THE EFFECT OF PIPER SARMENTOSUM AQUEOUS EXTRACT ON BUCCAL ULCER HEALING

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Abstract

Oral ulcer is a lesion with multifactorial causes and occurs worldwide. The lesion usually resolved within 14 days, but the pain may have an impact on the quality of patient's life. Therefore, having a natural derived remedy that can reduce healing time would be a great advantage. This study aims to investigate histological sections of buccal ulcer on rats treated with aqueous extract of *Piper sarmentosum* (AEPS). Glacial acetic acid was used to induce buccal ulcer on male Sprague Dawley rats. Control group received normal saline while the experimental group received AEPS for treatment. On certain days of post-ulcer induction, buccal ulcer tissue samples were harvested, sectioned and stained with Hematoxylin and Eosin (H&E). Histological slides were examined for inflammation and scored. The inflammation severity reduced from day 2 to day 12. In the experimental group there was a statistically significant differences of inflammation score, particularly on day 2 with a score of (2.8 ± 0.2). Neutrophils were less in the experimental group and the tissue debris clearance was faster compared to control group. Full reepithelization was observed on both treated tissue sections on day 12 with less severe inflammation. Topical application of AEPS is proven to have anti-inflammatory effect by reducing the number of neutrophils during inflammation phase of oral ulcer healing.

Keywords: *Anti-inflammatory, oral ulcer, ulcer healing*

Introduction

Oral ulcer or clinically known as recurrent aphthous ulcer (RAU) is a common lesion which occurs worldwide and does not discriminate between genders. This oral lesion is classified based on its physical manifestation. Minor RAU has one to five active ulcers at one time and major RAU has more than five ulcers. However, herpetiform RAU may occur up to 100 ulcers at a given time. The minor form and herpetiform lesions usually resolve within 2 weeks while its major form resolves within 6 weeks (1-3). There are many causes of oral ulcers, which can occur spontaneously, or as a manifestation of certain diseases, or as a result of traumatic injury (4). Other factors include the influence of genetic, smoking cessation and micronutrient deficiency (5, 6).

Preferred treatment for oral ulcers is topical drugs in the form of paste, gel, cream or mouth rinse. Systemic drugs are also available in the market and frequently prescribed to patients with major ulcers. Oral ulcer drugs can be categorised according to their pharmacological functions. For example, corticosteroids are used to shorten the inflammatory phase of a wound while anti-inflammatory drugs are to prevent formation and release of all inflammatory mediators. In addition, topical analgesics are used for pain relieved. Even though these drugs are effective in attenuating the symptoms of ulcers, they are also known to cause unwanted adverse reactions especially in those with history of allergic reaction. Oral candidiasis is usually implicated with topical agents while suppression of hypothalamic-pituitary-adrenal and nausea are due to usage of systemic corticosteroids. Moreover, pregnant women are at risk of having deformed child with the usage of anti-tumor necrosis factor alpha agent such as thalidomide (7, 8).

The main purpose of wound healing is to repair an injured tissue to its original state. This process occurs unidirectional with overlapped sequences. Whenever there is an injury, the first stage of healing, which is inflammation will occur. Acute inflammatory cells, such as neutrophils dominate the tissue and reach highest concentration in 24 hours (9). As the first leukocytes to reach the wound, circulating neutrophils are recruited to the wound site and protect the compromised site from normally residing pathogens (10). Thereafter macrophages are activated and engulfed

apoptotic neutrophils. Various cytokines are released by different cells to stimulate keratinocyte proliferation as well as migration for wound closure and deposition of fibroblast for restoration of connective tissue (11). In understanding the mechanisms and factors for healing, appropriate treatments are developed to accelerate wound recovery.

Nature provides a lot of benefits especially to health. In the past, our ancestors took advantage of the environment by using plants and herbs to treat illnesses and to maintain good health. The plants are usually consumed raw or cooked, boiled with water or pounded into paste. While the primary metabolites are important for the plants' physiological functions, their secondary metabolites render medicinal values. Even though the usage of natural resources is never dampened especially in the Asian continents, their usage currently gets attention as it is believed to give less harmful side effects compared to synthetic products.

Various studies were carried out to evaluate the antioxidant and anti-inflammatory capacity of plants on oral ulcer healing and many of them had been published (12-14). *Piper sarmentosum* is one of the herbaceous plants which can be found growing abundantly around Southeast Asian countries including Malaysia. Locally, it is known as 'kaduk' and can be seen growing as weed in shaded areas. Beside using it as food, the plant is also famous among the folklore for treating fever, toothache and as a cough reliever (15). Research on this plant shows it to have high antioxidants (16) and anti-inflammatory activities (17, 18). Various parts of the plant were used to investigate its phytochemicals as well as its therapeutic properties. Methanol extract of the whole plant was reported to contain a high antioxidant index among Thailand's edible plants (19).

At physiological level, reactive oxygen species (ROS) is beneficial to induce angiogenesis to support proliferation phase of wound healing process (20). However, if the amount is too high, ROS can harm the surrounding cells by initiating a chain reaction of lipid peroxidation and create a condition called oxidative stress. Several studies showed that level of malonaldehyde (MDA) which is the marker of lipid peroxidation, was upregulated in oral aphthous ulcer patients (21, 22). Our body has its own defence mechanism against ROS which is enzymatic antioxidant such as superoxide dismutase, catalase and peroxidase. Nevertheless, in a state of oxidative stress, these enzymatic antioxidants are not sufficient to combat the innumerable ROS. Hence exogenous antioxidant is needed to indemnify ROS imbalance.

Extraction from *Piper sarmentosum* plant had been shown to have healing property. However, in most of the studies that we encounter, this extract was used to give effect by systemic distribution. We have not yet come across any study that uses this extract for local lesion. Hence, this study is conducted to prove that topical application of aqueous extract of *Piper sarmentosum* (AEPS) is able to reduce inflammation of the ulcer induced on rats' buccal mucosa.

Materials and methods

Plant materials

Piper sarmentosum plants were collected around Kota Bharu, Kelantan, Malaysia. The plants were sent to the Forest Research Institute Malaysia (FRIM), Kepong, Kuala Lumpur, Malaysia for identification by plant taxonomist. The voucher specimen number given was 170612-11.

Aqueous extraction of Piper sarmentosum plant

Piper sarmentosum leaves were dried in a drying cabinet at 60°C for 3 days and grounded to smaller pieces using an electric mill. The aqueous extract of *Piper sarmentosum* (AEPS) was prepared by macerating the leaves with distilled water (1:20) at 80°C for 3 hours. After filtration and heat treatment (Eppendorf Concentrator Plus, Barkhausenweg 1, Hamburg, Germany) the extract was freeze dried for 3 days. The final product in powder form was kept in the dark at 4°C until use.

Animal study of buccal ulcer

Thirty-six male Sprague Dawley rats (200-240 g) were used in this study and were obtained from Animal Research and Service Centre, Universiti Sains Malaysia. The rats were separated into two groups; [1] control group: buccal ulcer induced and treated with normal saline (NS) and [2] experimental group: buccal ulcer induced and treated with AEPS. Before the experiment, the animals were acclimatized for a week. The animals were kept in a 12-hour light and 12-hour dark cycle. Food and water were available ad libitum. Ulcer induction was carried out according to Novianty et. al (2011) (23) with minor modifications. Cotton swab was initially pressed onto a hard surface to flatten it and soaked in glacial acetic acid. Then, it was pressed onto the left buccal mucosa of the rat for 40 seconds. Treatment with NS and AEPS were given the day after ulcer inducement, twice daily until day 12. Water was withheld after each treatment for about 20 minutes. The rats were euthanized on day 2, day 6 and day 12 post-ulcer inducement by excess inhalation of carbon dioxide gas.

Histological analysis of rat buccal ulcer tissue

Left buccal tissue was harvested and fixed in 10% neutral buffered formalin (R&M, Selangor, Malaysia). The tissues were then processed and sectioned 3.5 µm thick and were stained with Harris haematoxylin (Diapath, Via Pietro Savoldini, Martinengo BG, Italy) and eosin (RMstain, Petaling Jaya, Selangor, Malaysia). Stained tissue sections were observed under light microscope and severity of inflammation was scored according to Table 1. The differences in inflammation score between the two groups were tested with Kruskal-Wallis test and Mann-Whitney test. Significance of difference was set at $p < 0.05$.

Table 1: Scoring for inflammation

Inflammation score	Description
0	No inflammation
1	Slight chronic inflammation
2	Mild chronic inflammation
3	Mild inflammation
4	Severe inflammation

Results

Figure 1 shows histological changes of different treatment effects on buccal ulcer. Both NS treated sections and AEPS treated sections showed severe inflammation on day 2. The appearance of neutrophils was identified as multi-lobed nuclei and purplish granules. Normal saline section showed a semi-detached ulcerated region whereas in AEPS section, the ulcerated region was completely detached from the connective tissue. Both sections also showed abundance of inflammatory cells especially at the detached region. Granulation tissues formed was comprised of small blood vessels especially near the ulcer bed. On day 6, inflammatory cells were reduced compared to day 2 for both treatment tissue sections. Lesser tissue debris was seen and both ulcerated wounds were not completely closed. Normal saline sections showed less inflammatory cells at the ulcer bed while more tissue debris was seen. Epithelisation occurred and covered the ulcerated region on day 12.

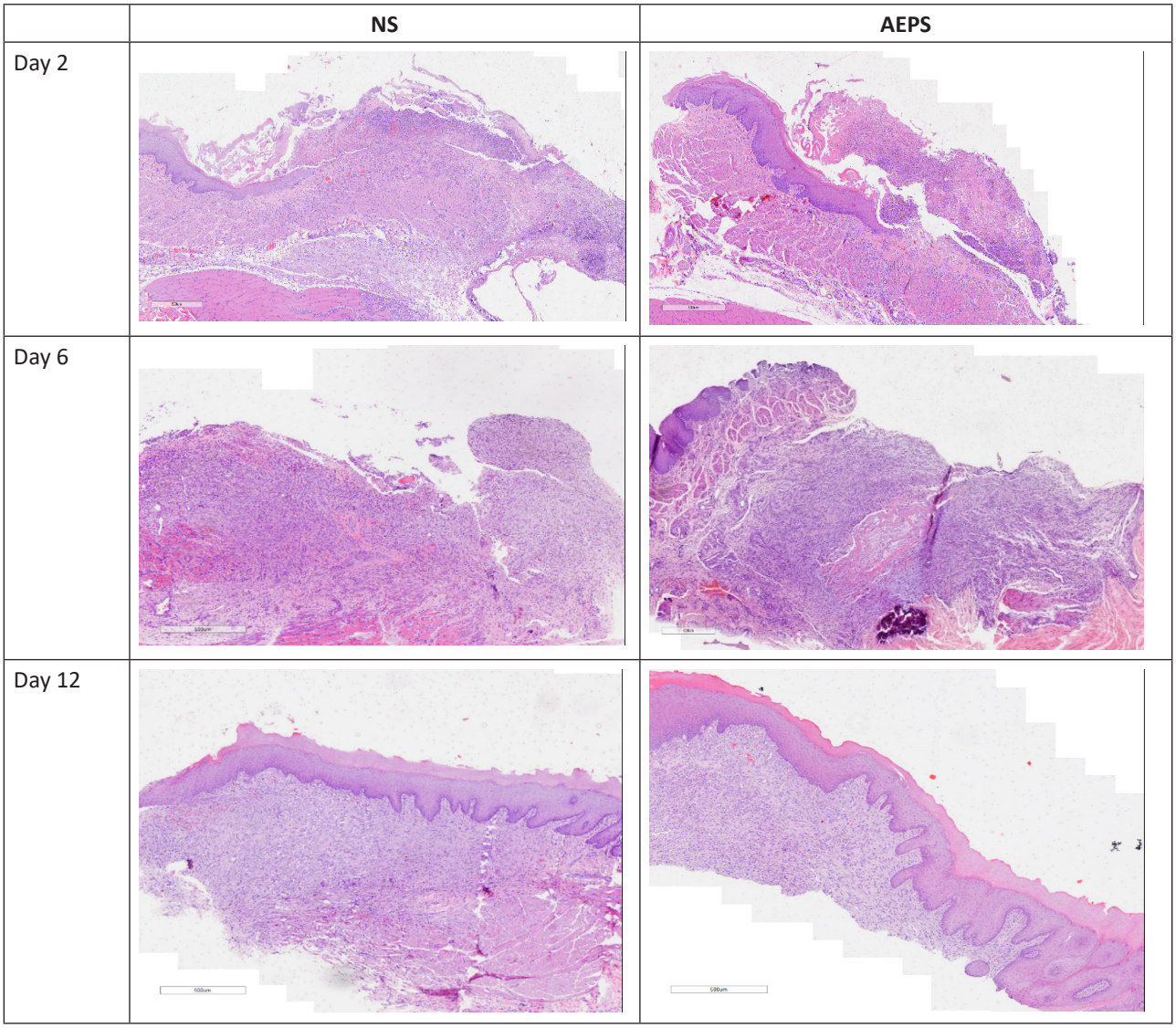

Figure 1: Comparison of histological sections of normal saline treated and aqueous extract of Piper sarmentosum treated tissue section (4x magnification). Day 2 showed ulcerated region on the buccal mucosa in which ulcerated region in AEPS treated section had completely detached from the connective tissue. Neutrophils were seen mosty on the detached region in AEPS section compared to normal saline section, in which neutrophils were abundant in ulcerated tissue. On day 6, full epithelisation had not occured yet and inflammatory infiltration was comparable between the two treatments. Full epithelisation occured on day 12 for both tissue sections with further reduction of inflammatory infiltration.
Abbreviations: NS, Normal saline; AEPS, Aqeuous extract Piper sarmentosum

Figure 2 shows the scoring for inflammation as compared to the type of treatments given to the rats on day 2, day 6, and day 12. As depicted in the figure, on day 12, the inflammation score gradually reduced. On days 2, 6 and 12, the inflammation score for AEPS treated rats was lower than the NS treated rats. As observed in Table 2, AEPS treated rats on day 2 showed a significantly lower inflammation score (2.8 ± 0.2) than the NS treated rats (3.8 ± 0.2). Interestingly, the inflammation score of AEPS treated rats on day 2 was lower than the score of NS treated rats on day 6. However, the inflammation scoring difference was not statistically significant. On the other hand, for both types of treatments, the inflammation score difference for day 2 and day 12 was significant ($p < 0.01$). Day 6 and day 12 also showed a decrease in the inflammatory scoring for AEPS treated tissue; however, the change was not statistically significant.

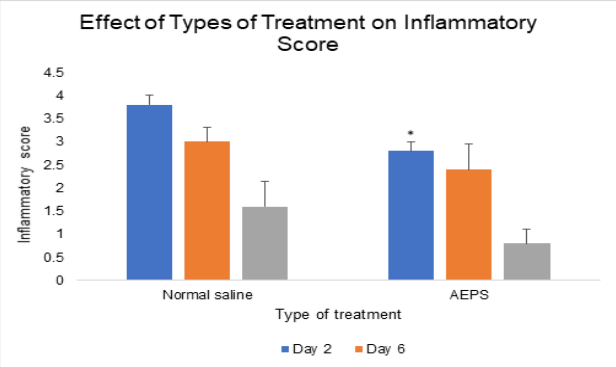

Figure 2: Inflammation score on normal saline (n = 18) and AEPS (n = 18) treated tissue sections according to days. Sections treated with AEPS showed a statistically reduction in inflammatory score on Day 2 compared to normal saline treated sections.* $p < 0.05$ in Mann-Whitney test.

Table 2: Inflammation score on normal saline (n = 18) and AEPS (n = 18) treated tissue sections according to days. Result shown as mean ± SEM. Sections treated with AEPS showed a reduction in inflammatory score compared to normal saline treated sections,especially on Day 2 and Day 12. * $p < 0.05$ in Mann-Whitney test. ** $p < 0.01$ in Mann-Whitney test.

Inflammatory score (mean ± SEM)		
Post-ulcer induction	**Normal saline**	**Aqeuous extract of *Piper sarmentosum***
Day 2	3.8 ± 0.2	2.8 ± 0.2*
Day 6	3.0 ± 0.3	2.4 ± 0.6
Day 12	1.6 ± 0.6**	0.8 ± 0.3**

Meanwhile, there were other changes observed in tissue sections. One of these was the dilation of blood vessels particularly on day 2 of the treatment, as shown in Figure 3. Dilated blood vessels were usually seen near the bed of ulcer wound. The next one was observed on day 6 in which many spindle cells were seen. However, the difference in NS sections was that the spindle cells were oriented in many directions, whereas on the AEPS treated sections, they were more abundant and most of them were oriented in a single direction. Moreover, on day 6 many small blood vessels were seen on the AEPS treated sections, especially beneath the basement membrane and some of them were dilated. On day 12, small blood vessels were seen below the basement membrane for both groups. The spindle cells were less seen on day 12 compared to day 6 for both groups. Collagen deposition seemed to be more abundant in AEPS treated tissue sections, which was stained pink beneath the epithelium.

Discussion

In the current study, the application of *Piper sarmentosum* aqueous extract on buccal mucosa ulcers was effective in reducing inflammation. An aphthous ulcer typically heals in 14 days. Therefore, day 2, day 6, and day 12 were chosen as time-points to depict inflammations at early, middle, and the end phase of healing. Since ulcer heals in a unidirectional manner, the inflammation score is expected to be reduced from day 2 to day 12. In the first few days of an injury, an overwhelming concentration of neutrophils was observed. However, AEPS treated sections showed a lower number of neutrophils which led to a better clearance of debris. This result proved that a high number of neutrophils was not vital for debris clearing, but an adequate amount was sufficient for that purpose. Furthermore, *Piper sarmentosum* has been known to possess antimicrobial activity (24-26), thus it is possible that this property may also have contributed for faster clearing of bacterial and tissue debris where it could enhance the process of inflammation activities. To further elucidate that neutrophils may play some roles in wound healing, a study using neutropenic mice revealed that wound closure was independent on neutrophil level, in which full re-epithelisation occurred in just 5 days compared to non-neutropenic mice which took a longer time (27). Hence the recent data from the current study showed that even though AEPS treated sections caused lesser neutrophils aggregation, it did not affect the overall process of wound closure.

Acute inflammation causes neutrophils accumulation at the site of injury. An abundance of neutrophils requires oxygen to carry their function for oxidative burst (28). This was evident in the current study in which oxygen was supplied to the hypoxic tissue through the formation of new blood vessels as observed in Figure 3. Moreover, in the process of oxidative burst, a superoxide molecule is produced after a reaction between NADPH oxidase and oxygen molecule. The superoxide molecule is one of the ROS that should be eliminated as it will further react to cause hydrogen peroxide generation. Hydrogen peroxide is one of the stimulus for angiogenesis (29) which is helpful in hypoxic condition due to abundancy of immune cells.

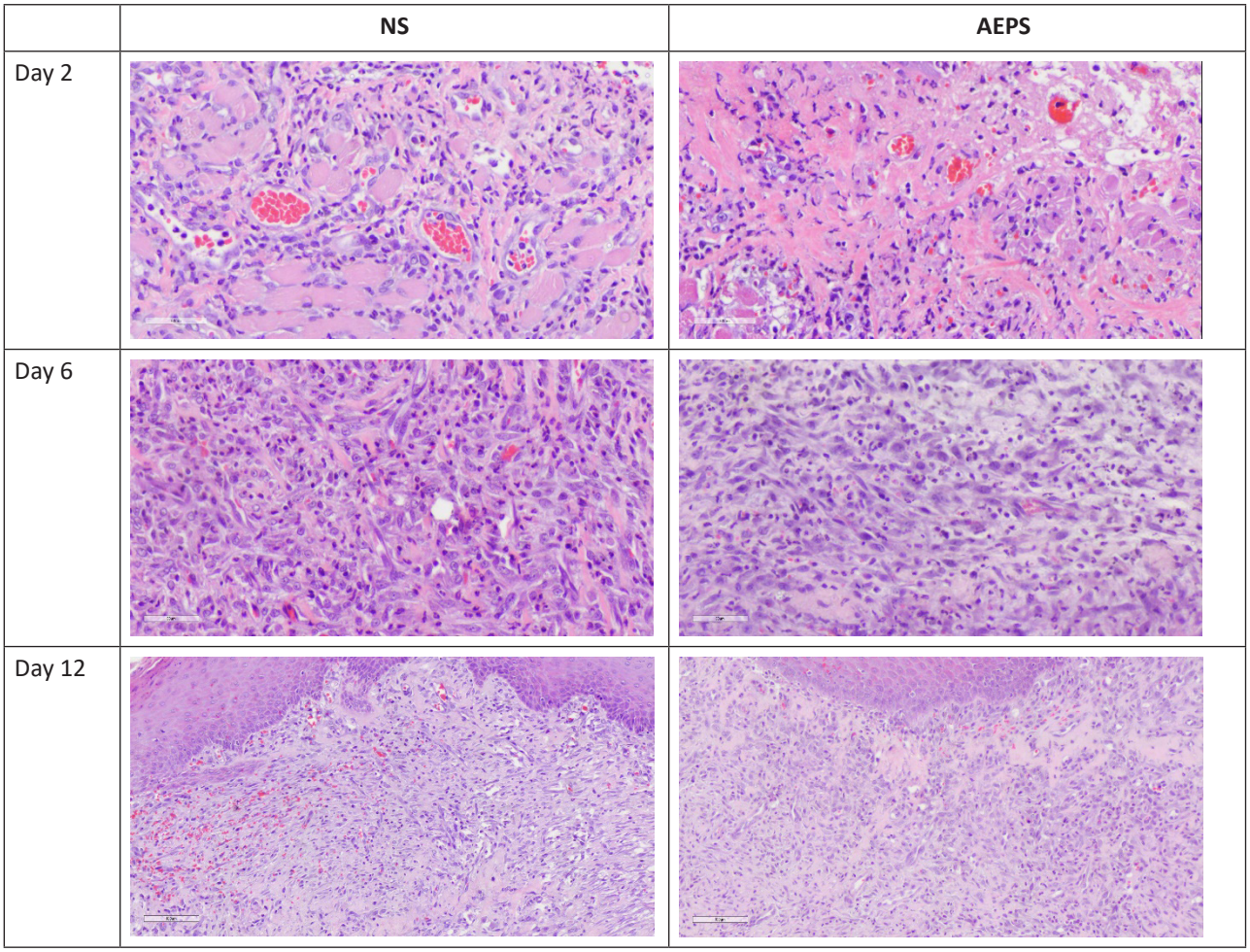

Figure 3: Observations of blood vessel and spindle cells on treated tissue sections. Day 2 tissue sections are magnified at 40x, Day 6 magnified at 20x while Day 12 magnified at 10x. Day 2 tissue sections showed dilated blood vessels (as shown by arrows). Normal saline sections showed some neutrophils inside a few of blood vessels. Day 6 showed different orientation of spindle cells observed. Day 12 showed differences in the presence blood vessels and spindle fibre.
Abbreviations: NS, Normal saline; AEPS, Aqeuous extract *Piper sarmentosum*

However, since this molecule can also initiate a chain reaction of lipid peroxidation, its production must be reduced and maintained.

Having the knowledge of the metabolites presence in plants is important as it may provide us with the first clue on the mechanism of action of the active metabolite. In the previous study, the main phytochemicals of *Piper sarmentosum* plant were found to be phenolic compound particularly flavonoids. By using the separation principle of high performance liquid chromatography (HPLC), isolation of AEPS compounds were found to be rich in vitexin and rutin as major flavonoids (30). Moreover, in a study using chemical based-assay oxygen radical absorbance capacity (ORAC), Jie Kang and colleagues found that vitexin showed the best antioxidant capacity among several other flavonoids (31). Other than that, few antioxidant assays proved that rutin showed promising antioxidant capacity (32). Although it was possible that these phytochemicals, either singly or synergistically, were responsible for the better inflammatory phase of AEPS treated ulcer healing, other phytochemicals that have yet to be elucidated could also contribute to the positive effect of the extract. Moreover, this plant contains other antioxidant compounds including carotenes, vitamin C, vitamin E, tannins and phenolics causing the plant to be one of those with the highest antioxidant activity (18).

Oxidative stress causes an imbalance level of prooxidant to antioxidant. In oral aphthous, the oxidant level does not only change systemically (13) but also locally (12). Hence topical medication would be of equal importance to prevent a delayed healing which would ultimately bring discomfort to the patients. As evident in the previous study, topical application of ethanolic extract of mauli banana stem onto oral ulcer showed antioxidant property by modulation of the SOD enzyme reduced MDA level (12).

A toxicity study showed AEPS did not cause any significant histological changes to liver and kidneys when the extract was given orally (33). Furthermore, LD_{50} for AEPS was found to be more than 10 g/kg (34). Hence, we postulated that

topical AEPS application may not cause any toxicity since the extract used was in a small amount and no death of rats was observed during the experiment.

Phytochemicals are not only potent anti-oxidants, but they have also been investigated as anti-inflammatory agents. Flavonoids are postulated to induce its anti-inflammatory activities using the same mechanism as non-steroidal anti-inflammatory drugs (NSAIDs). The drugs in NSAIDs class inhibited COX-1 and COX-2 activity which ultimately relieved inflammation (35). *Piper sarmentosum* was proven to provide a systemic anti-inflammatory effect in a carrageenan-induced paw edema test. The previous study demonstrated that AEPS was able to reduce the volume of paw edema in a dose dependent manner (17). In another study, supplementation of vitexin on activated neutrophils reduced TNF-α concentration as well as myeloperoxidase activity. These two parameters were inflammatory associated properties (36). The anti-inflammatory effect seen in this current study showed that AEPS could also give effect when applied locally, in terms of reducing neutrophil aggregation to the ulcer site.

Additionally, *Piper sarmentosum* extract exhibited a potent anti-inflammatory activity as indicated by the reduction of nitric oxide production. Nitric oxide is one of the inflammation markers. In this study, this extract inhibited nitric oxide concentration in a dose-dependent manner (37). Moreover, *Piper sarmentosum* was found to be among the Malaysian medicinal plants contributing to a moderate anti-inflammatory activity, with more than 50% nitric oxide inhibition (38).

An ulcer involves the sloughing off of epithelial layer, leaving an exposed lamina propria. Full epithelisation was observed on Day 12, however the connective tissue had not been fully restored. While normal rat's buccal mucosa showed a thin layer of lamina propria beneath the epithelium, ulcerated mucosa showed hyperplasia. A finding from the previous study showed that AEPS might have a minor effect on the proliferation of fibroblast. This observation is in concordance to an *in-vitro* study that showed AEPS leaves had little effect on fibroblast proliferation, although it showed a dose-dependent effect on the cells' growth (28). The observation also illustrated that connective tissue restoration was independent of re-epithelisation and full re-epithelisation did not necessarily indicate the tissue was completely healed, as the connective tissue took a longer duration to be restored to its original state.

Conclusion

The current study demonstrated that aqueous extract of *Piper sarmentosum* leaves enabled the reduction of severe inflammation on buccal ulcer. Topical application of AEPS showed a better inflammation score with lower neutrophils, suggesting that the ulcer would not progress to a chronic wound. The inflammation score for both treatments were reduced in time but AEPS treated sections showed a statistically significant difference compared to NS treated sections and was distinctly observed on day 2. The antioxidant activity of AEPS could also contribute to a better inflammation score and consequently reducing neutrophils number.

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Competing interests

The authors declare that they have no competing interests.

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Ethical clearance

This animal works in this study was carried out with the approval of Institutional Animal Care and Use Committee from Universiti Sains Malaysia (USM/Animal Ethics Approval/2016/ (99) (733)).

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