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Research Article

Formulation, Evaluation and Antibacterial Properties of Herbal Ointment Containing Methanolic Extract of C*linacanthus nutans* Leaves

Mahendran Sekar*, Nurashikin Abdul Rashid

Faculty of Pharmacy and Health Sciences, Universiti Kuala Lumpur Royal College of Medicine Perak, Ipoh - 30450, Malaysia.

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ABSTRACT

Background: *Clinacanthus nutans* is known as Sabah Snake grass belongs to the family of Acanthaceae. The plant was traditionally used for the treatment of skin rashes, insects and snake bites, lesions caused by herpes simplex virus, gout and diabetes in Malaysia, Thailand, Indonesia and China. *Staphylococcus aureus* and *Escherichia coli* are the main pathogens that cause skin infections. *Clinacanthus nutans* was also well known for antibacterial properties.

Objective: The main objective of the present study was to apply this antibacterial potential in the preparation of ointment.

Methods: The leaves of *Clinacanthus nutans* was extracted with 70% methanol by using soxhlet method. The methanolic extract of *Clinacanthus nutans* was investigated for qualitative phytochemical analysis and the ointment was formulated and evaluated using standard parameters. The antibacterial activity of the extract and ointment in different concentration were investigated by using two gram-positive bacteria (*Staphylococcus aureus and Bacillus cereus)* and two gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) through disc diffusion method.

Results: The qualitative analysis showed the presence of carbohydrates, steroids, glycoside, flavonoids, tannins, proteins and amino acids. The antibacterial potential of the formulated ointment was studied with three different concentrations (100, 250, 500 mg/mL. The ointment also showed significant antibacterial activity against all the tested organisms with zone of inhibition ranges from 8.00±2.00 to 18.67±2.09 mm. This activity was maintained when the extract was incorporated into the ointment base. The ointment was stable after two months.

Conclusion: In future, the extract can be used for the commercial production of *Clinacanthus nutans* ointment. Similarly, this ointment can be tested for skin related bacterial infections.

Keywords: *Clinacanthus nutans*; Sabah snake grass; Disc diffusion method; Antibacterial ointment

INTRODUCTION

Microorganisms such as bacteria can cause many types of skin-related diseases such as skin rashes, acne, eczema, psoriasis, dermatitis and etc. *Staphylococcus aureus* and *Escherichia coli* are the main pathogen that causes these skin infections[1]. Topical ointment containing antibacterial properties can be used in the treatment and prevention of this kind of bacteria causes the infection. Most of the available topical drugs used to treat skin-related diseases in the market are obtained by various synthetic processes which are using chemicals and have some kinds of side effect. The differences between the antibiotic drug and herbal antibiotic is that they are not just limited to the effect of one chemical but they have several constituents with the variety of healing properties and produce more synergistic effect to fight the bacteria. Topical ointment containing extract of medicinal plant is one alternative to treat the skin infection caused by bacteria and prevent the use of oral antibiotic which then can develop bacterial-resistant. An ointment is the viscous semi solid preparations that are use topically on the variety of body surface. It may or may not be medicated. Most of the medicated ointment preparation contains medicament ingredient which are dissolved, suspended or emulsified in the based and used topically for several purposes such as protectant, antiseptic, emollient, and astringent[2]. *Clinacanthus nutans* is well known because of its various medicinal uses**.** *Clinacanthus nutans* or Sabah snake grass is small shrub that belongs to the family of Acanthaceae and can be found in South East Asia primarily in Malaysia, Thailand and Indonesia. In Malaysia, it is called as "Belalai Gajah" and mostly can be found in Sabah state but now they are widely cultivated in all Malaysia state due to its beneficial value. *Clinacanthus nutans* is widely used in Malaysia as traditional medicine to treat many diseases such as gout, diabetes, hypertension, liver cancer, kidney syndrome and uterine fibroid. From the previous research, they found that the *Clinacanthus nutans* having many biological properties such as anti-inflammatory, cytotoxic, antioxidant and antimicrobial agents[3]. In Thailand, the leaves of *Clinacanthus nutans* traditionally used for the treatment of many skin-related diseases such as skin rashes, snake bites, herpes infection and varicella-zoster virus (VZV) lesions[4]. According to the recent study, *Clinacanthus nutans* was shown to have

*Author for Correspondence: mahendransekar@unikl.edu.my

Figure 1: Formulated ointment.

Table 1: Composition of the methanolic extract of *Clinacanthus nutans* leaves ointment.

Components	Amount (g)
Methanolic extract of *Clinacanthus nutans* leaves	2
Emulsifying wax	28
White soft paraffin	50
Liquid paraffin	20

Table 2: Qualitative phytochemicals analysis of the methanolic extract of *Clinacanthus nutans* leaves.

Qualitative phytochemical test	Results
Carbohydrates	+
Alkaloids	-
Steroids and sterols	+
Glycosides	+
Saponins	-
Flavonoids	+
Tannins	+
Proteins & amino acids	+

+ indicates present; - indicates absent

significant antiviral activities against herpes simplex virus type-2 and varicella zoster virus and has been used in Thailand as their primary health care programme[5]. The antimicrobial properties of this plant have been investigated in previous research on several microorganisms. According to the study done by Arullapan et al., 2014[3], the presence of flavonoids in *Clinacanthus nutans* could be the one that inhibit the microorganism. This flavonoids may have bacteriostatic (inhibitory) or bactericidal (killing) effects on microorganisms. This plant extract was reported to be more potent to Gram-positive bacteria (*Bacillus cereus and Staphylococcus aureus*) than gram-negative bacteria *(Escherichia coli and Salmonella enterica Thypimuri*um). However, there is no study has been reported in the formulation of antibacterial ointment containing *Clinacanthus nutans* leaves extract. Though, it was reported as potent antimicrobial properties. Hence, the present study aimed to formulate antibacterial ointment and evaluate its properties.

MATERIALS AND METHODS

Collection and identification

The leaves of *Clinacanthus nutans* were purchased from the local market, Ipoh District, Perak, Malaysia and identified.

Extraction

The leaves of *Clinacanthus nutans* were washed thoroughly with distilled water to remove and clean the adhering dust particles. Then the leaves were dried under the shade for 2 weeks. The dried plant materials were coarsely powdered using mechanical blender. 100 g of powdered leaves of *Clinacanthus nutans* were extracted by Soxhlet method using 2.5 L of 70% methanol as a solvent for 18-20 hrs. The methanolic extract of *Clinacanthus nutans* leaves was filtered and concentrated under controlled temperature and stored at 4 ^{o}C in a refrigerator till further use.

Qualitative Phytochemical Analysis

The methanolic extract of *Clinacanthus nutans* leaves were subjected to the phytochemical analysis by using various chemical tests to identify the phytoconstituents present in it[6].

Tests for carbohydrates

Molisch test: Take 1 ml of extract in the test tube and added with 1 mL of α-naphthol solution and few drops of concentrated sulphuric acid, purple or reddish violet colour gives positive result.

Fehling's test: Take 1 ml of extract in the test tube and to this added the equal quantities of Fehling's solution A and B, a brick precipitate indicates the presence of carbohydrate.

Tests for alkaloids

Mayer's test: Take 1 ml of extract in the test tube and add 2-3 drops of mayer's reagent, a dull white precipitate indicate the presence of alkaloids.

Dragendoff's test: Take 1 ml of the extract in test tube and add 2-3 drops of Dragendorff's reagent, orange precipitate gives positive result.

Wagner's test: Take 1 ml of extract in the test tube and 2 to 3 drops of Wagner's reagent is added, presence of reddish brown precipitate indicates the presence of alkaloids.

Table 3: Antibacterial activity of the methanolic extract of *Clinacanthus nutans* leaves.

	Gram Positive		Gram Negative	
Concentration (mg/mL)	*Staphylococcus aureus*	*Bacillus cereus*	*Escherichia coli*	*Pseudomonas aeruginosa*
	Zone of inhibition (mm)			
25	23.00 ± 2.65	8.00 ± 1.00	10.00 ± 2.65	9.33 ± 2.08
50	27.00 ± 2.00	11.33 ± 1.53	12.33 ± 1.53	10.67 ± 1.52
100	26.67 ± 3.51	15.00 ± 1.00	17.00 ± 2.00	13.00 ± 1.00
Ciprofloxacin (5 µg)	29.33 ± 2.51	26.00 ± 1.00	31.00 ± 2.00	29.67 ± 3.05

* Values are expressed as mean ± SD (n=3). P<0.05 between negative control disc and extract treated disc. P<0.05 between negative control disc and standard treated disc.

Table 4: Evaluation of formulated ointment containing methanolic extract of *Clinacanthus nutans* leaves.

Physicochemical parameters	Formulation
Colour and odour	Dark greenish
Odour	Characteristic
Loss of drying	0.16%
pH	6.4
Diffusion study	2 cm in 1 min
Stability study	Stable with pH 6.3

Tests for steroids and sterols

Salkowski test: Take 1 ml of extract and dissolved in the chloroform, the same amount of concentatred sulphuric acid is added. Bluish red to cherry red colour is observed in the chloroform layer and the green flourosence in the acid layer indicate the presence of steroids.

Tests for glycosides

Baljet's test: Take 1 ml of extract in the test tube and added with sodium picrate solution, yellow to orange colour indicates the presence of glycosides.

Keller-Kiliani test: The extract was dissolved in acetic acid containing trace of ferric chloride and transferred to the surface of concentrated sulphuric acid. Reddish brown colour is formed which gradually becomes blue indicating the presence of glycosides.

Tests for saponins

Foam test: Take 1 mL of extract and added with the distilled water. The sample was shake and the foam was observed.

Test for flavonoids

Shinoda test: Take 1 ml of the extract in the test tube and added with the magnesium turnings, followed by the addition of concentrated hydrochloric acid. A red colour indicates the presence of flavonoids.

Tests for tannins

Ferric chloride test: To the extract, ferric chloride was added. Dark blue or greenish black colour indicates the presence of tannins.

Potassium dichromate test: To the extract, potassium dichromate solution was added. A precipitate indicates the presence of tannins.

Test for proteins and amino acids:

Biuret test: To the extract, 1 mL of 40% sodium hydroxide and 2 drops of 1% copper sulphate solution were added. A violet colour indicates the presence of proteins.

Formulation of ointment[7]

The constituents of base were shown in Table 1. All the ingredients were weighed and melted in a beaker at 70 °C using heating mantle. The ingredients were stirred gently for 5 min by maintaining the temperature at 70 °C. Then, the methanolic extract of *Clinacanthus nutans* leaves was added and stirred well until homogenous mass is formed and transferred in the bottle. The ointment is prepared for 100 g (Table 1 and Fig. 1).

Evaluation of ointment

The evaluations were carried out on the ointment by using the following parameters

Colour and odour

Colour and odour of prepared ointment was examined by visual examination.

Loss on drying

1 g of ointment was placed in the petridish and heated in the water bath at 105 °C every 30 min until it get constant weight.

pH

The pH of ointment was determined by digital pH meter. 1 g of ointment was dissolved in 50 ml of distilled water and the pH was measured.

Diffusion study

The diffusion study was carried out by preparing agar nutrient medium of any concentration. It was poured into petridish. A hole bored at the centre and ointment was placed in it. The time taken for the ointment to get diffused was noted.

Stability study

The stability study was carried out for the prepared ointment at temperature of 37 ºC for 2 months.

Antibacterial screening

Test microorganisms

A panel of common pathogenic microorganisms were used in the study, which includes two gram-positive bacteria (*Staphylococcus aureus and Bacillus cereus*) and two gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*).

Disc-diffusion method

The antibacterial activity of the ointment and extract were evaluated using disc-diffusion method. A suspension of the tested microorganisms was uniformly swabbed on agar plates using sterile cotton swabs. Sterile blank discs were individually impregnated to the different concentrations of formulated ointment (100, 250 and 500 mg/ml) and extracts (25, 50 and 100 mg/ml) were placed onto the inoculated agar plates[8]. The plates will be inverted and incubated at 37 ºC for 24 h for bacteria. The antibacterial activity was measured by measuring diameter of the resulting zone of inhibition against the tested organisms.

Table 5: Antibacterial activity of the methanolic extract of *Clinacanthus nutans* leaves ointment.

	Gram Positive		Gram Negative	
Concentration (mg/mL)	*Staphylococcus aureus*	*Bacillus cereus*	*Escherichia coli*	*Pseudomonas aeruginosa*
	Zone of inhibition (mm)			
100	10.67 ± 3.05	12.00 ± 3.00	8.00 ± 2.00	13.33 ± 3.22
250	8.67 ± 1.53	13.33 ± 1.53	11.00 ± 2.00	16.33 ± 4.16
500	13.00 ± 2.00	18.67 ± 2.09	10.33 ± 3.06	17.67 ± 5.03
Ciprofloxacin (5 µg)	30.33 ± 3.06	28.67 ± 3.22	28.00 ± 3.00	30.33 ± 5.03

* Values are expressed as mean ± SD (n=3). P<0.05 between negative control disc and extract treated disc. P<0.05 between negative control disc and standard treated disc.

The test for positive control and negative control was performed in duplicate.

RESULTS

The preliminary phytochemical screening of methanolic extract of *Clinacanthus nutans* leaves was showed for the presence of carbohydrates, steroids, glycoside, flavonoids, tannins, proteins, amino acids and absence of alkaloids and saponins (Table 2). The methanolic extract of *Clinacanthus nutans* leaves showed the maximum antibacterial activity against *Staphylococcus aureus* as seen by zone of inhibition ranges from 23.00±2.65 to 26.67±3.51 mm (Table 3). The next best response for inhibition activity was seen against *Escherichia coli* (10.00±2.65 to 17.00±2.00 mm) followed by *Bacillus cereus* (8.00±1.00 to 15.00±1.00 mm) and *Pseudomonas aeruginosa* (9.33±2.08 to 13.00±1.00 mm), respectively. All the extracts were showed significant (P<0.05) zone of inhibition when increasing the concentrations of the extracts. However, the standard ciprofloxacin (5 µg) showed significantly increased zone of inhibition against all the tested organism. Based on the above results, the methanolic extract of *Clinacanthus nutans* leaves was used as active agent in the preparation of antibacterial ointment. The formulated ointment was evaluated for physicochemical parameters such as colour, odour and pH, the results were shown in Table 4. There is no colour changes and pH variation was observed during the stability study of the formulated ointment. This was good sign for ointment and it was acceptable. The antibacterial potential of the formulated ointment was studied with three different concentrations. The ointment also showed significant antibacterial activity against all the tested organisms with zone of inhibition ranges from 8.00±2.00 to 18.67±2.09 mm (Table 5). However, the standard ciprofloxacin showed significant (P<0.05) antibacterial activity with low concentration when compare to tested ointment containing methanolic extract of *Clinacanthus nutans* leaves.

DISCUSSION

Clinacanthus nutans is believed to have many beneficial effects in the treatment of various diseases such as skin rashes, scorpion and insect bites, diabetes mellitus, fever and diuretics among the Malaysian people[9-11]. Various phytochemicals presence in the leaves of this plant is responsible for the therapeutic effects. The infusion or decoction of dried leaves are used for the treatment for hepatitis infection[12, 13]. The antibacterial potential of the methanolic extract of *Clinacanthus nutans* leaves along with formulated ointment were determined by using disc diffusion method against *Staphylococcus aureus, Bacillus cereus, Escherichia coli and Pseudomonas aeruginosa*. From the result, the antibacterial activity of the methanolic extract of *Clinacanthus nutans* leaves showed greater zone of inhibition towards gram-positive bacteria compare to gram-negative bacteria. These results was supported by Templer and Brito 2009[14], stated that most of the infections are caused by the gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pyogenes.* Less common cause by the gram-negative bacteria such as *Escherichia coli* and *Pseudomonas* species. The methanolic extract of *Clinacanthus nutans* leaves showed significant antibacterial activity against all the tested microorganisms. This observation indicates that the activity due to the presence of large varieties of phytoconstitunets present in the extract. This results also well correlates with the earlier report[15]. Hence, the observed antibacterial activity of the ointment was due to the presence of active constituents of the extract and the activity also well maintained when it was converted to ointment. This was good sign to do further studies on that to make it as one of the commercial ointment for the treatment of bacterial infections.

CONCLUSION

The antibacterial activity of the methanolic extract of *Clinacanthus nutans* leaves was evaluated. This activity was maintained when the extract was incorporated into the ointment base for topical use in the treatment of common skin condition. The ointment was stable after two months. Further the extract can be used for the commercial production of *Clinacanthus nutans* ointment. Similarly, this ointment can be tested for skin related bacterial infections.

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CONFLICT OF INTEREST

All the authors declared that there is no conflict of interest.

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