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Antibacterial Activity of Topical Polyherbal Formulation on *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is pathogen organism associated with a variety of skin and soft tissue infection, including diabetic foot ulcer. The aim of this study was to evaluate the antibacterial activity of polyherbal combination as well as its topical formulation in water in oil cream. The extraction was conducted by maceration in ethanol 96%. Five mixtures consisting of honey, *Aloe vera*, and ethanolic of extract *Momordica charantia* fruit, *Curcuma longa* rhizoma, as well as *Smallantus sonchifolius* leaves at different composition have been prepared (F1 to F5). The prepared mixtures were screened for their antibacterial activity against *P. aeruginosa* ATCC 27853 by agar well diffusion method, using cream contained of Neomycin sulfate and bacitracin as positive control. All mixtures were effective against *P. aeruginosa* ATCC 27853. Formula 5 consists of honey, *Aloe vera*, *M. charantia* fruit extract, *C. longa* rhizoma extract, and *S. sonchifolius* leaves extract (2.5; 2.5; 5; 5 and 5% respectively) was the most effective mixture. The w/o creams contained 7% of glycerine showed higher activity.

Keywords: antibacterial, *Pseudomonas aeruginosa*, polyherbal formulation, topical, w/o cream.

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1. INTRODUCTION

Chronic foot ulcers are very common and serious complication of diabetes mellitus. These open wounds become rapidly colonized by microorganism, and often requires antimicrobial therapy. *Pseudomonas aeruginosa* is a pathogen microorganism that frequently causes severe tissue damage in diabetic foot ulcers.¹ However, treatment of infection disease becomes more challenging due the development of resistance to antibiotics.² In addition, some antibiotics cause side effects such as hypersensitivity and allergies. Because of the side effects and resistance of pathogen microorganism against antibiotic, recently much efforts have been conducted to obtain the drug from natural product.³

Indonesia is large producer of medicinal plants. There were many research reports on the antibacterial activity of medicinal plants, i.e. turmeric (*Curcuma longa* L.) rhizoma, honey, aloe vera, *Momordica charantia* fruit, as well as *Smallantus sonchifolius* leaves.³⁻⁸

The use of honey as traditional remedy for microbial infection has been done for a long time. The previous research have been demonstrated the effectiveness of honey against several human pathogens, including *Escherichia coli*, *Enterobacter aerogenes*, *Salmonella typhimurium*, and

Staphylococcus aureus. Shenoy et al. reported the activity of honey against *P. aeruginosa* isolated from infected wounds.⁹ *Aloe vera* being used extensively as antiseptic, as well as wound and burn healing. Different Aloe species would have various phytochemical contents, health benefits, and possible toxicities. It was reported that *A. vera* gel at various concentrations can be used as effective antibacterial agents in order to prevent and cure wound infected caused by *P. aeruginosa*.¹⁰

Momordica charantia (family Cucurbitaceae) are used in Asia, among its many uses, for treatment of skin infections. The fruits contain alkaloids, glycoside, saponin-like substances, resin, an aromatic volatile oil and mucilage. Previous studies have evaluated the antimicrobial activity, and it was concluded that unripe fruit show higher antibacterial activity against *P. aeruginosa* compared to the other parts of the plant.¹¹

The *C. longa* rhizome show more potent antimicrobial activity than the leaf extract. The phytochemical study of *C. longa* rhizome revealed the presence of various active constituents, i. e. alkaloid, tannins, flavonoids, glycoside and saponins.¹² Sometimes, the use of polyherbal combination with different mechanism of action can provide a higher effect.

The main objectives of this study were to evaluate and compare the antimicrobial activity of the polyherbal

combination (contains of honey, *Aloe vera*, extracts of *M. charantia* fruit extract, *C. longa* rhizoma, as well as *S. sonchifolius* leaves) against *P. aeruginosa*, and formulate an antibacterial w/o cream consisted of polyherbal combination.

2. EXPERIMENTAL DETAILS

Preparation of test compounds

The ingredients were purchased from commercial supplier and authenticated. All the ingredients, except honey and *A. vera*, were shade dried and powdered. Each of dried simplisia was further macerated in ethanol 96% for 5 days and filtered. The filtrate was vacuum evaporated to dryness. Full size of *A. vera* leaves were cut from the plant and the rind was removed. Honey was directly use as ingredient of polyherbal formulation. Phytochemical analysis

Preliminary phytochemical screening of test compounds was conducted based on the standard procedures (table 1).¹³ The extracts were subjected to phytochemical tests for determination the secondary metabolites such steroid, alkaloids, flavonoids, as well as tannins and phenolic compounds.

Table 1. Phytochemical screening methods

Secondary metabolite	Test method	Positive result
Steroid	Libermann-Buchard	An array of colour change
Alkaloid	Mayer's	white creamy precipitate
Flavonoid	Alkaline reagent	Yellow fluorescence
Tannin and phenolic compound	Ferric chloride	A dark green colour

Libermann-Burchard's test: The extract (50 mg) was dissolved in of 2 ml acetic anhydride. To this, 1 or 2 drops of concentrated sulphuric acid were added slowly along the sides of the test tube. Mayer's test: To a few ml of plant sample extract, two drops of Mayer's reagent were added along the sides of test tube. Alkaline reagent test: An aqueous solution of the extract was treated with 10% ammonium hydroxide solution. Ferric Chloride test: The extract (50 mg) was dissolved in 5 ml of distilled water. To this few drops of neutral 5% ferric chloride solution were added.¹³

Test microorganisms and control antibiotics

The standard strain of *Pseudomonas aeruginosa* (ATCC 27853) used as test organism was obtained from Microbiology Laboratory of Setia Budi University. A cream contains Neomycin sulfate 5 mg-bacitracin 250 iu in 5 gram cream was used as positive control. Tween 80 was used as negative controls.

Preparation of polyherbal formulation

All the ingredients were aseptically mixed to obtain the polyherbal combinations of Formula 1-5 as shown in table 2. Tween 80 was used as vehicle to enhance the delivery of active constituents.

Table 2. Composition of polyherbal formulation

Ingredients	Composition (%)				
	F1	F2	F3	F4	F5
Honey	5.0	-	5.0	-	2.5
<i>A. vera</i>	-	5.0	-	5.0	2.5
<i>S. sonchifolius</i> extract	5.0	5.0	-	7.5	5.0
<i>M. charantia</i> extract	5.0	5.0	7.5	-	5.0
<i>C. longa</i> extract	5.0	5.0	7.5	7.5	5.0
Tween 80	80.0	80.0	80.0	80.0	80.0

Antibacterial Activities

A loopful of strain was inoculated into 15 ml of Nutrient broth and incubated at 37°C for 24 hours to activate the strains. The inoculum's density was estimated visually to match the turbidity of McFarland 0.5 standard (approximately 10⁷ CFU/ml).¹⁴ The suspension of the bacteria was spread over the agar using a sterile cotton swab. The plates were allowed to dry for at least 15 minutes. A well of 5 mm diameter was cut on agar with sterile bore. The single test compounds (20%), polyherbal formulation (F1-F5), as well as the controls, were added (100 µl) to the wells using micropipettes. Subsequently the agar-plates were incubated overnight at 37°C. Tween 80 was used as negative controls and the control activity was deducted from the test. Diameters of zones of inhibition (ZI) were measured and recorded in millimeters. The experiments were conducted in triplicates³.

Preparation topical formulation

Two water in oil (w/o) cream bases consist of 20% of polyherbal formula with the highest antibacterial activity was prepared. Table 3 presents the formulas of w/o cream. The solid oil phase (cera alba and cetaceum) was first melted on 60°C. Purified water and olive oil were added alternately with constant stirring to get a homogen mixture, then triethanolamine was added. Methylparaben was dissolved in a portion of propylene glycol and added into a mixture. Glycerine, sorbitol and propylenglycol were added to the mixture alternately while stirring. Polyherbal extract was gradually added to the bases and and stirred continuously until the homogenous cream was obtained.¹⁴

Table 3. Formulas of w/o cream bases

Ingredients	Concentration (%)		
	Cream 1	Cream 2	Cream 3
Polyherbal combination	20	20	20
Glycerine	7	7	1
Sorbitol	1	7	7
Propylen glycol	7	1	7
Methyl paraben	0.1	0.1	0.1
Triethanolamine	2	2	2
Cera alba	5	5	5
Cetacium	10	10	10
Olive oil	35	35	35
Purified water ad	100	100	100

Organoleptic Characteristics and Spreadability

All the formulations (Cream 1-3) were tested for physical appearance, color, texture, and homogeneity. These characteristics were evaluated by visual observation. Homogeneity and texture were tested by pressing a small quantity of the formulated cream between the thumb and index finger. Spreadability of the formulations was determined by measuring the spreading diameter of 1 g of sample between two horizontal glass plates (10cm×20cm) after one minute. The standard weight applied to the upper plate was 25g.¹⁵

Antibacterial Activity

Agar well diffusion method was performed for extract cream formulations, as described previously.

Statistical Analysis

The diameter of zone of inhibition data was analyzed using one-way analysis of variance (ANOVA) and the differences among group means were analyzed using the Dunnett's multiple comparisons test. P value < 0.05 was considered as significant.

3. RESULTS AND DISCUSSION

Preparation of Extract

The extracts were prepared by simple maceration process, used ethanol 96% as solvent. Tabel 4 shows the yield of extraction results. Ethanol was choosen as extraction solvent due to its wide range of polarity. Ethanol could extracted the polar to nonpolar compounds from the dry herbs.

Table 4. The extraction results

Extract	Dry herbs (g)	Solvent (L)	Dry extract (g)	% yield
<i>M. charantia</i> fruit	1000	10	74.880	7.49
<i>S. sonchifolius</i> leaves	1000	10	152.670	15.27
<i>C. longa</i> rhizome	1000	10	135.855	13.59

Phytochemical analysis

Phytochemical analysis of honey, *A. vera*, *S. sonchifolius* leaves extract, *M. charantia* extract, as well as *C. longa* extract showing the presence of different active constituents in different extracts (Table 5).

Table 5. Phytochemical analysis of the ingredients

Ingredients	Alkaloid	Flavonoid & phenolic compound	Tannin	Steroid & Triterpen
Honey	-	+	+	+
Aloe vera	+	+	+	+
<i>M. charantia</i> fruit	+	+	+	+
<i>S. sonchifolius</i> leaves	+	+	+	+
<i>C. longa</i> rhizome	+	+	+	-

This result was in line with previous studies. *A. vera* leaf gel contains various compounds, i.e. anthraquinones, carbohydrates, chromones, arachidonic acid, linolenic acid, steroids (campesterol, cholesterol, sitosterol), triglycerides, triterpenoid, gibberillin, lignins, potassium sorbate, salicylic acid, uric acid, and many other constituents.¹⁶ Honey contains defined substances such as glucose, fructose, sucrose, minerals, vitamins, antioxidant, amino acid, and other products.¹⁷

It was reported that *S. sonchifolius* leaves contains di- and sesquiterpenes, among them mainly ent-kaurenic acid. Other components are polyphenolic antioxidants, such as hydroxycinnamic acids and chlorogenic acid. A sesquiterpene lactone named sonchifolin, as well as three known melampolides, polymatin B, uvedalin and enhydrin, were isolated from leaf extracts of yacon. Three major phytoalexins were isolated from this medicinal plants.¹⁸ *M. charantia* secondary metabolites are alkaloids, flavonoids, tanins, saponins, disogenin, proteins, calcium, copper, etc.¹⁹ *C. longa* extract contains alkaloids, tannin, flavonoid, glycoside and carbohydrate. There are reports showing that alkaloids and flavonoids are the responsible compounds for the antibacterial activities in higher plants.³

Antibacterial activity

Agar disk-diffusion testing is the official method used in many clinical microbiology laboratories for routine

antimicrobial susceptibility testing. In vitro antibacterial activity against *P. aeruginosa* were presented at figure 1.

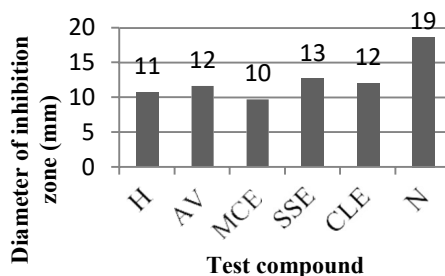


Figure 1. Antibacterial activity of single extract against *P. aeruginosa* (H: honey, AV: *Aloe vera*, MCE: *M. citrifolia* fruit extract, SSE: *S. sonchifolius* leaves extract, CLE: *C. longa* rhizome extract, N: Nebacetin cream)

In vitro antibacterial activity of polyherbal combinations were presented at Table 6.

Table 6. Antibacterial activity of polyherbal combination

Formula	Inhibitory zone diameter (mm)
Formula 1	11.44 ± 4.39
Formula 2	11.53 ± 3.45
Formula 3	11.80 ± 4.79
Formula 4	8.72 ± 1.30*
Formula 5	13.05 ± 3.54
Positive control	14.34 ± 5.94

*P<0.05 compared to positive control

The results of antimicrobial activity showed that all the ingredients play the important role to the acitivity. When used in combination, the antibacterial activity of these natural products were higher than when used as single compound. Removing *A.vera* and *M. charantia* fruit extract from the formula significantly reduced the antimicrobial activity.

The broad spectrum antimicrobial activity of honey has been demonstrated in various study. Honey reported exerts both bacteriostatic and bactericidal activities. The use of honey as effective wound treatment is increasing because it can markedly inhibit the activities of wound-isolated microorganism. The high sugar contents in honey plays a role in its antibacterial activity, due to the osmotic effect, in addition of its fairly acid property.

Other chemical constituents of honey (hydrogen peroxide and nitric oxide) also contribute to its antimicrobial activities.¹⁹⁻²⁰

A. vera contains six antiseptic agents, i.e. lupeol, salicylic acid, urea nitrogen, cinnamonic acid, phenols and sulfur. They all have inhibitory action on fungi, bacteria and viruses. The antibacterial activity of *A. vera* inner gel against both Gram-positive and Gram-negative bacteria has been demonstrated by several different methods. The anthraquinones constituent of *A. vera* have been reported to play a role in the antimicrobial activity. It was proposed that emodin, one of the anthraquinone, acts as antibacterial agent by inhibit the solute transport in membranes of bacteria.²¹⁻²²

M. charantia fruit extract contains phenolic and alkaloid compounds that contributed to its antibacterial activity. The antibacterial properties of *M. charantia* fruit also can be related to its high trans-nerolidol content. Major phytochemical constituents in *M. charantia* fruit with antibacterial activity are

sesquiterpens, phenylpropanoids, monoterpens, and transnerodilol.²³⁻²⁴

Catechol, terpenes and flavonoids were reported constituents are present in *S. sonchifolius* leaves. Phenolic compounds contributed in antibacterial activity of this plant. Another study reported that the antibacterial activity of *S. sonchifolius* can be attributed to enhydrin as polymatin B.²⁵⁻²⁶ The essential oil fraction from turmeric was reported to possess significant antibacterial activity at very low concentration (20 mg/disc) on pathogenic Gram-positive bacteria.²⁷

Semisolid topical dosage forms have been the subject of wide researchs, to improve the therapeutic efficacy of the

incorporated drug.²⁸ Creams are semisolid dosage forms aimed mainly for external use and usually consist of two immiscible phases. Water in oil cream consists aqueous internal phase and oily external phase. Drugs formulated as cream more effectively been deliberated and interact with skin and penetrate through biological membranes.²⁹

The organoleptic properties (including physical appearance, color, texture, and homogeneity), as well as spreadability of the cream 1-3 are presented in Table 7. All the creams had smooth texture, and they were all homogenous without phase separation.

Table 7. Organoleptic and spreadability properties

Properties	Cream 1	Cream 2	Cream 3
Physical appearance	opaque	opaque	opaque
Colour	brown	brown	brown
Texture	smooth	smooth	smooth
Homogeneity	homogeneous	homogeneous	homogeneous
Spreadability (mm)	42.75	40.75	42.75

Spreadability of semisolid formulations, represents the ability of the dosage form to spread on the skin, so plays an important role in the efficacy of a topical therapy.

Figure 2 presents the antibacterial activity of cream, consists of Formula 5 of polyherbal combination. Formula 5 was chosen due to the highest antibacterial activity against *P. aeruginosa*. The antibacterial activity of Cream 3 of topical polyherbal cream was lowered than other formulas (Cream 1 and 2).

Concentration of glycerine in Formula 1 and 2 was 7%, while in Cream 3 it was only 1%. Glycerine was reported showed antibacterial activity, so the reduction of glycerine concentration can reduce the antibacterial activity of the cream.

Although there was difference in concentration of sorbitol and propylenglycol in formulation of cream 1-3, it did not affect the antibacterial activity of the cream, due to the antibacterial activity of sorbitol and propylenglycol were lower than the antibacterial activity of glycerine.³⁰

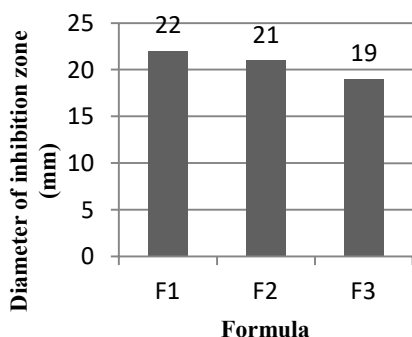


Figure 2. Antibacterial activity of polyherbal cream against *P. aeruginosa*

4. CONCLUSIONS

The present study revealed that all of polyherbal combination, except for formula 4, showed equal antibacterial activity to positive control. The polyherbal cream formulation contains higher concentration of glycerine showed higher antimicrobial activity against *P. aeruginosa*.

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