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Research Article

In Vitro Antibacterial Activity of Spanish Moss (*Tillandsia usneoides*) Crude Extract Against Skin Infection in Wound Healing

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ABSTRACT

Wound healing is a dynamic phenomenon that results in the restoration of anatomic continuity and function on which can be delayed by pathogenic bacteria. This research was designed to explore the antimicrobial efficacy of *Tillandsia usneoides* against skin infections in wound healing. Physical and chemical evaluations were done through phytochemical screening and thin layer chromatography. The effect of methanolic, ethanolic and aqueous extracts of *Tillandsia usneoides* against pathogenic bacteria were evaluated on antimicrobial activities using disc diffusion and broth dilution susceptibility assay. The excision bioassay analysis was used in examining the wound healing process in mice. The result of the qualitative phytochemical screening showed the presence of flavonoids and alkaloids. Thin layer chromatography revealed a high Rf value for flavonoids (0.75mm) and alkaloids (0.60mm). The antibacterial assay showed a high zone of inhibition (ZI) for both methanolic extract (>23mm, >22mm and >20mm) and ethanolic extract (>22mm, >22mm and >17mm) for *P.aeruginosa, S.aureus* and *S.epidermis* respectively. Resistance against aqueous extract was observed based on the lowest zone of inhibition (<4mm). Gentamicin was used as the positive control (>28mm) and DMSO as the negative control. The minimum inhibitory concentration and minimum bactericidal concentration results confirmed that methanolic and ethanolic extracts restrained the growth of tested bacteria in the range of 125 to 500mg/mL and showed bactericidal efficacy. Wound healing assay indicated that methanolic extract had a higher potency of wound closure (12 days; <1mm) compared to ethanolic and aqueous extracts (13days; >1.5mm and 13days: >1.8mm respectively). Povidone-Iodine was used as the gold standard (15days; <2mm) in the study. In conclusion, methanolic extract of *T. usneoides* has a great potential with regard to its antimicrobial and wound healing activity to be developed as a novel drug in the future.

Keywords: Antimicrobial, Spanish moss, Tillandsia usneoides, Wound Healing, Skin Infection

INTRODUCTION

Human skin is the largest organ colonized by a diverse milieu of microorganisms in which most of the microorganisms are harmless or even beneficial to their host[1]. The biology of the skin surface is highly variable depending on topographical location as well as several endogenous host factors and exogenous environmental factors. Hence, human skin is also the natural host for many bacterial species that colonize the skin as normal flora. *Staphylococcus aureus* and *Staphylococcus epidermis* are intermittent resident flora, yet they are accountable for a wide variety of bacterial pyodermas[2].

Wounds are a significant cause of morbidity worldwide. Previous studies have shown that for every one million patients with wound infection, at least 10,000 people die from microbial infections[3]. In developing countries, approximately 10% of the population will experience a wound during their lifetime[3]. According to the latest WHO data published in May 2014, skin disease in Malaysia makes up 0.49% of total skin diseases globally[4]. According to the Wound Healing Society, wounds are physical injuries which result in an opening or breaking of the skin which may cause disturbance to the normal skin as well as its functions[5]. Based on current estimates, almost 6 million people worldwide are suffering from chronic wounds. An unhealed wound produces inflammatory mediator that may cause pain and swelling at the wound site.

Antibiotic-resistant bacteria are typical bacteria that are not controlled or killed by antibiotics[6]. This type of bacteria can survive and even multiply in the presence of an antibiotic[7]. Most infections are prone to resistance to some antibacterial agents. Furthermore, it may result in severe diseases that may stem from a single wound. One example of such bacteria is *Staphylococcus aureus* which is currently almost resistant to the penicillin antibiotic[8]. This can inhibit the wound healing process.

Spanish Moss

Spanish moss (*Tillandsia usneoides*) is a tiny epiphyte flowering plant that grows on larger trees, native to West

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India. The name of the plant species is *usneoides* and it belongs to the Bromeliaceae family[9] as shown in Figure 1. The Spanish moss absorbs nutrients, namely calcium and water, from the air and rainfall. Moreover, the plant gains its nutrients directly from the air instead of from the soil, a host plant or trees[10]. This plant is colloquially known as the 'air plant'[11]. This plant hangs from trees in long, thick masses that may reach 20 inches in length. It is greyish-green in colour, narrowly linear, and measures up to two inches long. One of the functions of *T. usneoides* is for blood regulation[12]. In 2000, researchers at Northeast Louisiana University discovered the benefits of *T.usneoides* in controlling blood glucose level and for treating diabetes[13].

The Native Americans used Spanish moss as medication for a range of purposes. In addition, it is also used in contemporary herbal medicine among the Latin Americans[14]. The plant has also been used to treat hemorrhoids, abscesses and tumors as well as taken orally for heart, liver and lung ailments. Research has shown that *T. usneoides* exerts anti-viral, anti-inflammatory and analgesic activities[15]. Besides that, this plant makes up for a good herbal remedy. Furthermore, *T. usneoides* also contains a bio-active compound that may help control blood glucose levels. The compound is known as 3-hydroxy-methylglutaric acid or HMG, commonly used among diabetics as supplements[16]. Research has found that this plant can boost the strength of small blood vessels or capillaries in the skin as well as to protect the skin from damage *T. usneoides* can treat the effects of aging on the skin and the extract can inhibit the breakdown of skin cells[17].

Bacteria

S. aureus and *S.epidermidis* are gram-positive microorganisms commonly found in the skin which frequently cause surgical implant-related infections[18]. In addition, *Staphylococcal* wound toxicities can cause severe local and systemic complications including bacteraemia, metastatic infection and hypotension or organ failure[19]. Chronic wounds and burns are infected by *Staphylococci* as well as the gram-negative opportunistic pathogen *P. aeruginosa* in which the wound infection is related to ulcer enlargement or a delay in healing[20].

Wound Healing

The wound is a breakdown of the protective function in the skin which loses its continuity of epithelium or without loss of underlying connective tissues, for example the muscle, bone, and nerves[21]. A wound may heal by primary intention in order for the wound to be closed with sutures, or secondary intention where the injured tissues are restored by the development of connective tissue and re-growth of epithelium cells where the healing is a natural reparative process and are typically allowed to granulate.

Skin repair and regeneration

Skin repair is an instant physiological response of the injured tissue to restore normal functionality without any replacement of damaged tissue[22]. Dermal substitute is a wound healing which is followed by a regenerative pathway by reducing the wound contraction. Furthermore, in superficial layer injuries or burns, the wound healing process will develop as a regenerative paradigm[23].

Antimicrobial assay

Antimicrobial is an agent which kills microorganisms and inhibits the growth of bacteria. It is classified according to the mechanism of action of antibiotics based on its property[24]. Microbicidal are known as agents that kill microbes, whereas those that inhibit the growth of bacteria are called bacteriostatic. The application of antimicrobial agents is also known as antimicrobial chemotherapy[25]. MIC is commonly used in diagnostic laboratories, mainly to confirm resistance. It is also used as a research tool to determine the *in vitro* activity of a new antimicrobial and studies have been conducted to determine the MIC breakpoint[26]. This method can be determined by agar dilution or broth microdilution. In a clinical setting, the MIC is used to determine the amount of antibiotic that the patient will use[27]. Meanwhile, MBC is a determination of the MIC concentration which shows no growth of bacteria.

Gentamicin is an antibiotic commonly used to treat many types of bacterial infection. This antibiotic belongs to the aminoglycoside group which are bactericidal[28]. Furthermore, Gentamicin works against a wide range of bacterial infections, which mostly are gram-negative bacteria such as *Pseudomonas* and *Proteus*, as well as gram-positive bacteria *Staphylococcus.*

MATERIALS AND METHODS

Plant collection and preparation

T. usneoides was collected in Banting, Selangor. The plant was authenticated by Universiti Putra Malaysia with the voucher number, SK2959/16. The grass was carefully washed and oven dried for 1 hour at 60^{o}C[29]. Next, the grass was placed in a shaded area for further drying[29]. Once the grass had dried, it was grounded in the mixer grinder until it turned into a fine powder form[30].

Collection of bacteria

Bacterial culture of *S. aureus, P. aeruginosa*, and *S. epidermidis* were obtained from the microbiology laboratory, Management and Science University, Shah Alam. These bacteria were cultured in peptone water and followed by gram staining to confirm the species of bacteria[31].

Crude extraction

Dried powder of 50g crude extract was mixed with 500mL of 70% methanol, ethanol, and aqueous solvent for 24 hours at room temperature[32]. After a 24-hour immersion, the extract was evaporated in a rotary evaporator at 40^{o}C[33]. Once evaporated, the crude extract turned into semi-solid form.

Phytochemical screening

Detection of alkaloids (Mayer's test)

The crude extract was dissolved individually in diluted hydrochloric acid and then filtered. For the detection of alkaloids, Mayer's Test was employed. In this test, 1mL of aqueous extract and 1mL of Mayer's reagent, which was essentially a potassium mercuric iodine solution,

were added. Next, the reaction was observed based on the presence of alkaloids in a whitish or cream colour[34].

Detection of flavonoids (Alkaline Reagent Test)

For the detection of flavonoids, the test used was the Alkaline Reagent Test. 1mL of aqueous extract was treated with 1mL of sodium hydroxide solution. Subsequently, the reaction was observed based on the presence of flavonoids in a yellow-coloured form, which would turn colourless upon the addition of a few drops of acid[34].

Detection of saponins (Foam Test)

In this study, Foam Test was employed to detect saponins. 0.5g of crude extract was taken and diluted in 2mL of distilled water. The mixture was then shaken vigorously for 10 minutes, and the reaction was observed based on the presence of saponins through the formation of foam[34].

Detection of tannins (Gelatin Test)

The Gelatin Test was administered to detect the presence of tannins. For this purpose, 2-3mL of aqueous extract was used and 1mL of gelatin containing NaCl was added. The reaction was observed based on the presence of a white-coloured formation, indicating the presence of tannins[34].

Detection of carbohydrates (Molisch's Test)

To detect the presence of carbohydrates, Molisch's Test was employed. The extract was treated with two drops of alcoholic alpha-naphthol solution in a test tube. Then, the reaction was observed based on the formation of a violet ring at the junction, which indicated the presence of Carbohydrates[34].

Detection of phenols (Ferric Chloride Test)

The test used to detect the presence of phenols was the Ferric Chloride Test. In this test, the extract was treated with three drops of ferric chloride solution. The reaction was observed based on the formation of a bluish black colour, indicating the presence of phenols[34].

Detection of proteins and amino acids (Xanthoproteic Test)

For the detection of proteins and amino acids, the Xanthoproteic Test was used. The extract was treated with three drops of Nitric acid concentration and the presence of proteins was observed based on the resulting yellow-coloured formation[34].

Detection of steroid (Salkowski Test)

For the detection of steroid, the Salkowski test was performed. In this test, 2mL of extract were taken and 2mL of chloroform and 2mL of H_2S0_4 concentration were then added to the extract. Then, the test tube was shaken well to observe the chloroform layers which appeared in a red coloured-form and the acid layer which appeared in the form of a greenish yellow fluorescence[34].

Thin Layer Chromatography (TLC)

Thin layer chromatography is a technique which is used to separate non-volatile mixtures. It is performed on a sheet of glass coated with a thin layer of adsorbent material[35]. Silica gel is the standard material used in thin layer chromatography as it can be used to identify compounds which are present in a certain mixture. In this study, 3g of dried powder was measured, and 10mL of hexane was added to the powder. The powder was then heated in a water bath for 5 minutes and then filtrated. The amount of liquid was divided into three tubes labelled A, B and C. For tube A, the solution was mixed with 9mL of chloroform and 1mL of acetic acid. Then, the mixture was heated in a water bath for 10 minutes. For tube B, 4.9mL of chloroform, 4.9mL methanol, and 0.2mL acetic acid were added into the tube. This was followed by heating the tube in water bath at 37ºC for 10 minutes. Lastly, for tube C, the solution was mixed with 5mL of methanol and 5mL of distilled water and then heated for 10 minutes[36].

Developing a plate

TLC plates were developed in a beaker covered with an aluminium foil. Next, the solvent was poured into three different beakers labelled Solution A, Solution B and Solution C. The lower edge of the plate was dipped into the solvent. The solvent travelled up the matrix by capillary, moving the components of the samples at various rates due to the different degrees of interaction with the matrix and solubility in the developing solvent[37].

Disk diffusion assay method

Firstly, a few Muller-Hinton (MH) agar plates and sterile cotton swabs were prepared[38]. Next, wells were made on the different MH agar plates containing *S. aureus, P. aeruginosa* and *S. epidermis* bacteria[39]. Gentamicin antibiotic was applied as the positive control and *T. usneoides* crude extract from ethanol, methanol and aqueous were added to each agar plate containing the bacteria. Lastly, all agar plates were incubated at 37ºC for 24 hour[38].

Minimum inhibitory concentration (mic)

To determine the minimum inhibitory concentration (MIC), 21 tubes were used; 7 tubes were used for *T. usneoides* crude extract, and a series of different concentrations of crude extract with bacteria *S. aureus, P. aeruginosa* and *S. epidermis* (500, 250, 125, 62.5, 31.25, 15.62, 7.81 mg/mL) were prepared[40]. Next, each bacterial inoculum was adjusted to 0.5 McFarland standard. 0.5mL of bacterial inoculum and plant crude extract were dropped on each nutrient broth[41]. A positive control was prepared, which contained 3mL of nutrient broth and 0.5mL of inoculum while 3mL of nutrient broth was used as the negative control. Both tubes were incubated at 37ºC for 24 hours and the turbidity of the tubes at each concentration was observed[40].

Minimum bactericidal concentration (MBC)

The minimum bactericidal concentration of the crude extract on the bacterial culture was determined by measuring 1mL of the mixture onto the Muller-Hinton (MH) agar in order to obtain the value of MIC and inhibition of the bacterial culture. This was followed by a 24-hour incubation period at 37ºC[42]. The least concentration value of crude extract which resulted in no visible bacterial growth was taken as the minimum bactericidal concentration.

Wound healing measurement

Animal ethics were applied in the university ethics committee (AE/2016(1)/062). In this study, excision method was used on 45 mice to examine the wound

Figure 1: Spanish moss(*Tillandsia usneoides).*

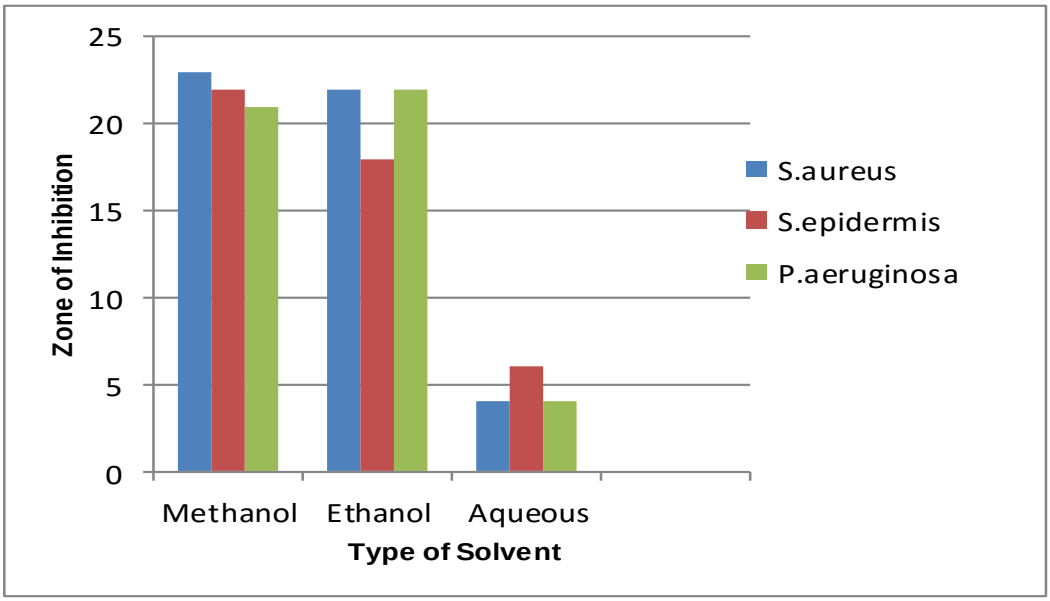

Figure 2: Zone of inhibition of bacteria using crude extracts of *T.usneoides.*

Table 1: Phytochemical screening of *T. usneoides.*

No.	Metabolites	Name of Test	Solvents		
			Methanol	Ethanol	Aqueous
1	Alkaloids	Mayers's Test	+	+	+
2	Flavonoids	Alkaline Reagent Test	+	+	+
3	Tannins	Gelatin Test	+	+	-
4	Saponins	Foam Test	-	+	-
5	Phenols	Ferric Chloride Test	+	+	-
6	Steroids	Salkowski Reagent	-	+	+
7	Protein and Amino acids	Xanthoproteic Test	+	-	-
8	Carbohydrates	Molisch's Test	-	-	-

healing process. First, the mice were shaved to remove their hair. After shaving, they were anesthetized with 1mL of intravenous ketamine hydrochloride (10mg/kg body weight) prior to the excision of wound[43]. A 6mm wound was created at the dorsal of the mice using a surgical blade, forceps, and scissors[44]. Next, *S. aureus*, *S. epidermis* and *P. aeruginosa* bacteria were applied on the wound of each subject. Following bacterial application, the crude extract of *T. usneoides* from 3 different types of solvent (methanolic, ethanolic and aqueous), distilled water (negative control) and an antibiotic (positive control) was applied onto 9 subjects each. Each subject was then observed for a period of 12 days[44] and the wound was measured in mm.

Statistical analysis

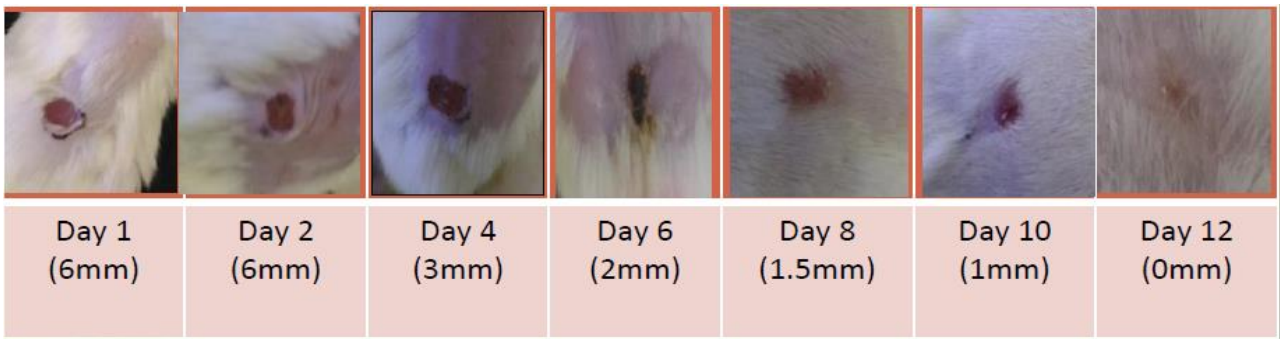

Figure 3: Wound healing measurement on methanolic extract.

Figure 4: Wound healing measurement on ethanolic extract.

Figure 5: Wound healing measurement on aqueous extract.

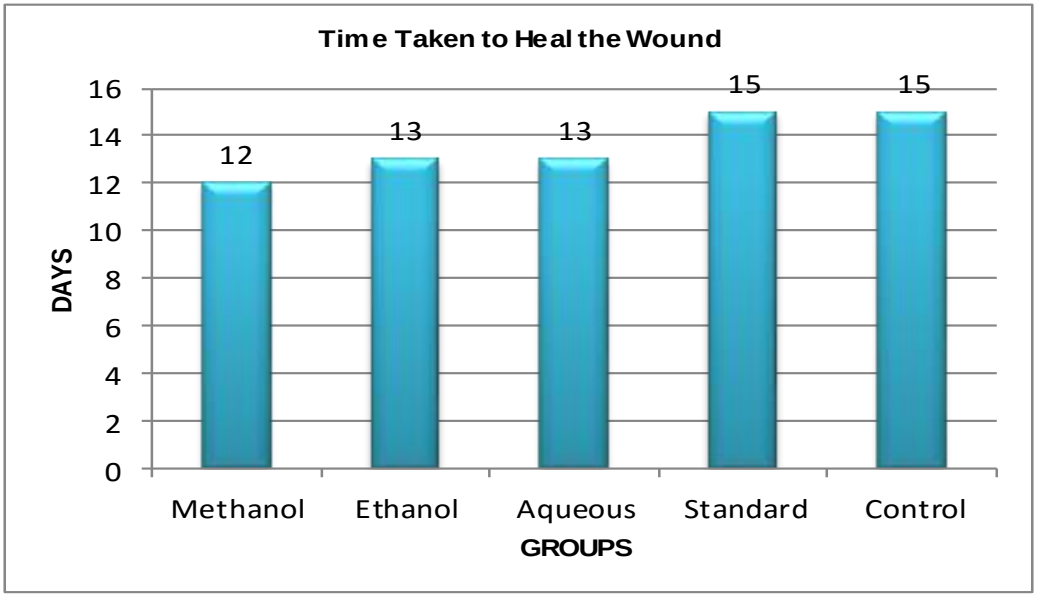

Figure 6: Graph of time taken to heal the wound.

Data were collected and analysed using SPSS (Statistical Package for Social Science) version 22.0. The Parametric test of ANOVA (One Way Analysis of Variance) was employed. Differences with a p value of <0.05 were considered to be statistically significant.

RESULTS

Phytochemical screening

In the present study, primary and secondary metabolites in *T. usneoides* plant were qualitatively and quantitatively analyzed. *T. usneoides* plant extracts such as methanol, ethanol and aqueous were studied. Out of these three extracts, ethanol showed the maximum number of plant constituents such as alkaloids, flavonoids, tannins, saponins, phenols and steroids. These results are exhibited in Table 1.

The test results were determined by the colour change as well as the presence or absence of precipitate or any ring formation at the top, bottom or between two layers in the test tube. The results were then compared to the standard reference to determine the presence or absence of phytochemical constituents.

Thin layer chromatography

Results revealed that a high Rf value for flavonoid (0.75mm) and alkaloid (0.60mm) was obtained. A previous study on *T. usneoides* concluded that flavonoid content was the highest, and alkaloid content was the second highest[45].

Disk diffusion

The disk diffusion was done using the Modified Kirby-Bauer Method. MH agar was done in triplicate sets. 500mg/mL and 1000mg/mL of *T. usneoides* concentration were used, and the result showed that the 500mg/mL concentration of *T. usneoides* showed the largest zone of inhibition. Furthermore, methanolic extract showed the largest zone of inhibition for *S. aureus*, which was 22mm, whereas the zone of inhibition for *S. epidermis* and *P. aeruginosa* was 20mm and 23mm, respectively. Similarly, ethanolic extract also showed the largest zone of inhibition for *S. aureus* with the measurement of 22mm, whereas for *S. epidermis* and *P. aeruginosa*, the zone of inhibition was 17mm and 22mm, respectively. On the other hand, the aqueous extract showed the lowest zone of inhibition for *S. aureus* which was 4mm, and the zone of inhibition for *S. epidermis* and *P. aeruginosa* was 4 mm and 3mm, respectively.

Minimum inhibition concentration and minimum bacterial concentration

MIC and MBC results confirmed that both methanolic and ethanolic extracts restrained the growth of tested bacteria at a concentration range of 125 to 500mg/mL and also demonstrated bactericidal efficacy.

Wound healing measurement

Wound healing assay indicated that methanolic extract has a higher potency of wound closure (12 days; <1mm) compared to ethanolic and aqueous extracts (13days; >1.5mm and 13days: >1.8mm, respectively). Povidone-Iodine was used as the gold standard (15days; <2mm) in the study.

The graph above shows the time taken for wound healing. Based on the results, methanolic extract showed a faster recovery time compared to the gold standard, which is the Povidone-Iodine.

DISCUSSION

Antimicrobial activity in the alcoholic extracts of *T. usneoides* can be primarily due to the presence of phytochemicals such as phenols, flavonoids, tannins, steroids and so on. These bio-active components exhibit their actions through various mechanisms. Tannins inhibit cell wall synthesis by forming irreversible complexes with prolene-rich proteins[46] while saponins causes leakage of proteins and certain enzymes from the cell[47]. Coincidentally, flavonoids are excellent antimicrobial substances as they are capable of complexing with extracellular and soluble proteins as well as bacterial cell walls[48]. Steroids, on the other hand, act on membrane lipids and leakage from bacterial liposomes become apparent[49].

In this research, *T. usneoides* showed antimicrobial activity at the concentration of 500mg/mL. Previous studies of Silva *et.al* (2013) on *T. usneoides* did not show any antimicrobial activity at the concentration of 50mg/mL and 100mg/mL. When the concentration was increased to 500mg/mL, the antimicrobial effect which was represented by the diameter of the zone of inhibition gradually increased. Hence, the earlier negative results do not necessarily justify the absence of bio-active compounds in the plants used in this study nor do they indicate their inactivity.

Methanolic and ethanolic extracts have a potential in terms of their antimicrobial activity against common skin bacteria compared to aqueous, which showed the smallest inhibition zone. Overall, these findings indicate that the prominent antimicrobial activity in *T. usneoides* is due to the presence of phenols in methanolic and ethanolic extracts. The presence of phenols indicates stronger antimicrobial properties.

The MIC for methanolic and ethanolic extracts against *S. aureus*, *S. epidermis*, and *P. aeruginosa* were the same at a concentration of 125 to 500mg/mL. Turbidity was observed at the lowest concentration, which indicated the presence of bacterial growth. However, at a concentration of 125 to 500mg/mL, the extracts were not only able to inhibit the growth of the tested bacteria but it could also kill the bacteria, thus justifying the potency of their inhibitory effects.

In general, the MIC for aqueous extract was fairly low against the tested bacteria (*S.aureus, S.epidermis* and *P. aeruginosa*) as the aqueous extract is a water-based solvent, which makes it difficult to kill or exert effects against these bacteria. Therefore, it can be concluded that the aqueous extract is not bactericidal against the tested bacteria *S.aureus, S.epidermis* and *P. aeruginosa*.

The investigation using the excision wound was conducted by calculating the contraction in the wound area over a period of 15 days as well as the calculation of the percentage of wound contraction on a weekly basis for a period of 15 days. The observed results revealed that

the methanolic and ethanolic extracts of *T. usneoides* had a significant wound healing effect on the excision wound with an area measuring 1mm compared to the control area, which was 3.5mm. The time taken for the wound to heal upon application of each extract was 12 days; this was faster compared to the control group which took about 15 days to recover. The aqueous extract of *T. usneoides* also exerted wound healing effect with a total area of 2mm. It took 13 days for the wound to heal upon application of aqueous extract, compared to the control group which took about 15 days to recover.

CONCLUSION

Spanish moss shows a significant antimicrobial activity against *S. aureus*, *S. epidermis*, and *P. aeruginosa*. Additionally, the findings of this study also revealed that the methanolic extract of *T. usneoides* exerts a significant wound healing effect. Conclusively, it can be inferred that the Spanish moss has the capability of being developed into a novel drug delivery system.

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CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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