ml) |
|---|---|---|
| *S. aureus* (B-2-1399) | 50 | >70 |
| *S. epidermidis* (B-2-1407) | 50 | >70 |

The antibacterial efficacy test of AP extract against *S. aureus* (B-2-1399) and *S. epidermidis* (B-2-1407) was performed by microdilution assay with MIC and MBC valued (mg/ml) as shown in Table 7. It exhibited that AP extract possessed the inhibitory activity against *S. aureus* and *S. epidermidis* with the MIC value of 50 mg/ml. In addition, AP extract gave the MBC against *S. aureus* and *S. epidermidis* with a value of >70 mg/ml. Nevertheless, the limitation of the solubility between the andrographolide compounds which consisted of AP extract presented the poor solubility in the cultured medium (Mueller Hinton broth, MHB). Thus, in this experiment, the highest concentration of AP extract which can be exhausted solute in broth medium was 70 mg/ml.

In comparison to other investigations, the MIC of this experiment is quite high. This could be related to extraction or the fact that the obtained source raw material contains less andrographolide than in other studies (Rafi et al., 2020).

**Development formulation**

s illustrated in Table 1, the foaming hand sanitizer was created by combining ingredients A, B, C, and D in specific proportions. The AP extract contains phytochemicals, including andrographolide, which are active ingredients to prevent microorganisms on hand while using the product. Low viscosity, soft foam, foaming retention, clear solutions, pleasant scents, and no drying after use are the ideal qualities for foaming hand sanitizer products. Syndet surfactant was used to make a variety of foamy hand sanitizer formulations, as indicated in Table 5. The formulation incorporates syndet surfactant due to its acidic pH, which helps maintain the stability of andrographolide under acidic conditions (Yan, Fang, & Du, 2018). It differs from the saponification process, which indicates an alkaline pH.

Table 6 Formulation of foaming hand sanitizer3

| **Ingredients** | | **Uses** | **Working formulation** | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Trade name** | **INCI name** | **(In formula)** | **Rx1** | **Rx2** | **Rx3** | **Rx4** | **Rx5** | **Rx6** |
| **Water** | Water | Solvent | 77.10 | 67.3 | 81.9 | 85.9 | 77.3 | 70.3 |
| **Disodium EDTA** | Disodium EDTA | Chelating agent | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| **Lactic acid** | Lactic acid | pH adjuster | 0.2 | - | 0.4 | 0.4 | - | - |
| **SulFoam** | Disodium Laureth Sulfosuccinate | Cleansing agent | 10 | 20 | 5 | 1 | 10 | 10 |
| **Cocamide DEA** | Cocamide diethanolamine | Foam booster | 3 | 3 | 3 | 3 | - | - |
| **Cocamidopropyl Betaine** | Cocamidopropyl Betaine | Foam booster | 8 | 8 | 8 | 8 | 8 | 8 |
| **Coco Wash™** | PEG-7 Glyceryl Cocoate | Cleansing agent | - | - | - | - | 3 | 10 |
| **DMDM Hydantoin** | DMDM Hydantoin | Preservative | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| **PEG-40 Hydrogenated Castor oil** | PEG-40 Hydrogenated Castor oil | Solubilizer | 1 | 1 | 1 | 1 | 1 | 1 |
| **CH. ONLY BL M10** | Fragrance | Fragrance | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |

**Evaluation of foaming hand sanitizer**

Viscosity profile of foaming hand sanitizer

The viscosity is important for a foaming product when applied with package pumper. The result showed formulations 1, 2, 4, 5, and 6 had no change in the viscosity profile when increasing the shear rate. This may imply that the rheological behavior of these formulations is Newtonian Fluids. However, Formulation 3 exhibited different viscosity behavior from the others. Because when increased, the shear rate affects the decrease in viscosity which manifests as shear thinning behavior or pseudoplastic fluid shown as in Figure 11. The viscosity of formulation 6 is nearly the commercial product. Therefore, the formulation was selected to develop with AP extract.

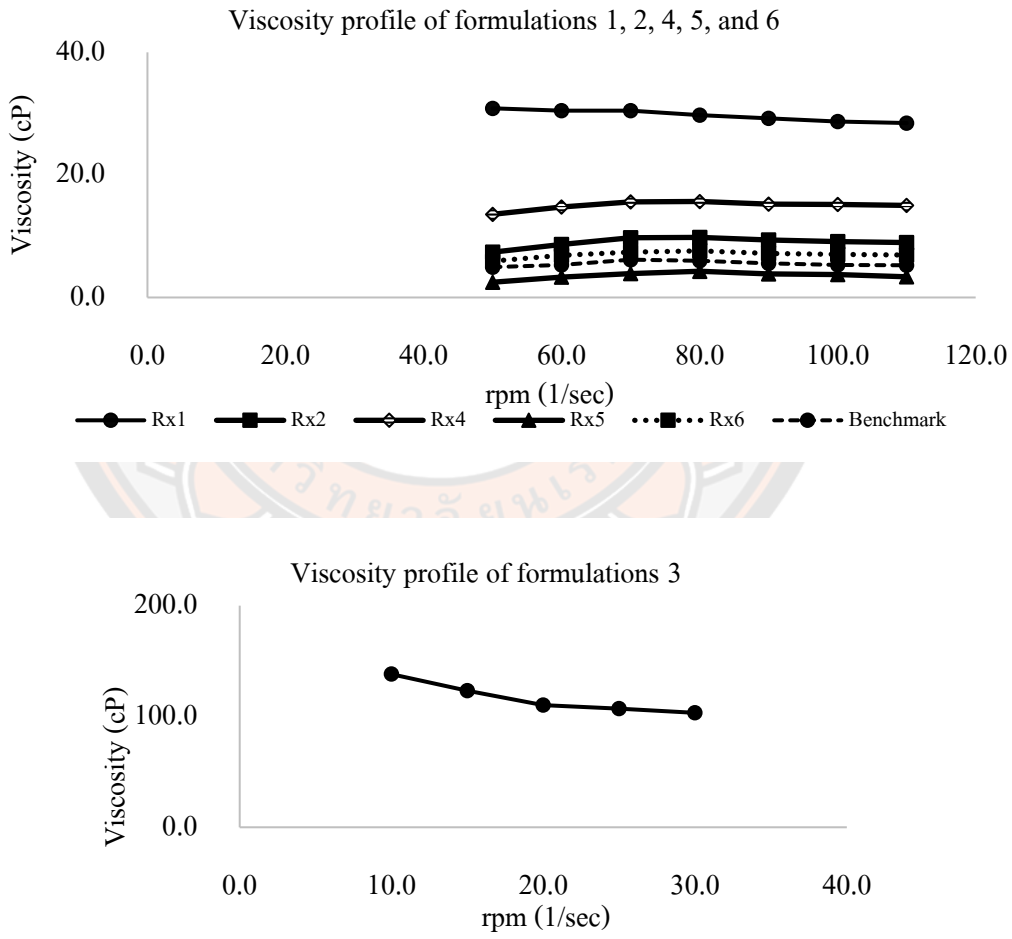



Figure 13 Viscosity profile of 6 formulations

Determination of Foamability and Foam stability

Foamability and foam stability were evaluated by recording the foam height and the foam retention, respectively. And the results illustrated that formulation 1-4, which uses Cocamide diethanolamine as the form booster surfactant, provided a foam height higher than PEG-7 Glyceryl cocoate, which using in formulation 5 and 6. This may imply that different surfactants provided a different amount of good foam, as shown in Figure 13.

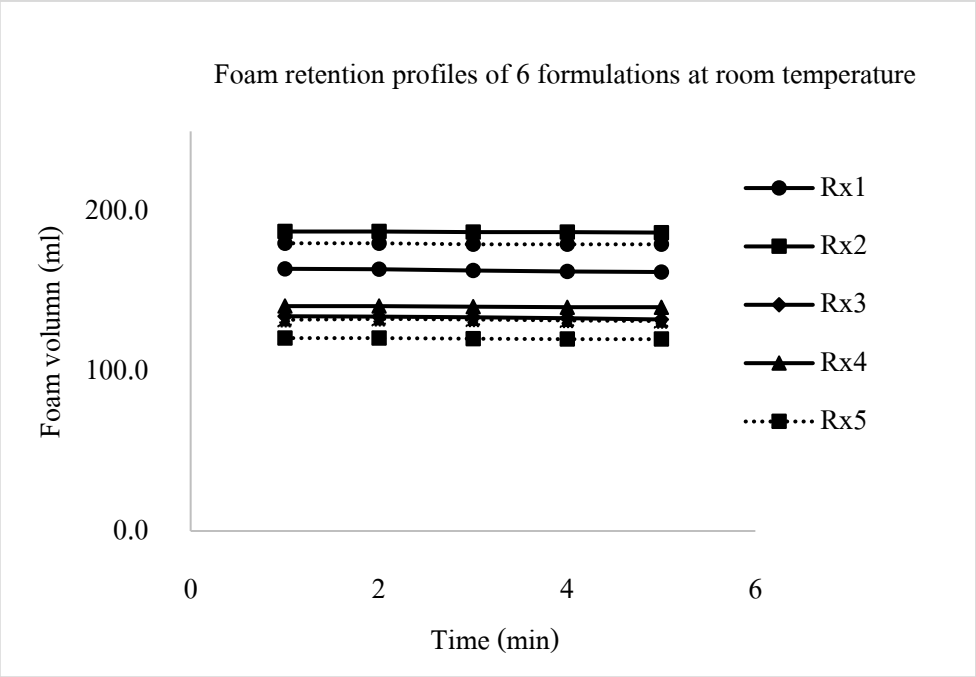
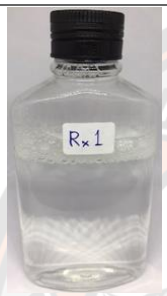
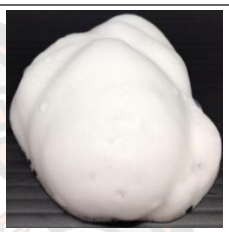
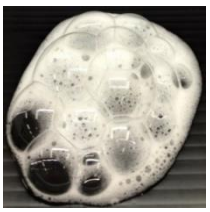


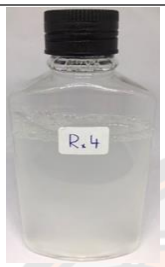
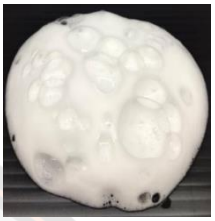
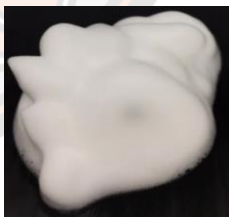

Figure 14 Foam retention profiles of 6 formulations at room temperature.

Physical appearance of 6 formulations

The formulation Rx6 showed the optimum criteria, Formulation 6 was selected to determine the physical appearance further as compared to the commercial product.

Table 7 Shows physical appearance of 6 formulations.

| Rx | Appearance of Product | Foam type | Feeling after wash | pH | Foam | ** |
|---|---|---|---|---|---|---|
| Rx1 |  | Small, dense | Dried | 5.64±0.02 |  | Disodium Laureth Sulfosuccinate (10%) |
| Rx2 |  | Small, dense | Dried | 5.55±0.01 |  | Disodium Laureth Sulfosuccinate (20%) |
| Rx3 |  | Big, airy | No dried | 5.58±0.07 |  | Disodium Laureth Sulfosuccinate (5%) |

| Rx | Appearance of Product | Foam type | Feeling after wash | pH | Foam | ** |
|---|---|---|---|---|---|---|
| Rx4 |  | Big, airy | No dried | 5.47±0.01 |  | Disodium Laureth Sulfosuccinate (1%) |
| Rx5 |  | Small, dense | No dried | 5.57±0.02 |  | PEG-7 Glyceryl Cocoate (3%) |
| Rx6 |  | Small, dense | No dried | 5.49±0.02 |  | PEG-7 Glyceryl Cocoate (10%) |
| Commercial product (Kirei Kirei®, JAPAN) |  | Small, dense | Dried | 9.29±0.01 |  | Saponification |

**Development formulation with AP extract**

The formulation of foaming hand sanitizer with AP extract was developed from Rx 6. The formulation was designated as RX 6.1, and Rx 6.2 were prepared, as shown in Table 8. The percent AP extract in the formulation was calculated by MIC value. Rx 6.1 and Rx 6.2 were prepared by incorporating 1% and 5% of PEG-40 Hydrogenated Castor oil. The PEG-40 hydrogenated castor oil was used as a solubilizer with 5 % (w/w) AP extract. The formulation shows the appearance of a drake green solution, and the pH of the formulation is 5.53±0.06, close to the skin's pH. The foam types of Rx 6.1 and 6.2 are dense and small. Both formulas are compliant.


Figure 15 Appearance of formulation Rx 6.2 (with AP extract) [A], Appearance of foam Rx 6.2 [B]

Table 8 Composition of formulations of foaming hand sanitizer with AP extract

| Ingredients | | Uses | Working formulation | |
|---|---|---|---|---|
| **Trade name** | **INCI name** | **(In formula)** | **Rx6.1** | **Rx6.2** |
| **Water** | Water | Solvent | 64.8 | 60.8 |
| **Disodium EDTA** | Disodium EDTA | Chelating agent | 0.1 | 0.1 |
| **SulFoam** | Disodium Laureth Sulfosuccinate | Cleansing agent | 10 | 10 |
| **Cocamidopropyl Betaine** | Cocamidopropyl Betaine | Cleansing agent, Foam booster | 8 | 8 |
| **Coco Wash™** | PEG-7 Glyceryl Cocoate | Cleansing agent | 10 | 10 |
| **DMDM Hydantoin** | DMDM Hydantoin | Preservative | 0.5 | 0.5 |
| **Thyme Kill™** | o-Cymen-5-ol | Preservative | - | 0.1 |
| **PEG-40 Hydrogenated Castor oil** | PEG-40 Hydrogenated Castor oil | Solubilizer | 1 | 5 |
| **CH. ONLY BL M10** | Fragrance | Fragrance | 0.3 | 0.3 |
| **AP extract** | *Andrographis paniculata* extract | Active ingredient | 5 | 5 |
| **Aloe vera extract** | Aloe vera extract | Humectant | 0.5 | 0.5 |

**Qualitative determination of antimicrobial activity in foaming hand sanitizer product**

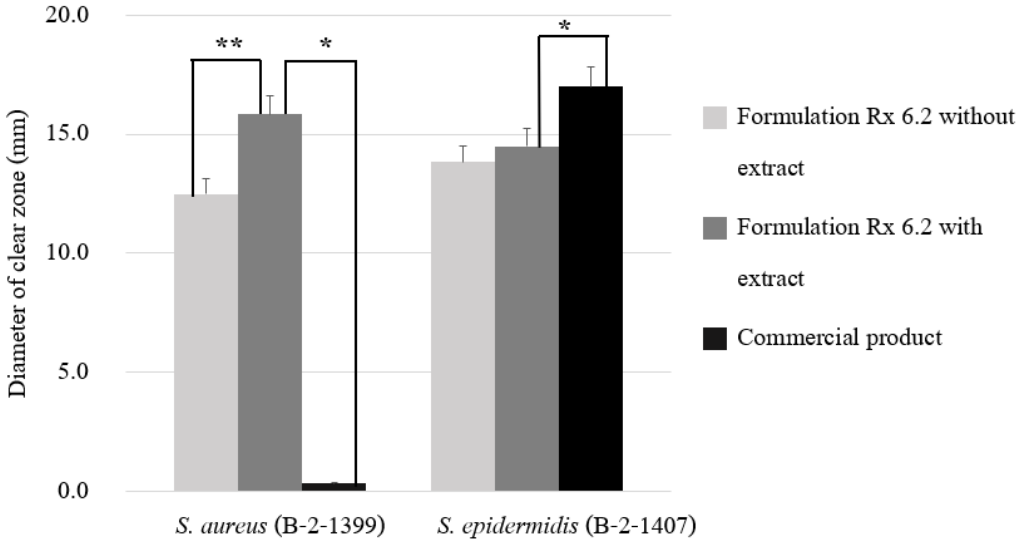



Figure 16 The qualitative determination antimicrobial activity of the formulation Rx 6.2 without AP extract (white bar), the formulation Rx 6.2 with AP extract (gray bar) and the commercial product (Kirei Kirei®, JAPAN) (black bar) against *S. aureus* (B-2-1399) and *S. epidermidis* (B-2-1407). Each bar represents mean ± SD of triplicate study. *$p$<0.5, when compared between each group (n = 3, Student's t-test).

The qualitative determination of the antimicrobial activity of a foaming hand sanitizer product utilized the disc diffusion method. The diameter of the clear zone (mm.) was expressed to evaluate the foaming hand sanitizer product against *S. aureus* (B-2-1399) and *S. epidermidis* (B-2-1407).

The results demonstrated that the formulation Rx 6.2 with AP extract, the formulation Rx 6.2 without AP extract and the commercial product (Kirei Kirei®, JAPAN) inhibited S. aureus by 15.8 ± 0.3 mm, 12.5 ± 0.9 mm., and 0.0 ± 0 mm, respectively. The Rx 6.2 with AP extract is considered to be significantly better than Rx 6.2 without EAE. The results revealed that the AP extract is potent in inhibiting *S. aureus*. While the Rx 6.2 with AP extract presented the inhibitory activity against *S. aureus* (15.8 ± 0.3 mm) which was better than commercial product (Kirei Kirei®, JAPAN) as the positive control significantly ($p$-value < 0.5) with the diameter of clear zone of 10.0 ± 0 mm.

The products that presented inhibitory activity against *S. epidermidis* were 14.5 ± 0.9 mm., 13.8 ± 0.6 mm., and 17.0 ± 0.9, respectively. In addition, the commercial product (Kirei Kirei®, JAPAN) with AP extract presented inhibitory activity against *S. epidermidis* (17.0 ± 0.9 mm.) which was significantly better than the formulation Rx 6.2 with AP extract ($p$-value < 0.5) with a diameter of clear zone of 14.5 ± 0.9 mm.

**Quantitative determination of antimicrobial activity in foaming hand sanitizer product**

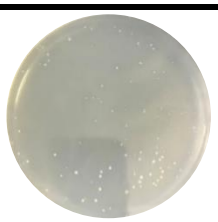
The product formulation Rx 6.2 cannot count colonies on agar or reduce bacterial numbers for tested Staphylococcus aureus ATCC 25923 bacterial strains in 1 min at 34°C under conditions. But The Rx 6.2 reduced bacterial numbers by more than 5 log10 reduction or more than 99.999% for tested bacterial strains *S. aureus* in 5 min at 34°C under conditions. This result indicated that the Rx 6.2 under test fulfilled the requirements of the EN 1276:2009 standard for the bactericidal activity of chemical disinfectants and antiseptic products.

However, commercial products that can inhibit bacteria may be caused by the formula. It has a relatively high alkalinity, which is another reason why it has better antimicrobial ability than Rx 6.2.

To prove that pH affects bactericidal activity. The Rx 7 was prepared to have an alkaline pH close to commercial products and the same composition as the Rx 6.2. The result found that Rx 7 with AP extract and Rx 7 without AP extract can reduce bacterial numbers by more than 5 log10 reductions or more than 99.999% for tested *S. aureus* bacterial strains in 1 min at 34°C under conditions similar to commercial products (Kirei Kirei ®, JAPAN) as shown in Table 10.

The Rx 6.2 has a limit of pH that requires acidic conditions. Because the active ingredient, such as andrographolide, is stable in acidic conditions, and its instability increases with the increase in alkaline strength (Yan et al., 2018). That is why formulation Rx 6.2 have to control pH below 6, and the alkaline conditions in the formulation can make skin dry after use.

Table 9 Antimicrobial activity test of the product using modified EN 1276: 2009 method.

| Test | 1 min contact time | 5-min contact time | Log10R | % Reduction |
|---|---|---|---|---|
| **Formulation 6.2 with AP extract (pH 5.5)** |  |  | 6.59 | >99.999 |
| **Formulation 6.2 without AP extract (pH 5.5)** |  |  | N/A | N/A |
| **Commercial product (Kirei Kirei ®, JAPAN) (pH 9.0)** |  | - | 8.66 | >99.999 |
| **Formulation 7 with AP extract (pH 9.0)** |  |  | 8.60 | >99.999 |
| **Formulation 7 without AP extract (pH 9.0)** |  |  | 8.60 | >99.999 |

*N/A = not applicable

Table 10 Efficacy of test product against tested bacteria using quantitative suspension test according to EN1276 at 1- and 5-min contact time.

| **Test Organisms** | Formulation Rx 6.2 (without AP extract) (1-min contact time) | | | |
|---|---|---|---|---|
| | Test 1 | Test 2 | Average | |
| | N | N | $Log_{10}R$ | % |
| ***Staphylococcus aureus*** | N/A | N/A | N/A | N/A |
| **Test Organisms** | Formulation Rx 6.2 (with AP extract) (5-min contact time) | | | |
| | Test 1 | Test 2 | Average | |
| | N | N | Log10R | % |
| ***Staphylococcus aureus*** | N/A | N/A | N/A | N/A |
| **Test Organisms** | Formulation Rx 6.2 (without AP extract) (1-min contact time) | | | |
| | Test 1 | Test 2 | Average | |
| | N | N | $Log_{10}R$ | % |
| ***Staphylococcus aureus*** | N/A | N/A | N/A | N/A |
| **Test Organisms** | Formulation Rx 6.2 (without AP extract) (5-min contact time) | | | |
| | Test 1 | Test 2 | Average | |
| | N | N | $Log_{10}R$ | % |
| ***Staphylococcus aureus*** | 138 | 97 | 6.59 | >99.999 |
| **Test Organisms** | Commercial product (Kirei Kirei®, JAPAN) (1-min contact time) | | | |
| | Test 1 | Test 2 | Average | |
| | N | N | $Log_{10}R$ | % |
| ***Staphylococcus aureus*** | 0 | 0 | 8.66 | >99.999 |

*N/A = not applicable

**Stability test**

The stability test was performed according to ICH guidelines for accelerated testing with required modifications. The formulation was stored in packaging and protected from light at 45 ± 2°C, 75%RH ± 5% for day 30 after the formulation ("Appendix C - Additional ICH and FDA guidelines," 2016). The Rx 6.2 showed no significant change in the color, pH, viscosity, foam height, and precipitate were observed at the end of the study. And the pH of the prepared Rx 6.2 was within the accepted range (5.0-5.7), as shown in Table 12. These pH values remain constant throughout the period of stability study. Moreover, for the chemical stability study, the percentage of andrographolide remaining after 30 days of storage for the Rx 6.2 formulation was approximately> 93%. The Rx 6.2 was found to be stable without significant change in the percentage of andrographolide contained in the product, as shown in Figure 16.

However, the chemical stability study of Rx 7 showed significant degradation. The percentage of andrographolide after three days for Rx 7 was approximately>88.6%. The percentage change of andrographolide was less than 11.4% for Rx 7, as shown in Figure. 17. The Rx 7 showed not good stability. Because active ingredients such as andrographolide are stable in acidic conditions, the andrographolide has an ester structure. Hydrolysis is easy, and its instability increases with the increase in alkaline strength (Yan et al., 2018).

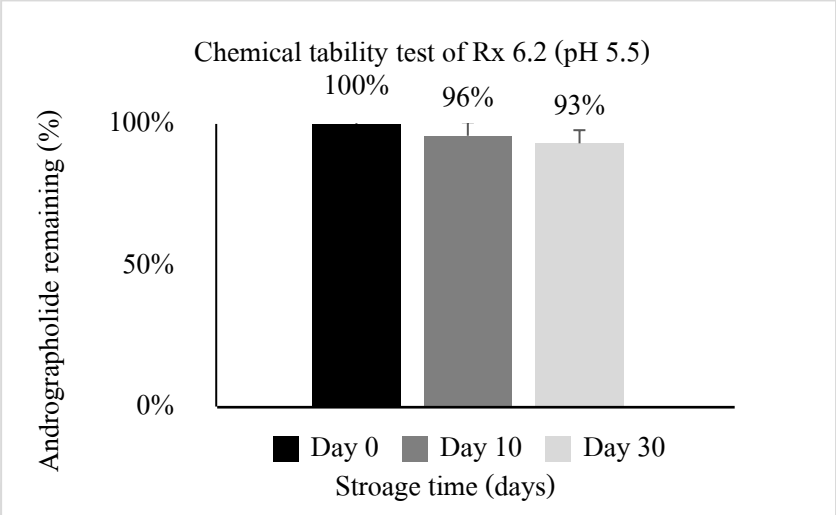
The foaming hand sanitizer containing AP extract from formulation Rx 6.2 showed a good appearance, low viscosity, soft foam, foaming retention, clear solutions, good smells, no drying after use, and relatively stable after preparation. And its good efficacy at preventing bacteria.

Table 11 Storage stability of foaming hand sanitizer

| Parameter | foaming hand sanitizer | | |
|---|---|---|---|
| | **0 day** | **10 days** | **30 days** |
| **Appearances** | Dark green liquid | Dark green liquid | Dark green liquid |
| **Color (L*, a*, b*)** | (2.66, 2.66, 1.09) | (2.45, 2.72, 1.26) | (2.21, 2.69, 1.10) |
| **Homogeneity** | Homogeneous | Homogeneous | Homogeneous |
| **pH** | 5.52±0.04 | 5.3±0.04 | 5.11±0.07 |
| **Viscosity (cP)** | 24.9±1.81 | 23.4±1.00 | 22.0±2.01 |
| **foam height (ml)** | 133.3±5.80 | 128.3±2.90 | 126.7±5.8 |
| **% Remaining of andrographolide in formulation Rx 6.2 (pH 5.5)** | 100% (14.57±0.92 µg/ml) | 96% (13.96±0.78 µg/ml) | 93% (13.57±0.80 µg/ml) |

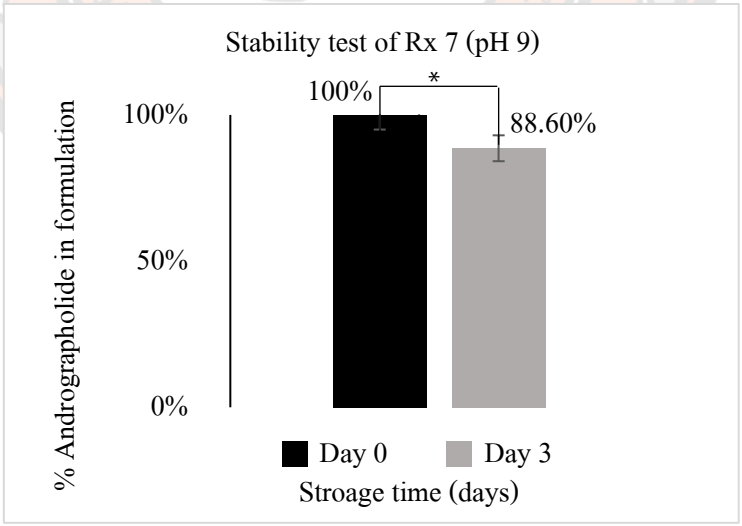
All results are represented as mean ± SD of triplicate study. *$p$<0.05, when compared between each group (n=3, Student's $t$-test)

Figure 17 The percentage of andrographolide remaining after 30 days of storage under accelerated conditions for formulation Rx 6.2.




All results are represented as mean ± SD of the triplicate study. *$p$<0.05, when compared between each group (n=3, Student's $t$-test)

Figure 18 The percentage of andrographolide remaining after 30 days of storage under accelerated conditions for formulation Rx 7.




All results are represented as mean ± SD of triplicate study. *$p$<0.05, when compared between each group (n=3, Student's $t$-test)

# CHAPTER V

# CONCLUSIONS

This study aimed to formulate a completely developed foaming hand sanitizer product containing *A. paniculata* extract, which has good antibacterial activity and stability. We formulated a foaming hand sanitizer by using AP extract. The AP extract was a sticky dark green paste with a percentage yield of 15.63%. For the quantification of andrographolide content, it was found that AP extract was observed at 4.3 min., which is present at the same retention time as the reference andrographolide standard and the andrographolide was found at 9.80% in the EAE. The determination of the antibacterial efficacy test was performed using the disc diffusion method and microdilution assay. The results showed that AP extract provided inhibitory activity against *S. aureus* and *S. epidermidis* to use as an alternative ingredient to formulate the foaming hand sanitizer.

The ideal quality of foaming hand sanitizer products includes low viscosity, soft foam, foaming retention, clear solutions, good smell, and no drying after use. The Rx 6.2 can answer every point of the ideal criteria. For the antimicrobial activity study of the product, the Rx 6.2 reduced bacteria by more than 99.999% for tested bacterial strains *S. aureus* in 5 min at 34°C under controlled conditions. This result indicated that the Rx 6.2 under test fulfilled the requirements of the EN 1276:2009 standard for the bactericidal activity of chemical disinfectants and antiseptic products.

The stability evaluation of the foaming hand sanitizer formulation showed positive results, as the formulation was found to be stable for up to 30 days at 5 ± 2°C, 75%RH ± 5%. No significant changes in the test parameters were detected during this period. Further research is required to investigate in clinical to ensure that the foaming hand sanitizer product shows antibacterial activity. However, there are supposed to be studies on safety

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