



Evaluation of Herbal Formulation using *Andrographis paniculata* Gel as an Antifungal Agent against Superficial Mycoses onto Animal Model

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ABSTRACT: The emergence of antifungal resistance warranted the need of novel agents from herbal formulation for the fungal treatment. A medicinal herb known as *Andrographis paniculata* (AP) is claimed to possess antimicrobial activities. Symptoms, such as skin lesion, itchiness, nail damage, and hair loss, are usually known as superficial mycoses or skin fungal infection. Therefore, the current study aims at evaluating the AP extract formulations antifungal effect on selected fungal pathogens, causing superficial mycoses, on the animal model. In the present study, a new herbal formulation was prepared using the AP ethanol extract and later formulated into a gel form. It is followed by its *in vivo* antifungal activity on selected dermatophytes, namely *Microsporum canis*, *M. gypseum*, *Trichophyton rubrum*, *T. interdigitale*, and *T. mentagrophyte*, using Sprague Dawley Rats. Based on parameters recorded concerning the wound healing, wound size reduction, and wound conditions scoring percentage, the *in vivo* antifungal study revealed the antifungal effects' existence in the treatment arm with the AP ethanol extract gel compared to the negative control (the untreated arm). Besides, an eradication of fungal elements in the treatment arm with the AP ethanol extract gel compared to the untreated arm had been shown in the histopathological examination wound sections using haematoxylin-eosin (H&E) and the Grocott-Gomori's Methenamine Silver (GMS) staining. The application of scientific approaches, such as *in vitro*, *in vivo* study and controlled clinical trials are among the specific challenges for herbal medicines research. Therefore, these findings have provided evidence of a potentially novel antifungal agent from herbal technologies and need further clinical studies onto human in ascertaining the new AP formulation use as an alternative treatment for superficial mycoses.

Keywords: *Andrographis paniculata*; antifungal; alternative treatment; herbal formulation; *in vivo*; superficial mycoses

Abbreviations: PDA, potato dextrose agar; H&E, haematoxylin-eosin; GMS, Grocott-Gomori's Methenamine Silver; G, growth; NG, no growth; MG, *Microsporum canis*; MG, *Microsporum gypseum*; TI, *Trichophyton interdigitale*; TR, *Trichophyton rubrum*; TM, *Trichophyton mentagrophyte*.

I. INTRODUCTION

Herbal technologies in the medicinal field have become an important contributor to the new diseases' treatment development. Nowadays, aromatic plants have various chemical compounds, contributing to the medicinal values all over the world. The herbal mixtures, known as polyherbal formulations, are commonly used since ancient times to increase the medicinal qualities concerning their synergistic therapeutic effects [1].

Although certain herbal formulations are still controversial concerning their cleanliness during processing, the herbal medicines use is still popular. A study on herbal medicines conducted by Amla and Baheda showed the aflatoxin presence [2]. However, herbal technologies development has become a safety control measure for most herbal products.

Besides, due to certain circumstances, the safe use of certain existing medicinal drugs is being questioned. These include the emergence of antifungal resistance and certain antifungal drugs side effects on users. These situations warrant a new antifungal herbal formulation development.

Andrographis paniculata is the herb used for the superficial mycoses treatment. It is widely planted in the Southern and South-east Asia, and popularly known locally as *hempedubumi*. The whole plants, especially the leaves and roots, are traditionally and famously-used for medicinal purposes in treating many types of infections.

The AP extract is known to combat certain medical conditions. Furthermore, the AP is known by the locals as antifungal, antihepatitis, antihepatotoxic, anti-

inflammatory, antimalarial, antipyretic, antisnakevenom, antithrombogenic, and antityphoid activities [3-5].

The superficial fungal pathogen causes dermatophytosis, affecting the stratum corneum layer. This fungal pathogen produces keratinase, which metabolises the stratum corneum layer, and therefore, lives on human keratins, such as hair, nail, and skin. Common clinical mycoses are superficially presented as a hair loss, itchiness, nail damage, and skin lesion. Dermatophyte fungi, such as *Trichophyton*, *Microsporum*, or *Epidermophyton*, also usually known as *tinea*, are commonly used in drugs to treat fungal infections. However, due to the dermatophyte fungi indiscriminate use, it leads to an antifungal resistance higher rate among the fungi.

Medicinal plants, other than the antifungal drug, are also a resource for most antifungal treatment, as they possess various therapeutic value agents. Furthermore, there is a need to develop novel antimicrobials, especially antifungal agent, from natural resources with the current alternative medicine craze. Since some antibiotics and antifungal drugs have serious drawbacks, such as fungistatic activity, limited action or resistance spectrum, and toxicity [6], the need for herbal technology research and innovation on antifungal agent from medicinal plants has now become a necessity.

II. MATERIALS AND METHODS

Fungal Inoculum Preparation. The fungi (*M. canis*, *M. gypseum*, *T. interdigitale*, *T. mentagrophyte*, and *T. rubrum*) isolation was maintained on the PDA plates for seven days. The spore suspension concentration was adjusted to 1×10^4 CFU/ml.

AP Gel Preparation. The freshly prepared AP ethanol extract was applied to the rats. The gel was prepared by adding 0.5 g of AP ethanol extract into 9.5 g of petroleum jelly to get 5.0% (w/w) of AP gel.

Animal Preparation. Upon obtaining the Animal Ethics Committee USM (AECUSM) approval (USM/Animal Ethics Approval/2013/(90)(536)), the Animal Research and Service Centre (ARASC) of USM Health Campus released the male Sprague Dawley (*Rattus norvegicus*) Rats for this study. The rats were between 8 to 12 weeks old, with the weight ranging from 250 to 350 g. They were kept in individually ventilated cages (IVC) at room temperature, with a 12-hour light-dark cycle. Furthermore, they were acclimatised for one week before the experiment. They were also given standard wood bedding and standard rat pellets (Altromin, Germany), with ad libitum water.

The rats were anaesthetised using ketamine (90 mg/kg) and xylazine (5 mg/kg) by intramuscular injection. Their back was shaved at the dorsal region using a standard electric shaving machine to create a wound and then cleansed with a povidone-iodine solution. An antibacterial scrub with 70% alcohol was used to disinfect the skin before inflicting the superficial wound, followed by gentle scarification using a sterile scalpel blade to obtain an approximately ten mm² wound area size. The scrapped skin depth was approximately 0.1 - 0.2 mm, involving only the epidermis, and just a short blood drawing (adapted from Darestani *et al.*, 2014) [7]. Each rat's dorsal area had three wound areas.

AP Antifungal Effect. Each wound area received 0.1 ml of each fungi suspension, including *M. canis* (n=4), *M. gypseum* (n=4), *T. interdigitale* (n=4), *T. mentagrophyte* (n=4), and *T. rubrum* (n=4). After that, the polyethylene film and TG® Fixtubuler net bandage covered the wound.

The treatment started after eight days of the inoculation. The wound represented the negative control (untreated), positive control with 0.25 g ketoconazole treatment (Yucomy Cream, Malaysia), and a wound with 0.25g AP gel treatments, respectively. The dressing was changed every three days, which were on day 3, 6, 9, 12, 15, and 18. The procedure was conducted under anaesthesia and aseptic conditions.

Wound Evaluation. The wound observation was carried out, and scoring system was used to evaluate the crust, dryness, erythema and exudate formation degree, using a scale of 1-3; 1 is the lowest and 3 is the highest. Averages from all four rats were used in all groups infected with different fungi. The scores were recorded on day 6, 9, 12, 15, 18, and 21 post-inoculation.

The wound diameter was recorded by tracing the wound boundaries using a clear paper, followed by recording its measurement (in mm²) using a graph paper. The wound contraction was calculated based on the wound reduction from the original wound size [8]. The wound healing percentage was calculated as follows:

$$\frac{(A_{Day 0} - A_{Day X})}{A_{Day 0}} \times 100$$

where X and A refer to days after wound creation and wound surface area, respectively.

On day 21, the rats were sacrificed. The wound swabs were collected and subjected to PDA culturing. It is followed by the histopathological examination (H&E and GMS staining) using the tissue biopsy of the skin section. The swab cultures were observed if they showed fungal growth after five incubation days, and the results were compared between the skin treated with ketoconazole, AP gel, and untreated arm.

Statistical Analysis. The Statistical Package for Social Science (SPSS) version 25 was opted to carry out the data analysis, including the General Linear Model that is a repeated measure analysis of variance (ANOVA) for each group. $p<0.05$ was considered statistically significant.

Microscopic Assessment of Wound Healing. In this assessment, healthy skin and infected skin margin were excised and fixed using a 10% neutral buffered formalin solution. After that, it was hydrated in ethanol (from 10% (v/v) to 100%), washed in xylene, and embedded in paraffin. Two sections were stained by GMS and H&E staining, respectively. The sections were assessed through the histopathological examination under a light microscope.

III. RESULTS

The Fungal Growth. After inoculating and treating the fungi with the AP gel and ketoconazole as a positive control, the fungal growth was observed and summarised in Table 1. No fungal growth was observed in the AP gel and ketoconazole treatment arm; however, while the fungal growth was recorded in the untreated wound. These results were consistent with the predicted

effect, as all untreated wounds showed fungal growth. All wounds treated with the AP gel resulted in zero fungal growth, similar to the positive control. These indicated that the AP gel was found to be susceptible towards the antifungal effect of all tested fungi.

Table 1: The growth of fungi after wound treatment.

Fungi Wound	MC	MG	TR	TI	TM
Wound 1	NG	NG	NG	NG	NG
Wound 2	NG	NG	NG	NG	NG
Wound 3	G	G	G	G	G

Note: The wounds were treated with; Wound 1: AP gel, Wound 2: ketoconazole, and Wound 3: untreated.

A. Wound evaluation

Crust. Figure 1 shows the actual treatment effect on crust formation. On day 6 after the wound creation, the crust formation was seen in all inoculated wounds, except for *T. rubrum*, showing the crust formation beginning on day 9 (Fig. 1(a)). The crust formation was seen for *M. canis* and *T. rubrum*, and for *M. gypseum* and *T. interdigitale* up to the day 12 and day 15, respectively (Fig. 2).

There was no significant difference ($p<0.05$) between untreated wounds and wounds treated with the AP gel and ketoconazole during the observed days. However, compared to wounds treated with the AP gel and ketoconazole, practically, most untreated wounds showed higher crust scores. Most untreated wounds delayed the crust formation elimination when compared to other treated wounds. The AP gel and ketoconazole treated wounds (the positive control) crust formation recorded practically similar scores to all inoculated fungi and between days observed.

The decreased crust formation was demonstrated for most wounds up to day 21. The decreased crust scores were seen for *T. mentagrophyte* on the day 12. However, the untreated and ketoconazole treated wounds scores increased on day 15, and later decreased again on the day 18 onwards (Fig. 1(b)).

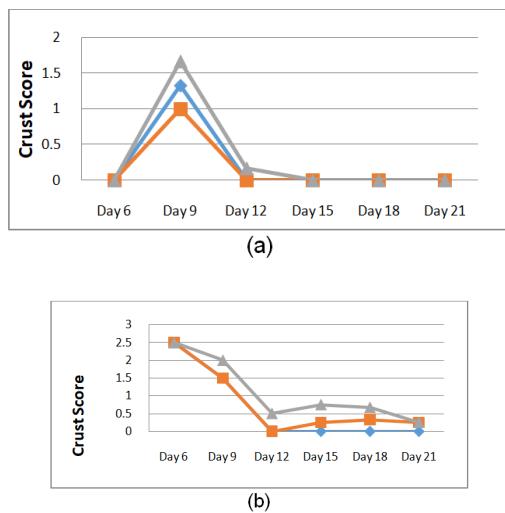


Fig. 1. Crust score of wounds inoculated with (a) TR, (b) TM. Data represent animals treated with (◆) AP gel, (■) ketoconazole, and (▲) untreated.

(A)

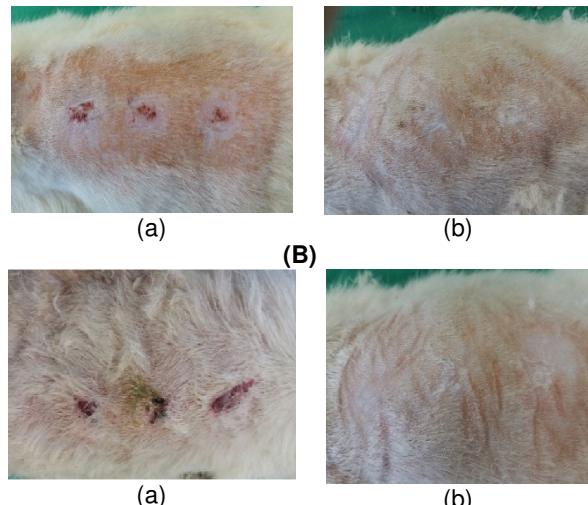
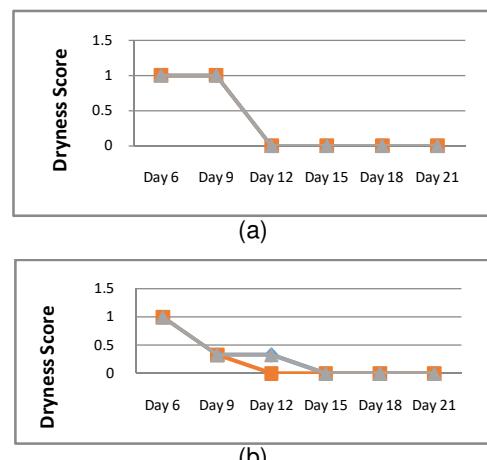


Fig. 2 (A) Crust formations of *M. canis* inoculated wounds at (a) day 6 and (b) day 12. **(B)** Crust formations of *M. gypseum* inoculated wounds at (a) day 12 and (b) day 15. The wounds were treated with; left (AP gel), middle (ketoconazole), and right (untreated).

Dryness. For dryness observation, Fig. 3 shows that some wounds recorded a decreased dryness score trend, while some others did not record any dryness effect. Among all tested fungi, *T. rubrum* and *T. interdigitale* showed dry effects throughout the study, proving functional healing activities, both in treated and untreated wounds. Other tested fungi recorded the dryness scores almost up to the day 9 (*M. canis*) and up to the day 12 (*M. gypseum* and *T. mentagrophyte*). On day 12, the scant dryness scores were noted in the untreated wound inoculated with *M. gypseum* and *T. mentagrophyte* (Fig. 4). During these days, there was no dryness presence in the wound treated with the AP gel and ketoconazole, except for the low dryness score observed in wounds treated with the AP gel and inoculated with *M. gypseum*, indicating the infection occurred but had been cleared due to the treatment.

There was no significant difference ($p<0.05$) in all inoculated wounds subjected to different treatments compared to untreated wounds. As shown in Fig. 4, there was no dryness score difference between the treated and non-treated wounds inoculated with the *M. canis* and *T. mentagrophyte*.



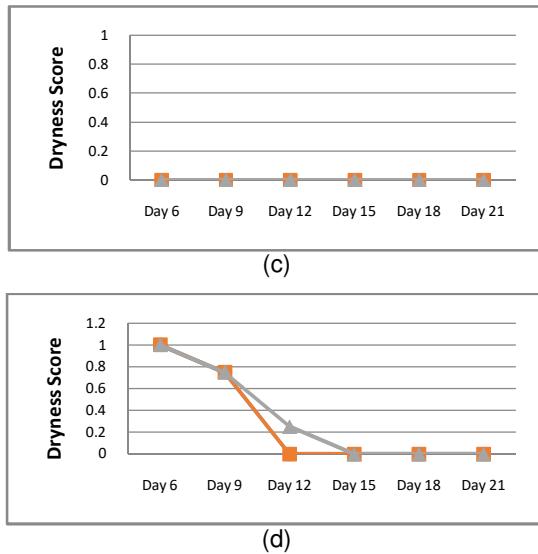


Fig. 3. Dryness score of wounds inoculated with (a) MC, (b) MG, (c) TI& TR, (d) TM. Data represent animals treated with (◊) AP gel, (■) ketoconazole, and (▲) untreated.

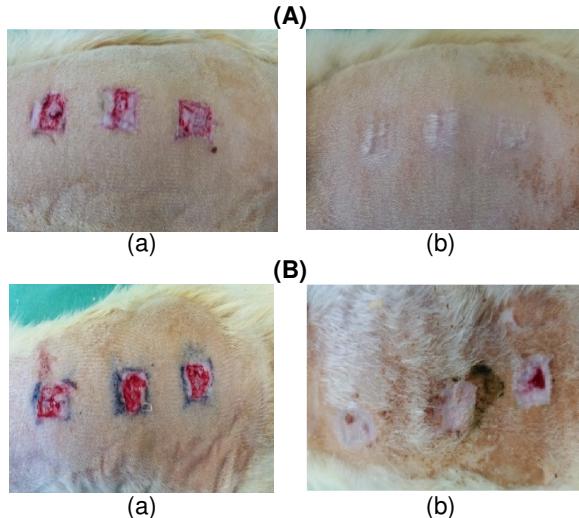


Fig. 4 (A) Dryness effect of *M. canis* inoculated wounds at (a) day 6 and (b) day 12. (B) Dryness effect of *T. mentagrophyte* inoculated wounds at (a) day 6 and (b) day 12. The wounds were treated with; left (AP gel), middle (ketoconazole), and right (untreated).

Exudate. Exudate formation was observed to quantify the treated and untreated wounds healing process. As shown in Fig. 6, exudate formation was seen in all *M. canis*, and *T. rubrum* inoculated wounds on day 6 after the inoculation. However, the exudate disappeared prior to the treatment commencement and maintained throughout the study. In the *T. rubrum* treated skin group, there was the hair growth formation on day 12, in contrast to untreated wounds, showing no hair growth. For *T. interdigitale* group, there were none exudate formations in all wounds throughout the study (Fig. 5(b)). The group inoculated with the *T. mentagrophyte* did not exudate prior to the treatment. Nevertheless, the exudate existence was observed on day 9 in wounds treated with ketoconazole and untreated wounds. The

wounds treated with the AP gel did not show any exudate formation throughout the study (Fig. 5(d)). A significant difference ($p<0.05$) was not found between all treated and untreated wounds, inoculated with all tested fungi. In the wounds inoculated with the *T. mentagrophyte*, a less exudate formation was displayed in the AP gel treated wounds compared to untreated wounds.

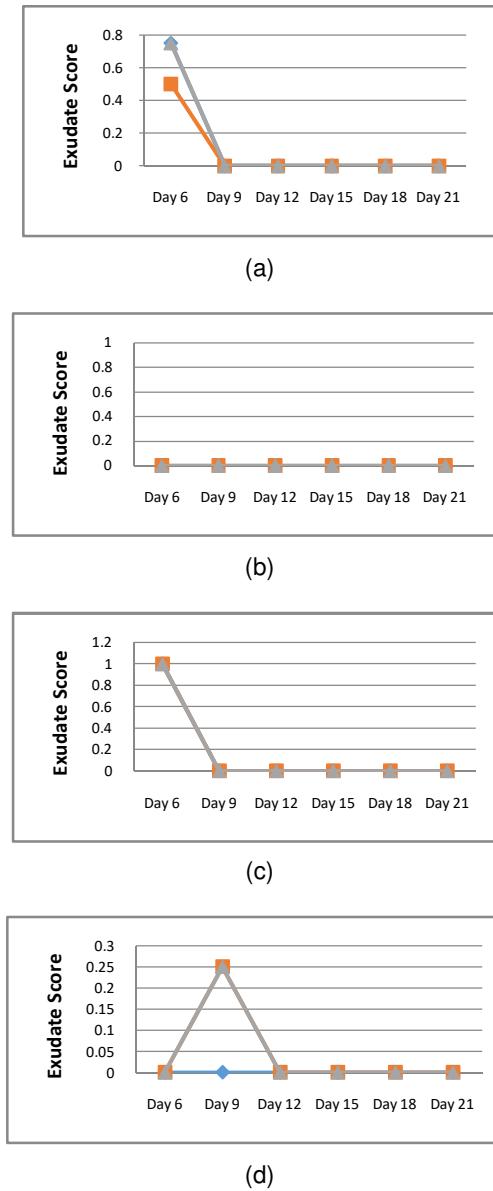
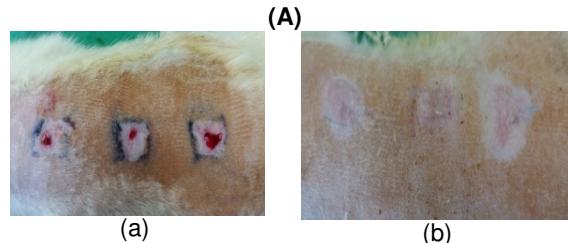


Fig. 5. Exudate scores in wounds inoculated with (a) MC, (b) TI, (c) TR, (d) TM. Data represent animals treated with (◊) AP gel, (■) ketoconazole, and (▲) untreated.



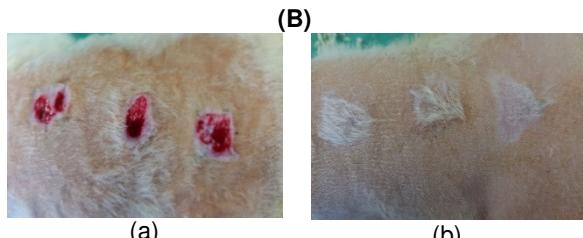
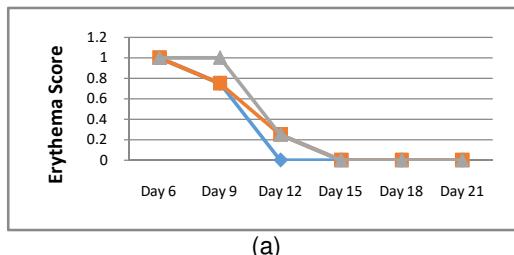


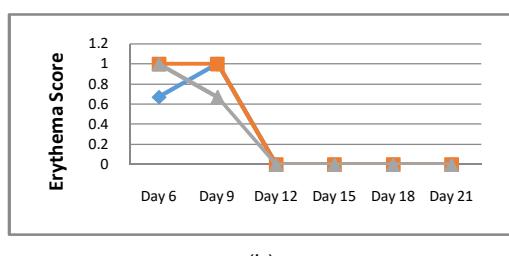
Fig. 6 (A) Exudate formation in *M. canis* inoculated wounds at (a) day 6 and (b) day 9. (B) Exudate formation in *T. rubrum* inoculated wounds on (a) day 6 and (b) day 12. The wounds were treated with; left (AP gel), middle (ketoconazole), and right (untreated).

Erythema. The wound redness (erythema) was quantified in the experiments to evaluate the treatment response, resulting in the erythema scores of most wounds inoculated with fungi starting on day 6 and up to day 9. These included the wounds inoculated with the *M. gypseum*, *T. interdigitale*, and *T. mentagrophyte*. Only in the *M. canis* that the erythema was observed until day 12 in ketoconazole treated and untreated wounds (Fig. 7(a)). In the wounds inoculated with the *T. rubrum* case, the erythema formation was only shown on day 6 after inoculation. After the treatment on day 8 and up to day 21, there was no erythema on all tested wounds (Fig. 7(d)).

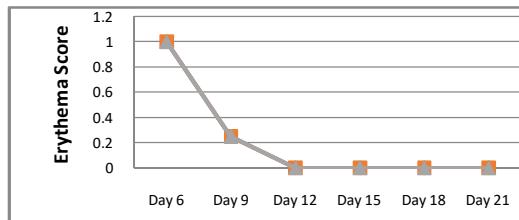
There was a decreased erythema score trend and also the score variability in rats infected with different fungi and treated with different treatments. There was no erythema seen in the AP gel treatment arm. Although there were no differences ($p<0.05$) among the erythema scores, in the *M. canis* inoculated wounds, without treatment and untreated wounds required a longer time to heal compared to wounds treated with the AP. On day 15, hair growth was observed for all treated wounds, while no hair growth recorded on untreated wounds (Fig. 8). The remaining wounds, inoculated with other fungi, showed practically similar erythema scores in all treated and untreated wounds (Fig. 7(b-e)). In the *T. interdigitale* group, on day 9, the wounds displayed practically similar erythema degree, with the healing process occurred throughout the study (Fig. 8).



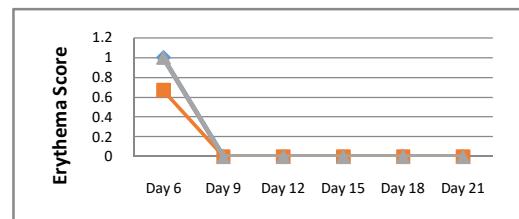
(a)



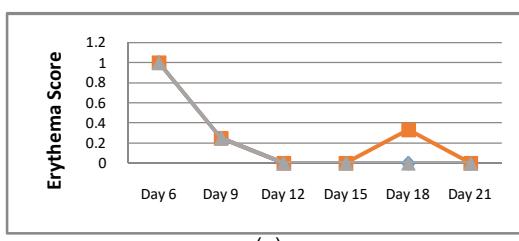
(b)



(c)



(d)



(e)

Fig. 7. Erythema score of wounds inoculated with (a) MC, (b) MG, (c) TI, (d) TR, (e) TM. Data represent animals treated with (▲) AP gel, (■) ketoconazole, and (△) untreated.

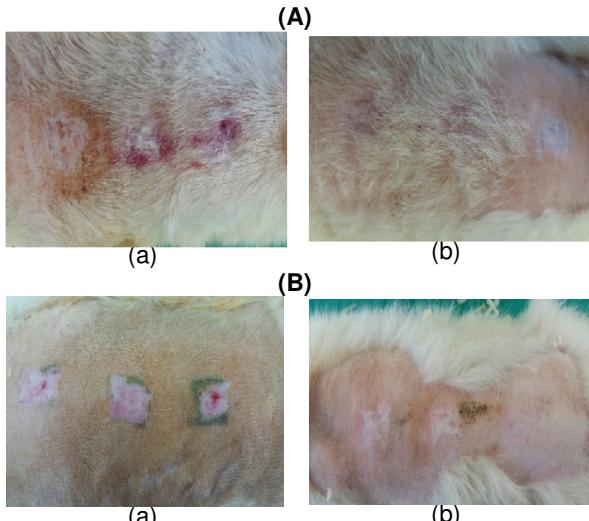


Fig. 8 (A) Erythema formation in *M. canis* inoculated wounds at (a) day 12 and (b) day 15. (B) Erythema formation in *T. interdigitale* inoculated wounds on (a) day 9 and (b) day 12. The wounds were treated with; left (AP gel), middle (ketoconazole), and right (untreated).

B. Percentage of wound healing

This study found a significant effect of wound healing processes of treated and untreated wounds in most fungi with inoculated wounds ($p<0.05$). The wound

healing percentage on day 6, 9, 12, 15, 18 and 21 is shown in Fig. 9.

There was no significant difference in all *M. canis*, and *M. gypseum* inoculated wounds on day 6 and 9. Full healing was seen in all treated wounds from day 15, except for wounds treated with ketoconazole and inoculated with *M. canis* and *T. mentagrophyte* on day 21. Almost all untreated wounds required a longer time to heal compared to the wounds treated with the AP gel. On day 21, all wounds exhibited a 100% healing effect. For wound inoculated with the *M. canis*, a significant difference was found in treated and untreated wounds on day 12, 15, and 18. Besides, bigger wound size was seen in the *M. canis* and *T. interdigitale* non-treatment arm (Fig. 10).

These findings revealed the AP gel effectiveness, with the same performance recorded as the positive control (ketoconazole). For the *M. gypseum* and *T. mentagrophyte*, wounds treated with the AP gel recorded a significant difference compared to untreated wounds on the day 12 and 15. On day 9, 12, and 15, the *T. interdigitale* inoculated wounds treated with the AP gel recorded a significant difference compared to the control arm. Most ketoconazole treated wounds also recorded a significant difference. The *T. rubrum* inoculated wounds displayed a less healing effect, which only recorded the statistical difference on the AP gel treated wound on day 9. However, most of the wound healing percentage tended to be recorded at a higher percentage in the AP gel treated wound compared to untreated wounds.

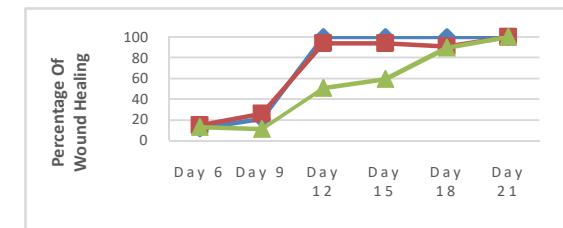
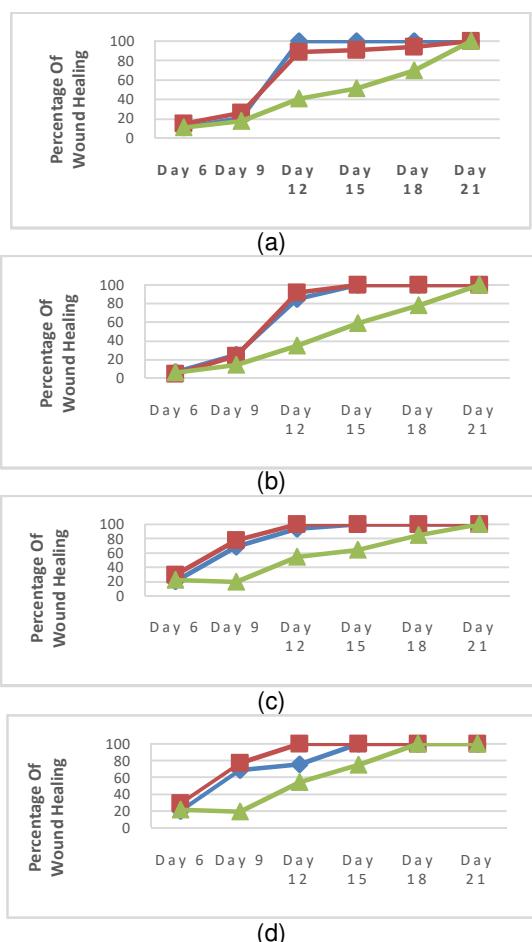


Fig. 9. Percentage of wound healing in wounds inoculated with (a) MC, (b) MG, (c) TI, (d) TR, (e) TM. Data represent animals treated with (◊) AP gel, (■) ketoconazole, and (▲) untreated. (A)

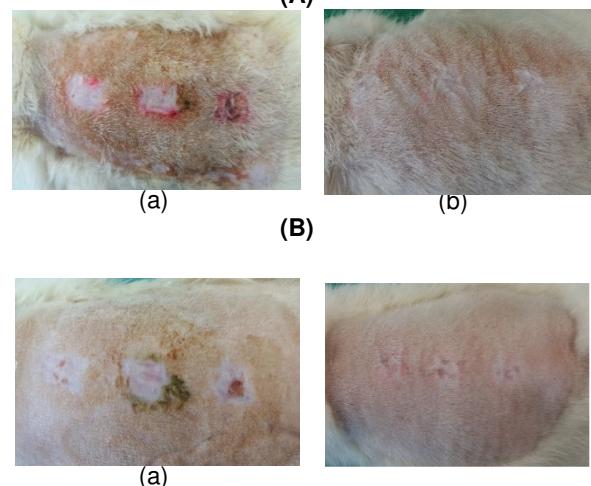


Fig. 10. (A) Wound healing in *M. canis* inoculated wounds at (a) day 12 and (b) day 21; (B) Wound healing in *T. interdigitale* inoculated wounds at (a) day 12 and (b) day 21. The wounds were treated with; left (AP gel), middle (ketoconazole), and right (untreated).

C. Histopathological evaluation

Based on the histopathological evaluation using the GMS staining, in untreated wound sections, they showed substantial fungal hyphae and spores. At the same time, there were scanty or none fungal hyphae in the treated wounds (Fig. 11(a)). An example of the tissue invasion by the fungal hyphae with an acute-angle branching was observed in the *M. canis* on-treatment arm (Fig. 11(b)) (MC).

There were no fungal elements seen in the wounds treated with the AP gel, denoting the infection clearance (Fig. 11(a)) (MC and TI). However, in the wounds inoculated with *M. gypseum*, *T. rubrum*, and *T. mentagrophyte*, on the day 21, there were scanty fungal elements seen (Fig. 11(a)) (MG, TR, and TM)). The non-viable fungi in the tissue might be present if the wounds have received antifungal medications.

In the skin treated with the AP gel and inoculated with the *M. canis*, staining with H&E showed the fibroblasts' proliferation enhancement (Fig. 12(a)). The increased proliferation was possibly resulting from the attracted inflammatory cells action towards the wound area. In the *T. interdigitale* infection treated with the AP gel, there was a thick and flat epidermis seen (Fig. 12(b)).

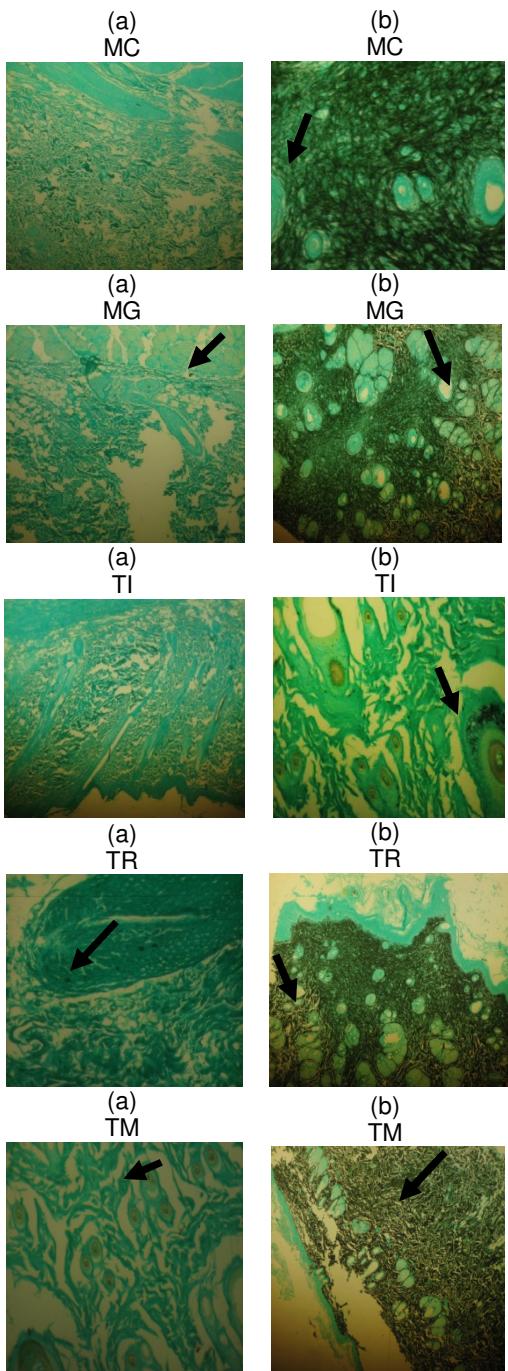


Fig. 11. GMS staining of infected wound treated with; (a) AP gel and (b) untreated. Magnification: 40x.

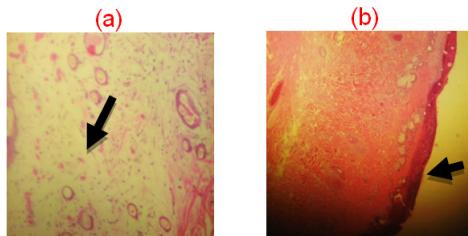


Fig.12. H&E staining of skin sections treated with AP gel; a) enhanced proliferation of fibroblasts (arrow), and (b) thick and flat epidermis (arrow). Magnification: 40x.

IV. DISCUSSION

This study aimed at determining the AP extract antifungal effect on the superficial fungal infection, causing dermatophytosis. In most cases, *M. canis* was the most common and followed by *M. gypseum*, *T. mentagrophytes*, *T. quinckeum*, and *T. verrucosum* [9].

Recently, for in vitro study, there was also a report by Jeenkeawpieam [10] who recorded the antifungal effect of AP extract onto *Talaromyces marneffei* (yeast phase), *Cryptococcus neoformans*, *Candida albicans* and *Aspergillus fumigatus*. On the other hand, in vivo research conducted in the present study showed the AP gel exhibited antifungal activities almost at the same degree of all parameters recorded; crust, dryness, erythema, and exudate formation. Besides, the statistical analysis recorded a significant difference in wound healing percentage between the AP gel treatment arm and non-treatment arm. The H&E staining in the histopathological study indicated the cell proliferation presence as actions towards the epithelialisation process and granular tissue formation. Thus, these results revealed that the AP gel is not only a potential antidermatophytosis formulation but also beneficial in the wound healing treatment.

There was a study investigating the petroleum jelly effect on *in vivo* full-thickness skin wound tested on the diabetic mice using two treatments; one with the petroleum jelly and simvastatin combination, and the other with the petroleum jelly alone [11]. The results showed that the treatment with the petroleum jelly and simvastatin combination exhibited a better healing effect compared to the petroleum jelly alone. The study was consistent with our study, showing the AP gel extract application might be significant for the wound healing process enhancement, instead of using other formulation bases, such as lotion and cream. These might be due to the wound healing process that improved the skin structure, with a thin and well-formed epidermis, a well-organised dermis, with more collagen fibroblasts, no inflammatory feature, and substantial blood vessels [12].

Another study by Ahmad [13] examined the topical gel of the mixture of *Allium sativum*, *Curcuma longa*, *Andrographis paniculata* and *Alpinia galanga* extracts combination exhibited synergistic effects of antifungal activity onto *T. rubrum*. Thus, this study also revealed the potential of AP to be used in treating *T. rubrum*.

Based on histopathological examinations, the results showed the favourable potential of the AP gel on fungal infection. These were proven by the substantial fungi hyphae presence in untreated wounds compared to skin with AP gel treated wounds, showing fungal elements scantily or absence. Nevertheless, scanty fungi still present in the skin section of certain skin swab cultures, despite the plate culture showed no fungal growth. It is possible due to the non-viable fungi presence. Concerning this condition, a study reported that the non-viable fungi presence in tissue frequently happens in chronic patients, endemic yeasts infections, such as in coccidioidomycosis, cryptococcosis, or histoplasmosis [14]. Besides, the same situation might occur due to antifungal medications, resulting in negative cultures. However, the tissue still contained fungal elements.

A study onto cats infected with dermatophytes had been conducted by Stuntebeck [15] and showed that the first negative fungal culture did not indicate that the cat was undergone mycological cure. This result showed that the fungal elements was remained inside the tissue although the culture was negative. In this case, the sampling technique need to be improved in order to avoid false-negative results.

Despite the scanty fungal elements present in some cases, our study had proven that prolonged treatment until 21 days could have eliminated the fungal elements and prevented a relapse. A study concerning the alkaloidal extract of the *Enantia chlorantha* showed a fungal loads decrease when compared to rats treated with a standard tioconazole 1% cream as the positive control, and normal saline as the negative control [16]. Another study using rat model reported that the treatment using the *A. verus* aqueous extract on the *T. verrucosum* infected rats showed antifungal activities, which observed the lesion scores reduction in higher extract concentrations [17].

V. CONCLUSION

Generally, according to the parameters recorded, most untreated wounds showed higher crust scores compared to wounds treated with the AP gel. There were also decreased erythema scores, increased AP gel treated wound healing percentage, and no exudate formation. Therefore, this study has elaborated the herbal formulation value on superficial mycoses treatments. The AP ethanol extract gel formulation, applied as a topical treatment, has been proven in this study to possess the ability as an antidermatophytes agent.

VI. FUTURE SCOPE

Since long ago until now, there was the gap between the traditional medicines' usage and the scientific research due to less interactions among traditional healers and modern health professionals. This present research provides useful input for herbal technologies to develop a new antifungal agent from medicinal plants. If the plants are well harnessed, and then formulated following the good manufacturing practice, they could be competitive with the existing antifungals in the market. This herbal product can, therefore, be used in the ringworm infection treatments, especially for the *M. canis*, *M. gypseum*, *T. interdigitale*, *T. mentagrophyte*, and *T. rubrum* management. Further clinical studies on the human, especially the patients, need to be carried out in ascertaining this herbal formulation use for the superficial mycoses' treatment.

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Conflict of Interest. The authors declare that they have no competing interests.

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