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HEALING EFFICACY OF METHANOLIC EXTRACT OF PILIOSTIGMA THONNINGII LEAVES IN WOUND

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Abstract: *Philostigma thonningii* (Schum.) is used in many parts of West Africa including Kebbi State, Nigeria for the management of wounds, chronic ulcers, gastric heart pain, and headache. It has been estimated that nearly 6 million people suffer from chronic wounds worldwide. This study aimed to investigate the wound healing activity of *Philostigma thonningii* leaves. The air-dried powdered leaves were extracted by maceration in methanol to obtain a crude methanolic extract (CME). The CME was screened phytochemically, then prepared into 2.5, 5 and 10% CME ointment by fusion before subjecting to wound healing assay using the wound excision model in Wister albino rats. The results showed a non-dependent dose healing effect owing to CME ointment since wound contraction at 2% and 5% CME treatment yielded higher percent contraction compared to that of 10% maximum dose administered. Comparatively, the treatment groups with CME showed better wound closure and re-epithelization of tissue repair and fibroblast in the granulation tissue formation than those in the untreated group of the negative control (in Group I, DW). However, the animals treated with 5% povidone iodine, PI (in Group II, Standard), showed a better wound contraction and arrangement of granulated tissue repair than those of both the CME treated group and the DW groups in the experiment. Consequently, there was no significant difference ($p > 0.05$) observed between wounds treated with CME (2.5, 5 and 10) % of *P. thonningii* leaves, except the standard treatment group with 5% PI, which showed significantly ($p<0.05$) wound healing activity. Conclusively, the observed wound healing activity of *P. thonningii* leaves may be attributed to a single or a synergistic effect of phytochemical constituents. The findings have also justified the traditional medicinal uses of *P. thonningii* leaves for the management of wounds.

Keywords: Contraction, Excision, Povidone-iodine, Re-epithelization, Wound healing

INTRODUCTION

In many developing countries like Nigeria, plant products play important roles in the treatment of wounds. A wound is a type of injury that happens relatively quickly in which skin is torn, cut, or punctured (an open wound), or where blunt force trauma causes a contusion (a closed wound). In pathology, it specifically refers to a sharp injury that damages the epidermis of the skin (Wikipedia). It has been estimated that nearly 6 million people suffer from chronic wounds worldwide (Branski *et al*., 2009). Wound healing which occurs in four phases - hemostasis, inflammation, proliferation, and remodeling (Schultze *et al*., 2011) is a complex and dynamic process with the

wound environment changing with the shifting health status of an individual (Guo and DiPietro 2010; Mercandetti and Cohen, 2021). Unhealed wounds constantly produce inflammatory mediators that produce pain and swelling at the wound site. Chronic wounds may even lead to multiple organ failures or the death of the patients (Järbrink *et al.*, 2016).

Plants have been shown to have potential for the management and treatment of various ailments including wounds. Crude extracts from different plants have been used to treat skin infections such as sores, bites, burns and lacerations (Chelsea Green, 2022). According to the world health organization (WHO, 2002) approximately 80% of the worlds' population depends on traditional medicine and a significant population in Africa still depend on herbal medicine for their health care needs (RBG Willis, 2017).

The presence of various bioactive constituents in plants has urged scientists to examine plants with potential wound healing properties (Akinpelu *et al.,* 2000). Besides being cheap, effective and affordable, these natural agents induce healing and regeneration of lost tissue by multiple mechanisms (FongnzossieFedoung *et al*., 2021; Sofowora, 1993).

Philostigma. thonningii (Schum.) belongs to the family fabaceae and it is also called Camel's foot tree, monkey bread, monkey biscuit tree, *Kalgo* (Hausa), *Rhodesian bauhinia*and wild bauhinia. The plant is commonly found in open woodland and wooded grasslands of sub-humid Africa at medium to low altitudes. Morphologically, its leaves petiole is 2-4cm long, blade mostly 5-15cm long and 6-16cm wide, bilobed apically about one-eighth to one-third of the length of the leaf, densely reticulate and rustypuberulous or pubescent beneath; each lobe with 5 or 6 main veins, and stipules of 3-6mm long (Dayamba, 2014). The stem bark is rough and longitudinally fissured, being creamy-brown when fresh and grey-brown later. Ethnobotanical information revealed that *P. thonningii*is widely used locally around the world. The leaves are used as food in the Benna-Tsemay and Hamar Districts of the South Omo Zone (Hailemariam *et al*., 2021; Paulos *et al*., 2016) and also consumed by animals as fodder (Tadesse *et al.* 2012, Amente, 2017, Tebkew *et al.,* 2018 and Ayenew 2019). The leaves are eaten and chewed by the Masai people to relieve thirst (Jemiseye *et al.,* 2019). The wood is suitable for poles, firewood, charcoal, carpentry and construction, to while the bark is used for making strings and ropes (Orwa *et al.,* 2009; Chidumayo, 2018). Macerated extract of *P. thonningii* is used by the Ethiopians to treat various diseases, such as fever, toothaches, wound healing, dysentery, cough, chest complaints, snake bites, skin infections, and stomachache (Jimoh and Oladiji, 2005; Orwa *et al.,* 2009). In Tanzania, the tender leaves are chewed and the juice swallowed to treat stomachache, coughs, and snakebite. The root is used to treat prolonged menstruation, hemorrhage and miscarriage in women and also for the treatment of cold (Ruffo *et al.,* 2002). A powder can be made from the dry pods for making nutritious porridge. Unripe pods can be used as a substitute for soap. Dry pods are roasted and ground into powder and mixed with tobacco powder and ashes of the red-leaf *Amaranthus* to make cooking soda (Coates Palgrave, 2002). Though several conventional drugs are known to promote wound healing, these drugs are however expensive with limited availability especially in rural communities (Ukwuani-kwaja *et al.,* 2019). This study was designed to investigate the wound healing potential of *P. thonningii* leaves using wistar rats.

MATERIALS AND METHODS Chemicals and Reagents

All chemical/reagents and solvents used in this study were analytical grade which included methanol, distilled water, povidone iodine ointment (5% w/w), white soft-paraffin, Lidocaine Hydrochloride Injection BP (1% w/v), methylated spirit, mayer's and dragend off's reagent, iron (III) chloride, Cons. H_2SO_4, Ammonia, acetic anhydride, ferric chloride solution, glacial acetic, lead acetate, amino acid solution, sodium hydroxide.

Plant Collection and Extraction

The leaves of *P. thoningii* were collected from Wammakko Local Government Area of Sokoto State, Nigeria during the dry season on April 2, 2021 with the help of a traditional healer. Taxonomic identification and

authentication were confirmed by Magaji of the Herbarium unit of the Department of Pharmacognosy and Ethnopharmacy, Usmanu Danfodiyo University, Sokoto. A voucher specimen of the plant sample was prepared and then compared for identification with the previously prepared specimen with number PCG/UDUS/Faba/0018. The collected barks were air dried for two weeks and later pulverized using mortar and pestle. Three hundred grams of powdered samplewas macerated in1.5 L ofabsolutemethanol for 48hrswith agitation after24 h. This was followed by filtration and the filtrate obtained was concentrated in an oven at 40 °C for 72 h to obtain a dark green residue. This was labeled as CME and stored at 4 °C for wound healing evaluation.

Qualitative Phytochemical Test

A wet phytochemical test was carried out on CME according to standard methods as outlined by Harborne (1983, 1992).

Preparation of *P. thonningii* CME-based Ointment The CME of *P. thonningii*leaves were mixed with soft melted translucent paraffin to obtain graded concentrations of 2.5%, 5%, and 10% (w/w) prepared in line with BP (British Pharmacopoeia, 2009).Briefly, 2.5g, 5g and 10 g of CME were incorporated into 97.5, 95, and 90 g portions of white soft-paraffin then melted over a water bath with constant stirring until mixture became homogeneous.The mixture was then stirred and allowed to cool and finally transferred to a clean container for topical application.

Experimental Animals

Ethical approval was sought and approved with reference PTAC/ES/CAF/OT/44-22 by the Health Research Ethics Committee (HREC) of the Usmanu Danfodiyo University, Sokoto. Healthy Wister albino rats of both sexes weighing between 110-175 g were obtained from the Animal Research Centre (ARC) of the Ahmadu Bello University (ABU) Zaria. The rats were housed under standard laboratory conditions and allowed to acclimatize for two weeks before commencement of the study. All animals were treated in accordance with the "Principle of Laboratory Animal Care'' (NIH publication no.85-23, revised1985)'.

Acute Dermal Toxicity Sudies

Following OECD guidelines no. 402, the acute dermal toxicity of the formulated CME ointment-base was performed by applying the ointment with the highest concentration of 10% (w/w) on the shaved back of the rat,

Experimental Design

The rats were grouped into five (I-V) groups of five animals per group as follows: Group I -Wounded rats without treatment (Negative Control); Group II - Wounded rats, treated with 5% w/w povidone-iodine ointment (Standard Control); Group III -Test group treated with 2.5% w/w *P. thonningii* CMEointment-base;Group IV - Test group treated with 5% w/w *P. thonningii* CME ointment-base; Group V- Test group treated with 10% w/w *P. thonningii* CME ointment-base

Excision Wound Model Assay

The method of Sumitra and Nidhi (2013) was adopted with slight modifications. The rats were anesthetized by administering 2% lidocaine (4 mg/kg S.C). The back of all the rats were shaved using a clipper and the exposed skin was scrubbed with methylated spirit. One circular full thickness wound of 1.5cm diameter was created at the dorsal area using a sterile sharp circular rod (mimicking biopsy punch) and the excised skin was removed with surgical scissors. Hemostasis was achieved by staining the wound with cotton wool soaked in normal saline. This was considered day zero. Thus, beginning from the first day, the rats were treated daily with the graded doses as described. Images of the wounds were captured and wound area was measured at day 0 and on days 5, 10, and 15 post wounding. The rate of healing was calculated using the relationship:

$$\% \text{ Wound Contraction} = \frac{woundareaonday0 - woundareaondaynx\ 100\%}{Woundareaonday\ 0}$$

Where n = days wound area measurement was taken.

Histological Examination

The wound specimens on 16thday including full-thickness skin layers were fixed in 4% buffered para formaldehyde and processed for light microscopy. Briefly, processed tissues were embedded in paraffin, 4µm tissue sections were stained with hematoxylin and eosin (HE) and Masson's trichrome staining kit. HE and Masson's trichrome sections were observed and photographed under an inverted microscope (H & E stain x 400).

Statistical Analysis

'IBM SPSS Statistics 26' was used for data analysis. Data were expressed as mean ± standard deviation of the mean while comparisons between groups were performed by one-way analysis of variance (ANOVA) with a posthoc test for significant differences at $p < 0.05$.

Results Phytochemical Test

The presence of saponins, tannins, cardiac glycosides, anthraquinones, alkaloids, steroids and triterpenoids were present while flavonoid was not detected in the CME of *P. thonningii* leaves as shown in Table 1.

Wound Healing Appearance

The result of the study showed that the CME of *P. thonningii* leaves (PT) significantly enhanced the healing process. There was also a moist healing environment maintained without infection in spite of the wound exposure

Table1. Preliminary phytochemical screening of CME leaves of *P. thonningii*

SECONDARY METABOLITE	QUALITATIVE TEST	OBSERVATION	INFERENCE
Saponins	Froth test	Formation of Froth 1cm height	++
Flavonoid	Shinoda's	Orange Color	-
Tannins	Lead acetate	Milky ppt.	++
	Ferric Chloride	Blue-black ppt	+
Phenols	Ferric chloride	Blue- black ppt	++
Anthraquinon	Bontrager's test	Rose Pink	+
^{es}Glycosides	Fehling's test	Brick-red ppt.	+
Triterpenoids	Lieberman Burchard	Reddish violet	+
	Salkowski's test	Reddish-brown	+
Alkaloids	Dragendorff's	Reddish ppt.	+
Steroids	Lieberman Burchard	Yellowish green fluorescence	+

+ = present in trace amount; ++ = present in moderate amount = Not detected

to the atmosphere. However, some of rats in the various groups treated with the graded doses of CME for days 5-7 developed a thick hard scab with fibrous tissue formed at the wound site beneath the scab (Plate 1). After the scab fell off, the fibrous tissue was resorbed and the healing process progressed effectively.

Rate of Wound Closure

There was a progressive improvement in the percentage of wound contraction in the treated excised wounds compared to the untreated control group (DW) throughout the study period (Plate I, Table 1 and Figure 1). On the 15th day post-wounding, a visible increase in the rate of wound contraction was observed in animals treated with the graded test doses of 2.5,5, and 10% CME of PT, which is equal to an average percentage wound contraction of 84, 84and 76% respectively, while less healing rate was observed in the negative control group with an average percentage contraction of 33.35%.

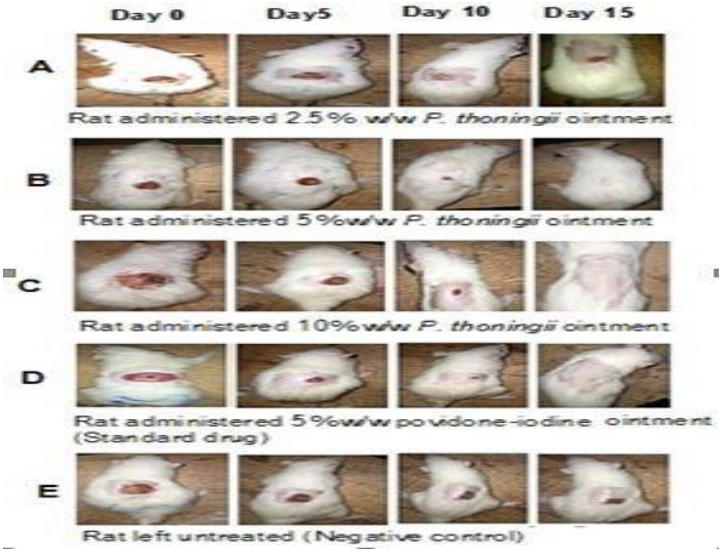

Plate 1. Selected images of wound healing pattern (wound contraction) from each group

The standard control group (5% povidone-iodine, PI) however, gave a higher wound contraction (90%) than those of all the treatment groups with CME of PT, having 76-84% wound closure (Table 2). Consequently, no significant difference ($p > 0.05$) was observed between wounds treated with 2.5, 5 and 10% CME of *P. thonningi* leaves. The standard treatment group of 5% povidoneiodine was the only significant value ($p<0.05$); however, there seems to be a more healing property exhibited by the least treatment concentration of 2.5% CME as oppose to higher doses, as shown in Figure 1. For instance, on day 10 the least dose of 2.5% gave a higher healing contraction of 48% while contractions recorded for 5% and 10% were 42.7% and 46.7%, respectively.

Table 2. Effect of CME of *P. thiningii* on excision wounds in rats

TREATMENT	WOUND CONTRACTION (%) DAY 5	DAY 10	DAY 15
Group I	6.7±7.7*	20.0±7.7*	33.4±15.4*
Group II	36.7±11.5	66.7±15.4	90.0±11.5
Group II	22.7±10.1^{+}	48.0±12.8^{+}	84.0±10.1^{+}
Group III	24.0±13.8^{+}	42.7±22.9^{+}	84.0±10.1^{+}
Group IV	10.7±7.6*	46.7±27.1^{+}	76.0±43.4^{+}

Values are mean ± SD of 5 replicates. Values with $^{+}$ and *superscripts are significant and non-significant when compared with Groups II (the standard control) respectively.

Group I - Wounded rats without treatment (Negative Control); Group II - Wounded rats, treated with 5% w/w povidone-iodine ointment (Standard Control); Group III Test group treated with 2.5% w/w *P. thonningii* CME ointment-base; Group IV - Test group treated with 5% w/w *P. thonningii* CME ointment-base; Group V- Test group treated with 10% w/w *P. thonningii* CME ointment-base.

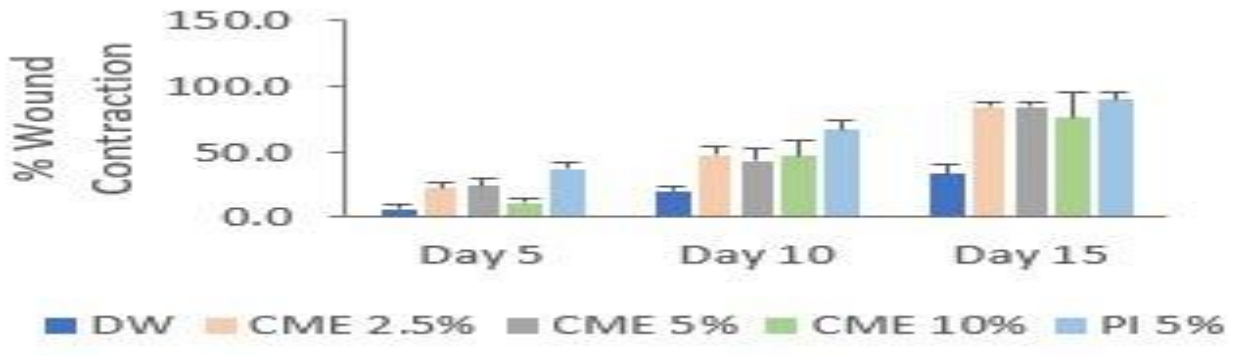

Figure 1: Wound healing rate of CME of *P. thonningii* leaves

Histological Study

Hematoxilyn and eosin sections showed pattern of tissue repair owing to the effect of the test samples of CME (Plate 2.). To further investigate the effects of CME of *P. thonningii* leaves on wound healing, angiogenesis and epithelialization were tested using HE staining and Masson's trichrome staining. Plate II shows the wounds in the control groups and the CME treated groups at the 16th day post-wounding. The DW group revealed distorted and thin keratin layers, stratified squamous layer thick in diameter with hyperplasia as well as hyperplastic dermis showing fibroblasts, inflammatory cells and scanty glands. By comparison, the group treated with CME showed thin.

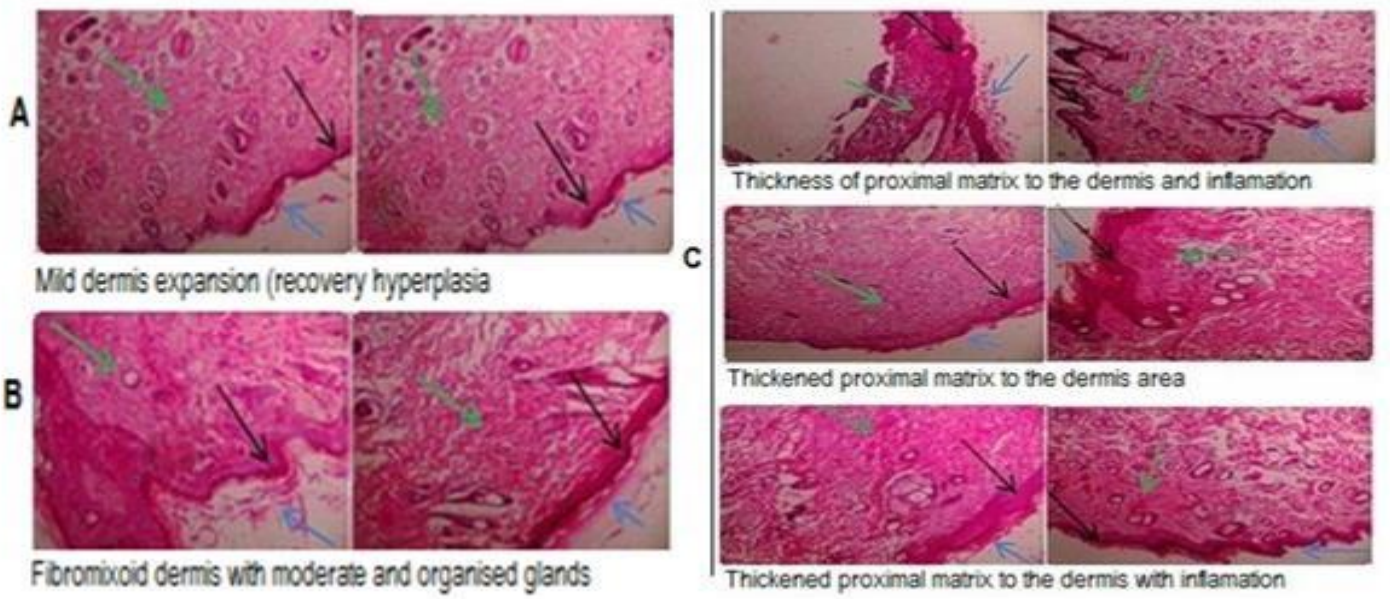

Plate 2. H&E-stained microscopy of rat skin sections of tissues from the healed area of the wounds (15th day post wounding) from the different groups, treated with different CME concentrations at x400 magnification (Blue arrow → keratinized layer; Black arrow →stratified squamous layer of epidermis; Green arrow →dermis layer) A-Group I: Negative Control, DW (distilled water treatment): Shows distorted and thin keratin layers, stratified squamous layer is thick in diameter, with hyperplasia and dermis is also hyperplastic, showing fibroblasts, inflammatory cells and scanty glands. B-Group II: Standard Control, PI (5% Povidone Iodine): Shows thin keratin layer, thin epidermis stratified squamous layer, hyperplastic fibromixoid dermis with moderate and organized glands. C-Group II, VI, and V: Test Concentrations of CME (2.5, 5, &10) %: Shows thin keratin layers,

moderately hyperplastic stratified squamous layer and hyperplastic dermis layer with glands, fibroblasts and inflammatory cells. All are noted repair pattern with best result at the 10%

keratin layers, moderately hyperplastic stratified squamous layer and hyperplastic dermis layer with glands, fibroblasts and inflammatory cells, which were noted as repair pattern in the skin injury on experimental rats and best result observed at 10% concentration. However, the standard drug control group (5% povidone iodine) invariably showed a better arrangement of granulated tissue, displaying a thin keratin layer, thin epidermis stratified squamous layer, hyperplastic fibromixoid dermis with moderate and organized glands. Thus, the results showed that the skin microstructure of the groups treated with 2.5, 5 and 10%CME compared favourably with the normal skin as observed in the PI control group concentration

DISCUSSION

It has been noted that plants have immense potentials for the management and treatment of wounds (Thakur *et al*., 2011). Wound healing has been shown to be a complex physiological process, consisting of outcome that comprises interaction of a variety of repair cells (epidermal cells, fibroblasts, endothelial cells, etc.), cytokines epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor (GMCSF) and extracellular matrix (Martin and Nunan, 2015).Tissue regeneration is mostly sustained by multiple mechanisms involving constituents of the plants that induce healing (Nayak and Pereira, 2006; Akinmoladun *et al*., 2007).These constituents include various chemical families like alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, and phenolic compounds (Edeoga *et al*., 2005). Ukwuani-Kwaja *et al.,* (2019), similarly detected the presence of saponins, tannins, alkaloids, anthraquinones, steroids and glycosides in the methanolic extract of *P. thonningii* leaves. The presence of these phytoconstituents which are largely phenolics as shown in this study,have been demonstrated to promote wound healing via various cellular-protein binding biochemical interactions (Dharmananda, 2003; Sanjay *et al*., 2018; Selvi *et al*., 2011; Scalbert 1991; Maikai and Kobo,2009).

Findings in this study suggest that the wound healing activity of *P. thonningii* is not dose dependent. The observed effects of 2.5 and 5% CME treatments were comparable but more effective in terms of wound closure and rate of general healing process than the 10% CME by day 15 post wounding. The mechanism is not well understood but presence of other antagonistic factors in the extract such as sugars may impact negatively on the wound healing process hence the lowest administered concentration of 2.5% CME was observed to offer a remarkable effect.

Histopathological observations on tissue sections of excised wounds agrees with earlier findings indicating three interrelated stages in wound healing process: inflammation, proliferation and remodeling (Peng *et al*., 2012). Thus, during the whole healing process, granulation tissue filling is a key step in wound healing (Zaccaria, 1996). Fibroblasts are the most important functional cells in granulation tissue formation (Diegelmann *et al*., 2003). More so, Li and Wang (2011) also established that during wound healing, fibroblasts migrate, proliferate and secrete a large number of collagen fibers and matrix components, which form granulation tissue together with new capillaries. Furthermore, Feng *et al*. (2014) noted that fibroblast also fills the tissue defect and create conditions for the coverage of epidermis. Our findings support earlier claims (Ukwuani-Kwaja *et al.,* 2019) that *P. thonningii* leaves CME based ointment promotes wound healing.

CONCLUSION

The methanolic crude extract (CME) of *P. thonningii* leaves exhibited wound healing effect at three different test concentrations of 2.5, 5 and 10%. Findings in this study showed significant wound healing activity at lower concentrations (2.5 and 5%) of CME., However, the wound healing process observed for the standard drug control group (5% povidone iodine) performed better when compared to the plant extract. Further purification of *P.*

thonningii constituents bioactive as wound healing agents is recommended for improved activity and as newer leads for wound healing drug formulations.

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