สำนักงานวิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER II

MATERIAL AND METHODS

1. Materials

1.1 Chemical

The following substances were commercially available:

Tea tree oil of pharmaceutical grade from Menthol Thai import export co., Ltd., Bangkok, Thailand; Tween 80® and triethanolamine were cosmetic grade purchased from Srichand United dispensary Co., Ltd., Bangkok, Thailand; tocopheryl acetate (vitamin E acetate), menthol crystal, methyl paraben, propyl paraben, propylene glycol, glycerin and Amerchol L-101® were pharmaceutical grade from S. Tong Chemical Co., Ltd., Bangkok, Thailand; Cremophore RH 40® was pharmaceutical grade obtained from Namsiang Company limited., Bangkok, Thailand; Calcium chloride and Calcium gluconate were analytical grade obtained from Merck, Germany.

Tea tree oil (TTO) purchased from Menthol Thai import export Co., Ltd., Bangkok, Thailand, which was supplied by Southern Cross Botanicals Ply, Australian & Newzealand. Batch RTTO1/4023 complied with the international standard for TTO, and contained 39.0% terpinen-4-ol, 20.6% γ-terpinine, 8.4% α-terpinene, 3.0% α-terpineol, 3.2% α-terpinolene, 3.8% 1,8- cineole, 2.7% α- pinene and 3.8% ρ-cymene. Levels of components were assessed by gas chromatography, performed by Certified Botanical Extracts, Australia & Newzealand.

Betel oil purchased from Thai Chaina Flavours and Fragrances Industry Co., Ltd., Bangkok, Thailand.

Tryptic soy agar and tryptic soy broth were microbiological grade obtained from Merck, Damstadt, Germany, Mueller hinton agar, Mueller hinton broth, brain

heart infusion agar, brain heart infusion broth, Sabouraud dextrose agar, Sabouraud dextrose broth and yeast extract were from Difco, Becton Dickinson and Company, France.

1.2 Equipments

- pH Meter (MP 230, Mettler Toledo, Switzerland)
- Viscometer (Brookfiled, USA)
- Magnetic stirrer (Model SP 46920-26, Barnstead/Hermolyne, USA)
- Hot air oven (Mammert, Germany)
- Incubator Model 6 (Thelco)
- Refrigerator
- Rotary evaporator (Büchi Rotavapor R-114, Switzerland)
- Hot plate (E.G.O., Germany)
- Vortex mixer (VORTEX-2 GENIE, USA)
- Autoclave HA-3D (Hirayama Manufacturing Coorperation, Japan)
- Micropipette (pipetman, Made in France)
- Pipette Aid (Easypet 4420,Eppendorf, Germany)
- Lamina flow (model LFV-60 MSSP)
- Colony counter (model 570, Suntex)

2. Methods

2.1 Isolation of polysaccharide gel (PG)

A polysaccharide gel (PG) was isolated from dried fruit-rind of durian (*Durio zibethinus* Murr.) by the following procedure. Waste of fresh durian fruit-hulls was collected, washed, blended and dried in hot oven at 70°C. A process of PG isolation was performed based on the method previously described by Pongsamart and Panmuang (1998). PG was purified by reprecipitating from a crude PG solution using acidic solution in ethanol. Product of PG was evaluated for determination of gel property and used for the preparation of antiseptic gel.

2.2 Properties of polysaccharide gel (PG)

2.2.1 Viscosity of PG

The viscosity of PG solutions in water at concentration 2.5% by weight was individually measured using small sample viscosity (Brookfield® viscometer). A volume of 8 ml PG solution was poured into cup, a cone was installed and the rate of shear was adjusted and the viscosity of PG solution was measured. The data were the mean value of the three determinations. The PG at 2.5% (w/v) in water that providing viscosity in the range of 400-800 cps was used in this study.

2.2.2 pH

PG solutions were prepared by dissolving PG in distilled water to make concentration of 2.5% by weight of PG. A pH meter individually measured the pH. The data reported were the mean value of three determinations.

2.2.3 Effect of sorbitol on viscosity of PG

The effect of sorbitol on viscosity of the PG solutions were examined by dropping sorbitol into 20 ml of 2.5% PG solution, the viscosity and volume of sorbitol were recorded. The graph was plotted between sorbitol concentration and viscosity of PG.

2.3 Properties of tea tree oil (TTO)

2.3.1 Solubility of tea tree oil (TTO)

Solubility test of 1% tea tree oil (TTO) was determined by using different solubilizing agents. Prepared 1% tea tree oil (TTO) by mixing 0.1 ml TTO in each of 9.90 ml of the following solvent mixture such as 20% ethanol, 0.5% Tween 20, 10% Tween 20, 0.5% Tween 60, 10% Tween 60, 0.5% Tween 80, 10% Tween 80, 0.1% DMSO, 10% Cremophore RH 40®, 15% Cremophore RH 40® in

deionized water, mixed for 3 min by using vortex mixer. The resulting mixtures of TTO in those solubilizing mixtures were observed.

2.3.2 Antimicrobial activity test

2.3.2.1 Microorganisms

All microorganisms were obtained from the Department of Microbiology, Faculty of Pharmaceutical Science, Chulalongkorn University. Nine bacteria, *Staphylococcus aureus* ATCC 6538P, *Staphylococcus epidermidis* ATCC 12228, *Micrococus luteus* ATCC 9341 and *Bacillus subtilis* ATCC 6633, were used as test gram positive bacteria and *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Proteus vulgaris* ATCC 13315, *Klebsiella pneumoniae* ATCC 10031 and *Pseudomonas aeruginosa* ATCC 9721 were used as test gram negative bacteria. All test bacteria were cultivated overnight on tryptic soy agar (TSA) slant. The bacteria were suspended in sterile normal saline (NSS). The culture was then adjusted to match turbidity of standard McFarland no. 0.5 before used. Two yeast strains, *Saccharomyces cerevisiae* ATCC 9763 and *Candida albicans* ATCC 10230 were used and cultivated on Sabouraud dextrose agar (SDA). The yeast suspension was prepared by the same procedure as described for bacteria cell cultures.

2.3.2.2 Preparation of test solution of tea tree oil (TTO)

Tea tree oil (TTO) was freshly prepared to make a series of two fold dilutions of various concentrations of tea tree oil (TTO) in 0.5% Tween 80 and tested in agar microbiological test.

2.3.2.3 Preparation of agar and broth media

Agar and broth media were prepared by dissolving the media in deionized water until dissolved and sterilized in autoclave at 15 pounds pressure (121°C) for 15 min.

Mueller hinton agar (MHA) and sabouraud dextrose agar (SDA) media were used in agar diffusion susceptibility test. All tested bacteria were inoculated on MHA plates. Two yeast strains were cultivated on SDA. For broth macrodilution susceptibility test, Mueller hinton broth (MHB) and sabouraud dextrose broth (SDB) were used as test media for testing bacteria and fungi, respectively.

2.3.2.4 Agar diffusion susceptibility test

Tea tree oil was prepared in a series of two fold dilution in 0.5% Tween 80. The petri dishes contained 25 ml of Mueller hinton agar (MHA) were inoculated with 1% microorganism suspension by seed layer method (Lorian, 1991). The sterile cork borers were bored into the seeded plates and filled with tested tea tree oil preparation 300 ml/well. The plates were incubated at 37°C for 24 hours, the diameter of inhibition zone was measured.

2.3.2.5 Broth macrodilution susceptibility test

MIC was determined by a broth macrodilution method as follows: in test tubes, pipette 4.0 ml of Mueller hinton broth (MHB) using two fold dilution of various concentration of tea tree oil in 0.5% Tween 80 and 0.5 ml of suspension of tested microorganism. The tubes were incubated at 37°C for 24 hours. MIC was the minimal concentration of tea tree oil that inhibited viable growth, and MBC was the minimal concentration of tea tree oil in tube showing no turbidity of microbial growth that demonstrated no growth on tea tree oil free agar media after incubation at 37°C overnight.

2.4 Preparation antiseptic PG gel

2.4.1 Formula 1: Tea tree oil-PG gel

Each formula of antiseptic gel using polysaccharide gel (PG) as a gelling agent and also an antibacterial agent including tea tree oil (TTO), second

active ingredient used as an antimicrobial agent, was prepared and evaluated the effect of different TTO concentration in finished product.

Ingredient	Function	Amount (g)
PG	Gelling agent, antibacterial agent	2.5
Tea tree oil (TTO)	Antimicrobial agent, Essential oil	0.7-1.0
Vitamin E acetate	Antiaging, Antioxidant	0.1
Amerchol L-101®	Emollient	0.5
Sorbitol	Humectants	5-10
Propylene glycol	Humectants	5-15
Glycerin	Humectants	5.0
Cremophore RH-40®	Solubilizing agent	5-13
Tween 80	Solubilizing agent	1-1.5
Menthol	Cool sensation	0.5-1.2
$CaCl_2$ (0.05M)	Electrolyte	0.1-0.2
Paraben concentrate	Preservative	1.0
Triethanolamine (TEA)	Adjust pH to 2.9 ± 0.1	-
Sterile water	to make total weight	100

2.4.2 Formula 2: Tea tree oil/Betel oil-PG gel

Each formula of antiseptic gel using polysaccharide gel (PG) as a gelling agent and also antibacterial agents including two other active ingredients, tea tree oil (TTO) and betel oil, was prepared and evaluated the antimicrobial activity of products of different concentration of TTO and BO in finished product.

Ingredient	Function	Amount (g)
PG	Gelling agent, antibacterial agent	2.5
Tea tree oil (TTO)	Antimicrobial agent, Essential oil	0.7-1.0
Betel oil	Antimicrobial agent, Essential oil	0.2-0.5
Vitamin E acetate	Antiaging, Antioxidant	0.1
Amerchol L-101®	Emollient	0.5
Sorbitol	Humectants	5-10

Propylene glycol	Humectants	5-15
Glycerin	Humectants	5.0
Cremophore RH-40®	Solubilizing agent	5-13
Menthol	Cool sensation	0.5-1.2
$CaCl_2$ (0.05M)	Electrolyte	0.1-0.2
Paraben concentrate	Preservative	1.0
Triethanolamine (TEA)	Adjust pH to 2.9 ± 0.1	-
Sterile water	to make total weight	100

2.4.3 Formula 3: PG-gel base

A formula of vitamin E gel using polysaccharide gel (PG) using as a gelling agent (Lertchaiporn, 2003) was prepared a used as a product control.

Ingredient	**Function**	**Amount (g)**
PG	Gelling agent	2.5
Vitamin E acetate	Antiaging, Antioxidant	0.1
Amerchol L-101®	Emollient	0.5
Sorbitol	Humectants	5-10
Propylene glycol	Humectants	5-15.0
Glycerin	Humectants	5.0
Cremophore RH-40®	Solubilizing agent	5-13.0
Tween 80	Solubilizing agent	1-1.5
Menthol	Cool sensation	0.5-1.2
$CaCl_2$ (0.05M)	Electrolyte	0.1-0.2
Paraben concentrate	Preservative	1.0
Triethanolamine (TEA)	Adjust pH to 2.9 ± 0.1	-
Sterile water	to make total weight	100

Preparation Procedures

All ingredients were tested for controlling microorganisms. PG powder was dispersed in an appropriate amount of sterile water with continuous stirring until uniform. The oil ingredients were mixed with solubilizing agent,

emollient, menthol and humectants by pouring oil phase into water phase with continuous stirring until a homogenous clear mixture was obtained. The mixture was slowly added into the PG solution in morter and mixed continuously, 0.05 M calcium gluconate solution was added and mixed until homogeneous and paraben concentrate was added. The pH of PG product was adjusted to a desired pH with triethanolamine to pH 2.9 ± 0.1. Distilled water was added to make a total weight to 100 grams with continuous stirring. The viscosity of gel product was measured and stability of each PG gel preparation was evaluated. The antiseptic PG gel selected product was also tested for antimicrobial activity.

2.5 Stability test of antiseptic PG gel products

The PG gel product was separated into two parts. The first part was tested by freeze-thaw method (Lechman *et al.*, 1986), the product was kept at 45°C for 48 hrs and changed to temperature at -4°C for 48 hrs (1 cycle). The procedure was performed for 6 cycles. The second parts were tested by storing at ambient temperature for 30 days. The antiseptic PG gel products were also tested physical properties such as stability, air bubbles, color, pH and viscosity after freshly prepared and after 6 cycles run by freeze-thaw method and also after stored 30 days.

2.6 Efficacy of antiseptic PG gel

2.6.1 Time-kill analysis

Time-kill analysis is an extension of the MHB that provides information of the rates at which organisms are killed. The inoculated Mueller hinton broth (MHB) media contained antiseptic PG gel product at 0.5 time dilution were incubated at 37°C for 24 hours. At the indicated time of incubation, the culture of 0.05 ml was removed at 0 min, 15 min, 30 min, 1 hour, 2 hours, 5 hours and 24 hours and inoculated on agar media after serial dilutions in NSS by drop plate method (Lorian, 1991). The number of viable colonies was counted after incubation overnight at 37°C. Viable colonies were calculated to determine colony-forming unit (CFU) per milliliter and a graph of time against the logarithm of the viable count was plotted. Cultivation of bacteria in NSS and media (MHB) were used as control, bacterial survival patterns

in media contained antiseptic PG gel product and control (NSS) were compared. The antiseptic PG gel preparation in this study is free preservative.

2.6.2 Agar diffusion method

Antiseptic PG gel product was freshly prepared. The petri dishes contained 25 ml of Mueller hinton agar (MHA) were inoculated with 1% microorganism suspension by seed layer method (Lorian, 1991). The sterile cork borers were bored into the seeded plates and filled with antiseptic gel preparations, 2.5% PG-gel base (NO.12), 1% tea tree oil-PG gel (NO.33), 1.5% tea tree oil-PG gel (NO.35) and 1% tea tree oil/0.2% betel oil-PG gel (NO.43), 150 ml/well and NSS (normal saline) was used as control. The plates were incubated at 37°C for 24 hours, the diameter of inhibition zone was measured. The antiseptic PG gel preparation tested in this study was prepared without preservative.

All ingredients in the formula of the PG gel preparation were also tested for antimicrobial activity by using the same method. Each ingredient dilution was prepared in sterile water, except for 1.2% menthol, which was dissolved in 15% propylene glycol. The cups filled with sterile water, 15% propylene glycol, 13% Cremophore RH 40®, 0.5% Amerchol L-101®, 0.1% vitamin E acetate and 1.2% menthol.

2.6.3 In-vivo handwashing test (Hammer, Carson, 2004)

The number of test organisms released from artificially contaminated hand was assessed before and after using antiseptic PG gel product. The prevalue is the number of colony forming unit (CFU) sampled from the fingertips before treatment, and the postvalue is the number after treatment with antiseptic PG gel product. The test was repeated for 3 treatments. Fingers used were an index finger, a middle finger and a ring finger. All fingers had short fingernail and intact skin, and were not used any antibacterial soap or toiletries on hand for 24 hours before test.

(1) Procedure for test of antiseptic PG gel product

The prevalue and postvalue were determined for each antiseptic PG gel and PG gel-base. The hands were washed for 1 min with tap water to remove natural transient microorganisms and then dried for 1 min. immediately, and then the dried fingertips were rubbed with 2 ml antiseptic PG gel product for 1 min. The hands were air dried for 1 min, after drying the fingertips were pressed on the agar plate of MHA for 30 second. The aim of the assessment was to determine the number of the colony count of organisms before and after treatment of hands (one petri-dish per hand). The number of colony forming unit (CFU) of microorganisms was counted using the colony counter after incubation at 37°C for overnight. The viable colony counts were performed using the colony counter and represented by score number. Treatment used three formulations such as, the PG-gel base (NO.12), tea tree oil-PG gel (NO.33) and tea tree oil/betel oil-PG gel (NO.43), tap water was used as control. The test in this study followed the processes test of antiseptic PG gel product in table1.

(2) Procedure for test of gel commercial and antiseptic PG gel product

The prevalue and postvalue were determined for each antiseptic PG gel and gel commercial in 10 subjects (8 female and 2 male). The hands were washed for 1 min with tap water to remove natural transient microorganisms and then dried for 1 min. immediately, after dried the fingertips were rubbed with 2 ml gel product for 1 min. The hands were air dried for 1 min, after drying the fingertips were pressed on the agar plate of MHA for 15 second. The aim of the assessment was to determine the number of the colony count of organisms before and after treatment of hands (one petri-dish per hand). The number of colony forming unit (CFU) of microorganisms was counted using the colony counter after incubation at 37°C for overnight. The viable colony counts were performed using the colony counter. Treatment used the three formulations, the gel commercial, tea tree oil-PG gel (NO.33) and tea tree oil/betel oil-PG gel (NO.43),compared with commercial clean feel® product and tap water was control.

Table 1. In-vivo studies of antiseptic PG gel product by hand washing test was preformed for prevalue of CFU using the following steps arrangement

Processes	Finger pressed on MHA agar plates for 30 seconds				
	Step 1	Step 2	Step 3	Step 4	Step 5
1	Before washing hands	After washing hands with water	After rubbing hands with PG-gel base (NO.12)	After rubbing hands with Tea tree oil-PG gel (NO.33)	After rubbing hands with Tea tree oil/Betel oil-PG gel (NO.43)
2	Before washing hands	After washing hands with water	After rubbing hands with Tea tree oil-PG gel (NO.33)	After rubbing hands with Tea tree oil/Betel oil-PG gel (NO.43)	After rubbing hands with PG-gel base (NO.12)
3	Before washing hands	After washing hands with water	After rubbing hands with Tea tree oil/Betel oil-PG gel (NO.43)	After rubbing hands with PG-gel base (NO.12)	After rubbing hands with Tea tree oil-PG gel (NO.33)

2.6.4 Perception Analysis

The instrument used in this study was volunteers. Ten healthy adult volunteers (8 female and 2 male), aged between 20-27 years old were asked to evaluated antiseptic tea tree oil-PG gel (NO.33) and tea tree oil/betel oil-PG gel (NO.43) before and after applying the product.

Physical appearance before applying the product: color, odour, viscosity, smoothness, firmness and clearness.

Perception after applying the product: coolness, spreadability, sticky while applying, time of disappearance, sticky after disappearance, residue, moist perception and overall satisfaction after use.

The opinions were analyzed by interpreting the opinion frequency according to the following criteria: 1 = poor, 2 = fair, 3 = good and 4 = excellent. The questionnaires are presented in Appendix G.

2.7 Statistic analysis

The values of variance were performed with one-way analysis of variance (ANOVA). The results were considered significant at $p< 0.05$; equal variance assumed using LSD.