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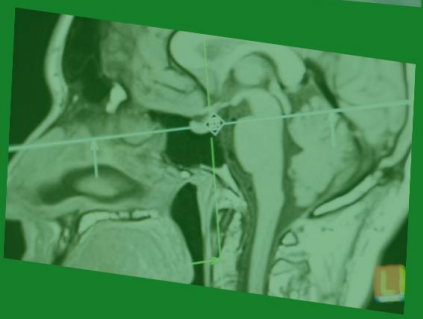

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Wound healing activity of *Muntingiacalabura* (Aratiles) ethanolic extract on Sprague Dawley rats

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Abstract

Wound healing is the cellular and biochemical reactions that restore an injured tissue's damaged structures and affected functions. Flavonoids, saponins, tannins are phytochemicals that have favorable effects on wound healing. These phytochemicals are mainly found in *Muntingiacalabura* leaves. This study determined the wound healing effect of the ethanolic extract of *M. calabura* leaves in varying concentrations applied topically using an excision wound model in Sprague Dawley rats. Estimation of hydroxyproline levels was used to indicate collagen synthesis; histopathological study and measurement of wound contraction also supported the property of the extracts in wound healing process. Results revealed that both concentrations hastened wound contraction. Concentration of hydroxyproline in 10% *M. calabura*ethanolic extract is higher than the 5% *M. calabura*ethanolic extract. Histpathoological study revealed that in 5% *M. calabura* a skin tissue showed the beginning of re-epithelialization, but still with ulceration while the 10% *M. calabura* reveals a skin tissue with area of healed ulceration and a thin layer of re-epithelialization. The wound contraction measurement also showed that 5% *M. calabura* was healed on the14th day while in 10% *M. calabura* the wound was healed on the13th day. There were significant differences between the test groups compared to the negative control (p-value <0.05). The optimum concentration of the extract that facilitated wound healing is 5% *M. calabura*ethanolic extract based on the parameters used. The study revealed that ethanolic extract from *M. calabura*leaves can facilitate wound healing activity with the same efficacy when compared to standard treatment.

Keywords*: Muntingiacalabura, wound healing, excision wound model, hydroxyproline*

INTRODUCTION

Skin is known to serve as a protective barrier against the environment. Wounds can cause an opening or breaking of the skin and healing after damage in the skin is needed to restore the structure and function of the injured tissue (Agarwal, Singh, Gaurav, Goel, & Khanna, 2009). Thakur, Jain, Pathak, and Sandhu (2011) described wound healing as cellular and biochemical reactions that restore an injured tissue's damaged structures and affected functions. This involves cell to cell and cell to matrix interactions. Inflammation, proliferation, and remodeling are the three stages of wound healing. Nowadays, herbal medicines have been socially-accepted similarly to those chemically manufactured drugs with their specialized functions, this is because plants have been used before by different tribal and folk groups in many countries for wound and burn treatments. Their potential on such diseases lies in the phytochemical constituents, such as lkaloids, flavonoids, saponins, tannins, terpenoids and phenolic compounds that allow them to induce physiological reactions in the human body (Thakur et al.,2011). *Muntingiacalabura* belongs to the family Elaeocarpaceae, which is a common roadside tree in Malaysia (Janick&Paull, 2008). Filipinos generally call it "aratiles" which is also found in most house backyards. However, it has dozens of native names since it is widely cultivated in warm areas of the Asian region, including cay trungin Vietnam; buahcheriah and kerukupsiamsim in Malaysia. *M. calabura* is a small evergreen, fast growing tree. The tree itself measures 7-12 m tall with tiered and slightly drooping branches. It has serrated leaves measuring 2.5-15 cm long and 1-6.5 cm wide while the flowers are small, white and slightly malodorous. It gives rise to 1.0 to 1.5 cm light red rounded fruit that is smooth, fleshy and sweet. It is the plant of choice since its various parts were documented for their medicinal use.

Its leaves, barks and flowers are believed to possess medicinal value and are rich in flavonoids, flavones and flavanones, rendering to its potent antitumor activities(Chen, Lee, Duh, & Chen, 2005). Scientifically, this plant has been proven with antinociceptive, anti-inflammatory and anti-pyretic properties (Zakaria et al., 2007), potential antimicrobial activity (Zakaria et al., 2010), potent antityrosinase, antioxidant activities and cardioprotective effect (Ibrahim, Abdulla, Abdelwahab, Al-bayaty, & Majid, 2012). It was proven to possess the antioxidant property (Gupta, Kumar, Pal, Banerjee, & Sawhney, 2005), due to its flavonoid content which

promotes wound healing process. Flavonoids help eliminate free radicals generated at the site of injury whichimpair the healing process by damaging cell membranes, nucleotide, proteins and lipids. In addition to this, other polyphenolic bioflavonoids such as tannins, proanthocyanidins, and cathecins have alsobeen reported by past researchers to possess wound healing potential (Gupta et al., 2005).

Figure 1. *Muntingia calabura* (Aratiles) leaves

There are no published data pertaining to the wound healing activity of *M. calabura* hence, this study aims to determine the wound healing potential ofthe ethanolic leaf extract of *M. calabura* on an in vivo technique, specifically it determined the phytochemical substances present in the plant leaves and compare the wound healing activity of 5% and 10% *M. calabura* ethanolic leaf extract that are suitable in treating the wound of rats. For further elaboration, assessment of wound healing was done by estimation of hydroxyproline, histopathological study and measurement of wound contraction. Moreover, this study aims to further utilize the medicinal value of the plant which has bioactive constituents that are of great significance in therapeutic treatment. If proven to be effective, it is of great help to those who prefer low cost at the same time, locally available alternative medicine that would aid in wound healing process.

MATERIALS AND METHODS

Collection and identification of plantspecimens

Leaves of *M. calabura* L. werecollected from Sampaga East, Batangas City and were authenticated at the Herbarium of the National Museum (Appendix A).

Preparation of ethanolic leaf extract

One hundred grams of the fresh anddry leaves were added to 1000 mL of ethanol and water. They were then left for48 hours (James & Friday, 2010). The resulting mixture was filtered using Whatman paper No.1 for three times and concentrated in a rotary evaporator at 50- 60°C (Barua et al., 2009).

Phytochemical analysisSaponins

The preliminary screening for the presence of saponins was based on a studyconducted by Nayak, Sandiford, andMaxwell (2007). It was done using froth test.The ethanolic leaf extract (300 mg) was boiled with 5 mL of water for 2 minutes. Themixture was stirred vigorously after it is cooled, and was left to stand for 3 minutes. The formation of frothing indicates thepresence of saponins.

Tannins

Presence of tannins in the ethanolic leaf extract was based on a study done by Kumar, Reddy, Rao and Nethaji (2014). In 10 ml distilled water, 0.25 g of the solvent extract was dissolved, then filtered. The intense green purple, blue or black colorindicated the presence of tannins after the filtrate has been added with 1% aqueous iron chloride ($FeCl_3$) solution.

Flavonoids

The test for flavonoids was based onthe study of Kumar et al. (2014). To remove the fatty material (lipid layer) from the 0.5 g of the extract, it was shaken with petroleum ether. Twenty milliliters of 80% ethanol was added to dissolve the defatted residue and then filtered. Formation of yellow color was observed when 4 ml of 1% aluminium chloride in methanol was added to the test tube containing the filtrate.

Ointment preparation

The ethanolic leaf extract (10g) was mixed with simple ointment base (petrolatum) of about 90g that served as theointment base to get 10% ointment. Theethanolic extract (5g) was mixed

with simpleointment base (petrolatum) of about 95g that served as the ointment base to get 5% ointment (Barua et al., 2009).

Preparation of animals

Male and female Sprague Dawley rats of similar ages, weighing 150-250grams were used for the study, which were authenticated by a veterinarian. They were housed at LPU animal house at controlled conditions with 25+/- 3 C, RH of 50 +/- 5% and were kept under 10/14h light/darkcycles with free access to food and water *ad libitum.* Animals were housed individually in polyprolene cages with sterile paddy husk bedding (Barua et al., 2009). The animals were divided into five groups with three rats each (James & Friday, 2010).

Acute dermal toxicity

The acute dermal toxicity testing of the ethanolic extractwas based on the Organization of Economic and CooperativeDevelopment of the United Nations Guidelines no. 402 (OECD Guidelines no. 402). Increasing concentrations of the experimental ointment were applied at the lumbar portion of the rats. Erythema andpruritus of the skin of rats were observed after 24 hours. All the experimental rats that underwent toxicity test remained alive at theend of the study (Gadgoli et al., 2012).

Excision wound model

Intravenous ketamine hydrochloride was used to anesthesize the animals (180m kg body wt) (Nayak et al., 2007). The dorsal fur was shaved and outlined to prepare the animals for an induced excision wound of circular area 250 mm^2 and 2 mm deep (Nayak, Marshall, & Godwin, 2010).

Experimental procedure

The rats were grouped randomly into five groups with three rats each. Povidone iodine served as the standard drug derived from the study of James &Friday (2010). The ethanolic leaf extract were formulated into ointment and applied once a day with one gram of the ointment (James & Friday, 2010). The groupings were as follows:

Wound healing assessment

Group 1: Positive control (Standard drug, 10% ointment-based povidone iodine).
Group 2: Positive control (Standard drug, 5% ointment-based povidone iodine)
Group 3: Negative control (Rats without treatment)
Group 4: Test Group (10% ointment-based M. calabura ethanolic extract)
Group 5: Test Group (5% ointment-based M. calabura ethanolic extract)

Estimation of hydroxyproline

Hydroxyproline was estimated using dry granulation of tissue from both control and treated groups. Sodium hydroxide, in the presence of copper sulfate was used to oxidize hydroxyproline present in the neutralized acid hydrolysate. It was complexed with *p*-dimethylaminobenzaldehyde to develop pink color that was measured spectrophotometrically at 560 nm (Carlson& Ruegg, 2009).

Histopathological study

On day 11, granulation tissue was obtained from the test and control group animals. To better appreciate collagen deposition, hematoxylin, eosin stain was used to stain the fibers purple and red, respectively (Nayak et al., 2010).

Wound contraction

To monitor the wound, the border of the healing area was traced using tracing paper. The tracing paper was then traced out on a 1 mm^2 graph sheet. The % wound closure was calculated using measurementsthat were gathered every 2 days (Mekonnen, Sidamo, Asres, & Engidawor,2013).

$$\text{Wound Contraction} = \frac{\text{Wound area on day}^0 - \text{Wound area on day}^n}{\text{Wound area on day}^0} \times 100$$

Statistical Analysis

The data obtained in the study were subjected to one-way analysis of variance (ANOVA) to determine the significant differences between the means of three or more unrelated groups similar to alternate days 1 to 11 used to evaluate the changes of wound extraction of lab used rats. The groups were analyzed using Dunnet's t-test to determine the significant differences ofthe wound healing activity of ethanol extractand control. A p-value <0.05 was considered significant. All the values were expressed as mean ± SEM.

RESULTS AND DISCUSSION

Ethanolic extract

Five hundred grams of leaves were used and 15 grams of viscid, greenish blackethanolic extract was obtained. In the study of Nayak et al. (2010), they also obtained 15 grams of ethanolic extract using 600ml ethanol and 300g fine powdered leaves.

Figure 2. Ethanolic extract of *Muntingi calabura*

Table 1
Phytochemical Analysis on Methanolic Extract of *Muntingia calabura* Leaves

PhytochemicalConstituents	Result
Tannins	+
Flavonoids	+
Saponins	+

Table 1 shows the results of the phytochemical analysis

done in the ethanolic leaf extract of *M. calabura* leaves. An intense blackening color was observedin figure 3A after the addition of 1% aqueous iron chloride ($FeCl_3$) solution whichindicated positive result for tannins. Afterthe addition of 4 ml of 1% aluminum chloride, the filtrate turned yellow seen in figure 3B and it indicates the presence of flavonoids in the extract. Presence of flavonols, flavones and chalcones wereconfirmed in the formation of yellow color (Kumar et al., 2014). Ethanolic leaf extract was also boiled with water and mixture was cooled and mixed vigorously, the formation of frothing observed in figure 3C indicates the presence of saponin (Nayak et al., 2007),and this was observed in the analysis done in this study.

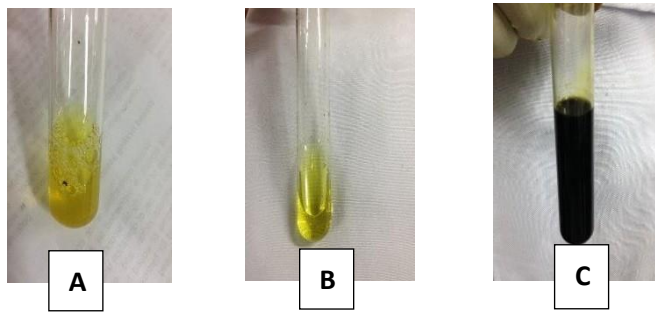

Figure 3. Results of Phytochemical Analysis A.Tannins B.FlavonoidsC.Saponins

Acute dermal toxicity

After application of ointment, the toxicity of increasing concentrations (5 and 10%) of ethanolic leaf extract of *M. calabura* were tested by observing erythema and pruritus.

Table 2
Toxic Effect of Increasing Concentrations of Ethanol Extract

***M. calabura* Ethanolic Extract Concentration**	**Erythema**	**Pruritus**	**No. of Death**
5%	Negative	Negative	0
10%	Negative	Negative	0

Table 2 presents the topical effect of ethanolic leaf extract on the shaved dorsal back of the rats for 24 hours. After the application

of 5% and 10% concentrations of ethanolic leaf extract of *M. calabura,* no erythema and pruritus were observed. It was also observed that no rat died after 24 hours of application of the ethanolic leaf extract. This shows that the rats did not exhibit manifestations of dermal toxicity and 5-10% concentration of ethanolic leaf extract were found to be non-toxic when applied topically on the skin of the rats and possible to be used for further studies. This is supported by the study of Gadgoli et al. (2012), where no signs of redness or itching on the shaved back of albino rats indicating that there is no observed toxicity when they used the methanolic extract of stem bark.

M. elengi and were found to be safe with no mortality observed at the highest dose concentration. This implies that the extract was safe to use since no side effects orcomplications were observed.

Wound contraction and epithelialization period

The effect of varying concentrations between *M. calabura* ethanolic leaf extract and povidone iodine ointment in excisionwound model are shown in Figure 4. In this parameter, the mean percentage of wound area of all three test animals in each group was calculated on the 11 post woundingdays. On the 3rd day, Group I treated with 10% Povidone Iodine and Group II treated with 5% Povidone Iodine had wound contraction of 20.55% and 22.01% while on the 5th day, wound contraction increased to 36.14% and 34.25%, respectively. After the 7th day, the contraction continued to increase to 53.42% and 62.96%, then60.38% and 68.35% on 9th day, and finally on 11th day the wound contraction were 73.79% and 74.83%, respectively. There has been a constant reduction rate using the standard drug (povidone iodine) and thisimplies that when compared to the negative control the rate was hastened when povidone iodine was used.This is supported by the study of Angel et al. (2008) where the application of povidone iodine was proven to be effective at reducing bacterial number and decreasing wound infections. In Groups IV and V administered with 5% and 10% *M. calabura* ethanolic extracts separately, the wound contraction were 28.09% and 26.91% onthe 3rd day and it increased to 44.79% and 41.92% on the 5th day. On the 7th day, the wound contraction of Group IV and V were 45.65% and 53.62% respectively, while on 9th day it increased to 62.25% and 67.45% and on the 11th day the contraction rates were 82.23% and 85.58% respectively.When compared to negative control, it can be inferred that the wound contraction rate was increased using the varying concentrations of

ethanolic leaf extract. Thisimplies the effectivity of the ethanolic leaf extract in accelerating the wound contraction rate in rats. This can be attributed to the presence of tannins andsaponins, which are known to have astringent properties causes soft tissuecontraction (Ashok & Upadhyaya, 2012).

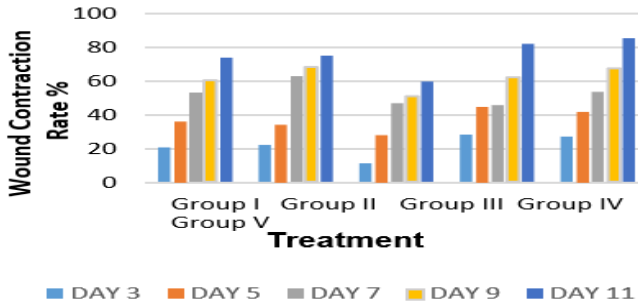

Figure 4. Effect of varying concentration between *M. calabura* ethanolic extract and Povidone Iodine ointment

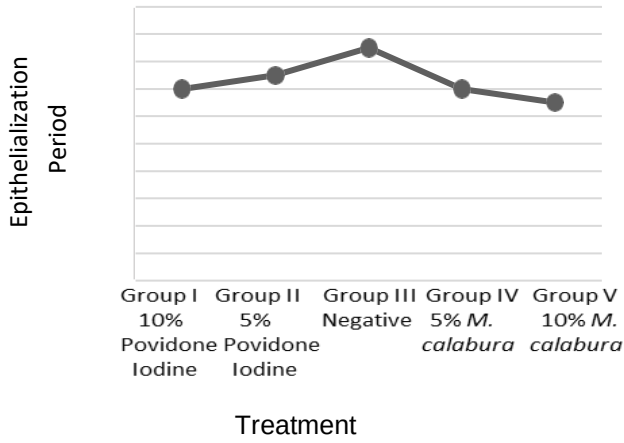

Figure 5. Epithelialization Period between *M. calabura* ethanolic extract and Povidone Iodine ointment

However, on the 7th and 9th day, the concentrations of povidone iodine had a better contraction rate than Groups IV and V. This decreased rate is possibly due to presence of tannins in the extract, which counteracts localization by its anti-inflammatory properties (Zakaria et al., 2007). Groups I, II, IV and V had fluctuating rates of contraction. The wound contraction rate exhibited by the negative control group is possibly due to the natural healingprocess of the rats that is supported by the study of Agarwal et al. (2009), where woundhealing is said to be a response to an injury to restore the function and integrity of the tissues that were damaged. These figures support the hypothesis that *M. calabura* possesses wound healing properties. On the 11th post wounding day, when ranked, it could be deduced that 10% *M. calabura* ethanolic extract as the most effectiveconcentration at which its full wound healing potential could be observed. When compared the standard drug, the ethanolic leaf extract had slower initial contraction butproved more effective after 11 days. The period of epithelialization shown in Figure 5.Group I treated with 10% Povidone Iodine and Group II treated with 5% Povidone Iodine had epithelialization period of 14 and 15 days, respectively; Group III had 17 dayswhile the Groups IV and V administered with 5% and 10% *M. calabura* ethanolic leaf extract had 14 and 13 days, respectively.This demonstrates that 10% *M. calabura* yielded the shortest period of time where wound closure was achieved. Since the period of epithelization was calculated asthe number of days required for falling of theeschar (Gadgoli et al., 2012), if epithelialization will be the sole basis of treatment effectiveness, it can be impliedthat the ethanolic extract of *M. calabura* is more potent than the standard drug. This could be due to tannins being able to form aprotective layer over the exposed tissue; this keeps it from being further infected (Ashok et al., 2012).

Post-wounding of experimental rats is shown in Figure 6. The actual picture of wound immediately after infliction was revealed in Figure 6A. Fresh and wide concavity with the measurement of 500mm^2 in diameter and 2 mm deep was excised to all rats in a uniform size. Clinical feature from the third day can be seen in Figure 6B.The wound was starting to dry up, a slight decrease in size can be noted. In Figure 6C, a significant contraction of the wound was observed with a fully dried eschar. With the treatment completed, Figure 6D shows the absence of the eschar with a completelyhealed area. Through adequate treatment eschar formation was observed indicatingthat the wound healing process was in progress. After treatment, the absence of the eschar indicated complete wound contraction. A continuous healing process could be observed within

14 days of treatment.

Table 3 shows the wound sizes of the different groups as the healing progresses from day 1 up to day 11. It can be gleaned from the data that from 500mm^2 diameter of wound, it gradually decreases insize to 144mm^2 and 117mm^2 for Group Iand Group II (10% and 5% Povidone Iodine)respectively, and for the Group III (negative control) the wound contraction size was 180mm^2 on 11th day. On the other hand, Group IV and V (5% and 10% *M.* calabura ethanolic leaf extract) had 96mm^2 and88mm^2 respectively.

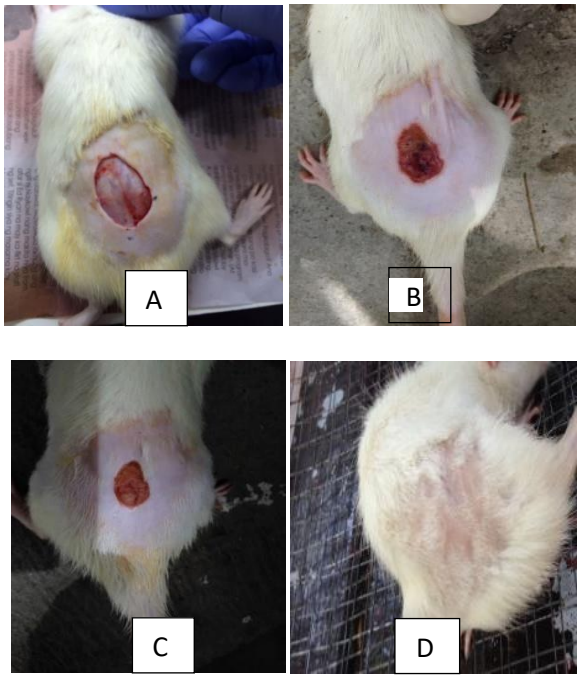

Figure 6. Post wounding of experimental rats. A. Woundafter infliction; B. Clinical feature from the third day; C. Significant wound contraction; D. Absence ofeschar

These results showed the different progression of the wound healing by the decreasing size of the woundinflicted. It can be implied

that the wound healing process is facilitated by both thestandard drug (povidone iodine) and the ethanolic leaf extract as compared to the natural healing process that occurred in the negative control. This implies that the enhanced decrease in wound size of groupstreated with different concentrations of ethanolic leaf extract can be attributed to its phytochemical constituent which isflavonoids.

Table 3
Wound sizes of different groupsas the healing progresses from day 1 up to day 11

Day	Group I10% Povidonelodine (mm^2)	Group II5% Povidonelodine (mm^2)	GroupIII Negative (mm^2)	Group IV5% *M. calabura* (mm^2)	Group V10% *M. calabura* (mm^2)
1	500	500	500	500	500
3	399	390	400	350	330
5	360	364	336	273	300
7	256	198	264	260	273
9	240	154	240	160	204
11	144	117	180	96	88

Table 4
Comparison on the Wound ContractionEffect of *M. calabura* Ethanolic Leaf Extract and Povidone Iodine against Negative Control at Day 3

Group	p-value	Interpretation
Group I: Povidone Iodine10%	0.373	Not Significant
Group II: Povidone Iodine5%	0.247	Not Significant
Group IV: *M. calabura* Ethanolic Extract 5%	0.026	Significant
GroupV: *M. calabura* Ethanolic Extract 10%	0.043	Significant

**significant level at p value <0.05*

As per the study of Agarwal et al. (2009), flavonoids were known to promote wound healing process primarily due to its anti-microbial and astringent properties which is responsible for the increased rateof epithelialization and wound contraction. Thus, the ethanolic leaf extract has actually hastened the process of wound healing in rats. Table 4 presents the comparison onthe wound contraction effect of *M. calabura* ethanolic leaf extract and povidone iodine against negative control at Day 3. Group I and Group II resulted to p-values of 0.373 and 0.247, respectively. This shows that wound contraction effect at day 3 is similar with the group without treatment. This couldbe attributed to the natural wound healing and the activity has no significant difference with the negative control group. According to Thakur et al. (2011), the phases of wound healing normally progress in a predictable and timely manner. Group IV treated with 5% *M. calabura* ethanolic leaf extract and Group V treated with 10% *M. calabura* ethanolic leaf extract when compared to the negative control gave the p-values of 0.026 and 0.043, respectively; thus, indicating the significant difference at Day 3. The increased rate of wound contractions from Groups IV and V are considered significant because the extracts from the said groups prevent other microorganisms to impair the wound healing process by the presence of tannins. Tannins scavenges the freeradicals that impair wound healing and the additional wound healing activities coming from the presence of flavonoids and saponins also exhibit antimicrobial property.Wound healing process was improved bythe rapid contraction rate which was the result of varying high concentrations of phytochemical constituents present on the extracts given to both groups. Biochemicallyactive constituents of *M. calabura* such as saponins which acts through its antioxidant and also antimicrobial property, that appears to be the one responsible for wound contraction and rate of epithelialization (Thakur et al., 2011). Tannins has the ability to chelate metal ions, specifically iron that disrupts the membrane of *S. aureus*, thus, contributing to its antimicrobial activity (Zakaria et al., 2010). Also in the study of James and Friday (2010), flavonoids have astringent and anti-microbial property which could be responsible for the increased rate of epithelialization and wound contraction rate.

Table 5 reveals the comparison on the wound contraction effect of *M. calabura* ethanolic extract against negative control at day 5. Group I and Group II resulted to a p- value of 0.5356 and 0.7556, respectively.This shows that wound contraction effect at day 5 is similar with the group without treatment. It also shows that Group IV has ap-value 0.031 while Group V has 0.0966 without treatment.

It also shows without treatment. It also shows that Group IV has ap-value 0.031 while Group V has 0.0966, indicating that there is only significant wound healing activity on group treated with5% *M. calabura* ethanol extract.

Table 5
Comparison on the Wound Contraction Effectof *M. calabura* Ethanolic Extract and Povidone Iodine against Negative Control at Day 5

Group	p-value	Interpretation
Group I: Standard Drug(Povidone Iodine 10%)	0.7063	Not Significant
Group II: Standard Drug(Povidone Iodine 5%)	0.0392	Significant
Group IV: *M. calabura* Ethanolic Extract 5%	0.9997	Not Significant
Group V*: M. calabura* Ethanolic Extract 10%	0.6828	Not Significant

**significant level at p value <0.05*

This impliesthat 5% ethanolic extract of *M. calabura* could be enough to allow the wound to contract in contrast with the other concentration used and with the standard drug concentrations applied. Conversely, the 10% concentration of the extract have the same wound healing activity when compared pair-wise to the 5% and 10%povidone iodine.

Table 6
Comparison on the Wound Contraction Effect of *M. calabura* Ethanolic Extract and Povidone Iodine against Negative Control at Day 7

Group	p-value	Interpretation
Group I: StandardDrug (Povidone Iodine 10%)	0.5356	Not Significant
Group II: StandardDrug (Povidone Iodine 5%)	0.7556	Not Significant
Group IV: *M. calabura* EthanolicExtract 5%	0.031	Significant
Group V: *M. calabura* EthanolicExtract 10%	0.0966	Not Significant

**significant level at p value <0.05*

Table 6 shows the comparison of the wound contraction effect of *M. calabura* ethanolic extract against negative control at day 7. Group I and Group II resulted to p- values of 0.7063 and 0.0392, respectively. This shows that wound contraction effect at Day 7 on group treated with 10% povidone iodine is similar to the negative control whilethe group with 5% povidone iodine has significance compare to negative control. This reveals that only Group II treated with 5% povidone iodine has a p-value <0.05 which indicates a significant wound contraction. Groups treated with 5% and 10% *M. calabura* ethanolic leaf extract has no significant difference having p-values of 0.9997 and 0.6828, respectively. This suggests that 5% povidone iodine could be used to aid the wound healing process in contrast with the other concentrations used and with the ethanolic extracts of *M. calabura* applied. According to Angel et al. (2008), povidone-iodine increased its bactericidal activity in lower concentrations. Furthermore, there is not enough evidence to conclude the positive or negative outcome on wound healing despite its efficacy in reducing bacterial load in both acute and chronic wounds.

Table 7
Comparison on the Wound Contraction Effect of M. calabura Ethanolic Extract and Povidone Iodine against Negative Control at Day 9

Group	p-value	Interpretation
Group I: Standard Drug(Povidone Iodine 10%)	0.0824	Not Significant
GroupII:Standard Drug(Povidone Iodine 5%)	0.055	Not Significant
Group IV: *M. calabura* Ethanolic Extract 5%	0.0018	Significant
Group V: *M. calabura* Ethanolic Extract 10%	0.0003	Significant

**significant level at p value <0.05*

Table 7 presents the comparison on the wound contraction effect of *M. calabura* ethanolic extract against negative control at day 9. Group I and II showed no significance having p-values of 0.0824 and 0.055. On the other hand, the data with p- value of <0.05 was obtained and consideredsignificant for the wound contraction as seen in Group IV and V, having 5% and 10% *M. calabura* ethanolic leaf extracts respectively. This implies that 5% and 10% *M. calabura* ethanolic leaf extract have greater efficacy in wound healing compared to the negative control at day 9. The phytochemical constituents of *M. calabura* ethanolic extract (10%) such as flavonoids, tannins and saponins were responsible for its wound healing potential as they remove free radicals, exhibit anti-inflammatory and antimicrobial property (Thakur et al., 2011).

Table 8
Comparison on the Wound Contraction Effect of M. calabura Ethanolic Extract and Povidone Iodine against Negative Control at Day 11

Group	p-value	Interpretation
Group I: Standard Drug (Povidone Iodine 10%)	0.3933	Not Significant
Group II: Standard Drug (Povidone Iodine 5%)	0.0237	Significant
Group IV: *M. calabura* Ethanolic Extract 5%	0.233	Not Significant
Group V: *M. calabura* Ethanolic Extract 10%	0.0349	Significant

**significant level at p value <0.05*

Table 8 reveals the significant difference in all treated groups that took place at Day 11. It shows that the group treated with 10% povidone iodine and 5% *M. calabura* ethanolic leaf extract has p-values of 0.3933 and 0.233, respectively.On the other hand, group treated with 5% povidone iodine and 10% *M. calabura*ethanolic leaf extract showed significant p- value of 0.0237 and 0.0349, respectively.

This data showed that the wound contraction of Group II and Group V has a greater value compared to the negative control. This implies that the wound healing potential of *M. calabura* leaf extract

may be responsibly due to the phytochemical constituents present in the plant extract. Flavonoids promote wound healing process primarily due to its astringent and antimicrobial properties that appears to be responsible for wound contraction (Nayak etal., 2009). Tannins and saponins are also present. They are responsible for scavenging free radicals and are also shown to have antioxidant and antimicrobial property (Thakur et al., 2011). Based on the data gathered from different time intervals, the results implies that ethanolic extract of M.*calabura* leaves improved period of epithelialization, wound contraction, and has significant statistical changes. This may suggest that there could be more efficacies on ethanolic extract than positive control (Povidone Iodine) in incision wound model. In the study of Gadgoli et al. (2012), their results indicated that formulation is better than the standard drug which is Betadine in wound healing potential. Based on the study of Angel et al. (2008), it is established that there was a reduction in wound healing rates and only an escalation in the tensile strength of wounds when povidone iodine was used.

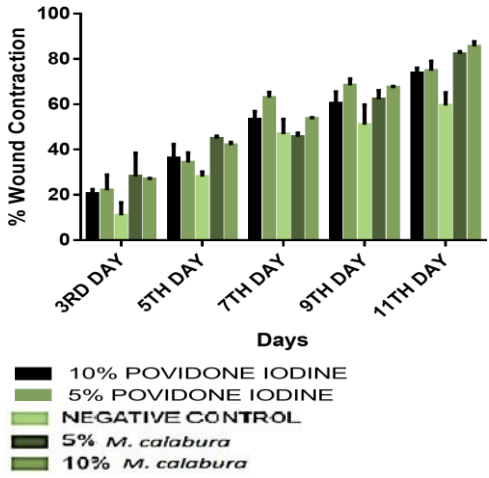

Figure 7. Mean contraction rate (%) of wound between each group

The trend of wound contraction rate of each group is demonstrated in Figure 6. Group I was given with the standard drug (10% Povidone Iodine) and showed noticeable wound contraction

during the time period. On the 3rd and 5th day, wound contraction was accelerated in groups treated with 5% and 10% *M. calabura* ethanolic leaf extracts. This suggest the enhancement of the wound healing process when 5% and 10% *M. calabura* ethanolic leaf extract was used. This can be attributedto the presence of flavonoids which are known to be efficient antioxidants thatscavenges free radicals (Gupta et. al.,2005). However, on the 7th and 9th day, the standard drug, had a better contraction rate than Groups IV and V. This decreased rate is possibly due to presence of tannins in the extract that has anti-inflammatory properties, which counteracts the localization (Zakaria et al., 2007). Theconcentrations of 5% and 10% ethanolic leaf extract showed significance in the last day of evaluation of wound contraction in the wound healing process. However, this claim requires further study to be conclusivebecause the study is limited to the concentrations from 5% up to 10% only. Any increase or decrease in the concentrations may contribute to the capability of the component to influence the process of healing. This is supported by the study of Das (2013) where the wound healing activity is demonstrated using the aqueous extract of the leaf of *Stevia rebaudiana* and found to be effective in the functional recovery of the wound healing by dose dependent manner.

Table 9 shows the comparison of the standard drug (Povidone Iodine) to the varying concentration of *M. calabura* ethanolic leaf extract as well as to the negative control at the final day of wound contraction evaluation which is Day 11. Groups treated with varying concentration of Povidone iodine compared to the other groups showed no significance; thus, suggesting that both the standard drug and the *M. calabura* ethanolic extract of varying concentrations promote wound healing.

Aside from this, groups treated with different concentrations of *M. calabura* ethanol leaf extract showed significant result compared to the negative control, which implies that the ethanolic extract of *M. calabura* with varying concentrations has potential ability in the wound healing process as it hastens the wound contraction. Varying concentrations of *M. calabura* ethanolic extract (5% and 10%) showed no significance indicating that both concentrations have efficacy in accelerating wound healing. Since both concentrations were found effective, higher concentration (10%) of extract is preferred because of greater potential for wound healing as seen in the results. These results could be attributed to the biochemically active components of the leaf extract such as flavonoids, tannins, and saponins. Flavonoids promote wound healing process primarily due to its astringent and antimicrobial property that is responsible for wound contraction and

accelerated rate of epithelializatio (Agarwal et al., 2009), while saponins exhibit antioxidant property and tannins scavenge free radicals as discussed in the study of Thakur et al. (2011).

Histopathological study

Histopathological examinations of skin and tissues from the control groups and groups treated with different concentrations of ethanolic leaf extracts are shown in Figure 8. The positive control with 10% povidone iodine (Figure 8A) revealed a skin tissue with an area of healed ulceration; thus, indicating an extensive granulation tissue formation in the dermis. The ulcerated area is sealed off by fibrosis. A lower concentration of 5% povidone iodine (Figure 8B) reveals a similar result to that of 10% but without the presence of fibrosisthat sealed off the ulcer. There is skin ulceration with extensive granulation tissue formation in the dermis. It can be observed that the effectiveness of Povidone Iodine is dependent on the used concentration. It canbe inferred with these results that 10% povidone iodine can be the recommended concentration since it yielded better histopathological results. It is shown in Figure 8C that for the negative control, (rats without any treatment applied), the histopathologic results revealed a skin tissue with an ulcerated lesion exemplified by the discontinuity of the epidermal lining indicating skin ulceration with extensive granular tissue formation in the dermis. Destruction of stratum corneum signifies clear picture of non-healing wound and means that there is already a discontinuity and complete loss of the delayed woundhealing.

For the treatment group, 5% *M. calabura*, as shown in Figure 7D, revealed a skin tissue showing the beginning of re-epithelialization, but still with ulceration, this indicates the start of the epidermal re- epithelialization stage. A higher treatment group concentration (10% *M. calabura*), reveals a skin tissue with area of healedulceration and a thin layer of re- epithelialization as shown in Figure 7E; thus, indicating epidermal re- epithelialization and extensive granulation tissue formation in the dermis.

In the study of Nayak et al. (2010), the granulation tissue of animals treated with the extract exhibited denser pattern of collagen deposition, and fewer numbers of inflammatory cells as compared to animals of control group. However, all histopathology specimen, revealed that the underlying dermis showed fibrous tissue proliferation, neovascularization and some inflammatory cell infiltrates. It can be impliedthat the phytochemical components of the extract contributed to the emergence of fibroblasts and collagen fibers.

Table 9
Multiple Comparison of the WoundContraction Effect of different Concentration of EthanolicExtract of *M. calabura*

	Concentration	p-value	Interpretation
Group I: Povidone Iodine10%	Group II (5% Povidone Iodine)	>0.9999	Not significant
	Group III (Negative Control)	0.1834	Not significant
	Group IV (5% Ethanolic *M. calabura*)	0.7275	Not significant
	Group V (10% Ethanolic *M. calabura)*	0.3807	Not significant
Group II: Povidone Iodine 5%	Group III (Negative Control)	0.1282	Not significant
	Group IV (5% Ethanolic *M. calabura*)	0.8224	Not significant
	Group V (10% Ethanolic *M. calabura)*	0.4855	Not significant
Group III: Negative Control	Group IV (5% Ethanolic *M. calabura*)	0.0048	Significant
	Group V (10% Ethanolic *M. calabura)*	0.0008	Significant
Group IV:5% Ethanolic *M. calabura*	Group V (10% Ethanolic *M. calabura)*	0.9935	Not significant

**significant level at p value <0.05*

According to Nayak et al. (2007), during the stage of wound healing, some characteristics of the proliferation phase were also observed such as presence ofinflammatory cells and fibroblasts and increased number of epithelial cells that produced the granulation tissue. In wound healing, the production of differentcomponents is essential for faster recovery of the skin. Migrating epithelium suggests that the wound is closed and is continuing the regeneration of the wound area.

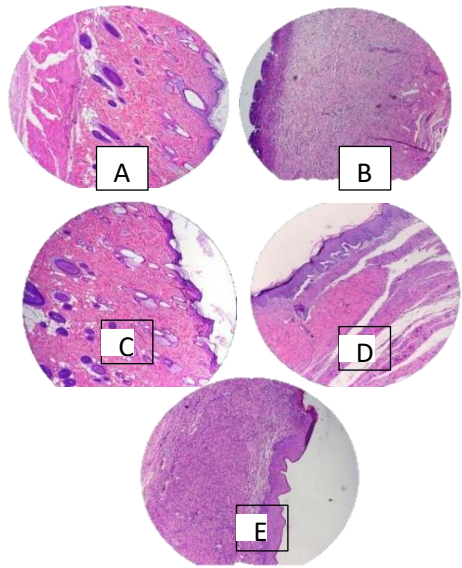

Figure 8. Histopathological Examination: Group I (10% Povidonelodine); B. Group II (5% Povidone Iodine); C. Group III (Negative);D. Group IV (5% *M. calabura* ethanolic extract); E. Group V (10% *M. calabura*ethanolic extract

Hydroxyproline estimation

Hydroxyproline absorbance levels which have been measured spectrophotometrically on each grouping were used as the basis of collagen content. Moreover, this explains that the higher the absorbances, the higher the concentration of hydroxyproline on each grouping. It can be gleaned on Table 10 that the absorbance of group II with 5 % povidone iodine and group IV with 5% *M. calabura* ethanolic leaf extract, have significant close values which are 1.2847 and

1.2313, respectively. On the other hand, group I with 10% povidone iodine and group V with 10% *M. calabura* ethanolic extract adjacently gave absorbance of 1.3667 and 1.3483, respectively. Furthermore, compared to the absorbance of the negative control which is 1.1533, hydroxyproline level absorbance results of the other groupings are significantly higher which proves that collagen syntheses are increased. This implies that the higher absorbance levels observed in groups IV and V indicates that greater concentration of hydroxyproline were measured; thus, increased collagen content were found in groups treated with 5% and 10% *M. calabura* ethanolic leaf extract. The study of Agarwal et al. (2009), indicates that when an injury is introduced, the extracellular protein in the tissue granulation of a healing wound will increase simultaneously, wherein, it strengthens and is responsible for the tissue matrix integrity.

Table 10
Effect of varying concentration between M. calabura ethanolic extract and Povidone Iodine ointment based in the hydroxyproline estimation

Groups	Mean Absorbance
Group I: 10% Povidone Iodine	1.3667
Group II: 5% Povidone Iodine	1.2847
Group III: Negative Control	1.1533
Group IV: 5% *M. calabura* ethanolic extract	1.2313
Group V: 10% *M. calabura* ethanolic extract	1.3483

**significant level at p value <0.05*

Table 11 reveals the significant differences to all treated groups that took place at the last day of evaluation. Statistically, it is shown that the group with the varying concentrations of *M. calabura* ethanolic leaf extract and povidone iodine treatment having p-value of less than 0.05 showed a significant result. In the present study, a significant increase in the hydroxyproline content of the granulation tissue of the animal treated with *M. calabura*ethanolic leaf extract was recorded compared with control groups, thus indicating positive effectiveness of the ethanolic leaf extract on collagen synthesis and hence, on wound healing. Hydroxyproline is an amino acid which is an integral part of the collagen fiber and is used as a biochemical marker for tissue collagen (Nayak et al., 2010)

Table 11
Comparison on the Effect of *M. calabura* Ethanolic Extract and Povidone Iodine against Negative Control

Group	p-value	Interpretation
Group I: Standard Drug (Povidone Iodine 10%)	0.000	Significant
Group II: Standard Drug (Povidone Iodine5%)	0.000	Significant
Group IV: *M. calabura* Ethanolic Extract 5%	0.000	Significant
Group V*: M. calabura* Ethanolic Extract 10%	0.000	Significant

**significant level at p value <0.05*

Table 12 shows the multiple comparison of the standard drug (povidone iodine) to the varying concentrations of *M. calabura* ethanolic leaf extract as well as to the negative control at the final day of evaluation. Groups treated with varyingconcentrations of povidone iodine comparedto the other groups is significant with p- value of less than 0.05; thus, suggestingthat both the standard drug and the *M. calabura* ethanolic extract of varying concentrations promote wound healing. On the other hand, groups treated with differentconcentration of *M. calabura* ethanolic extract shows significant result as well with p-value of less than 0.05 compared to the negative control, which implies that the ethanolic extract of *M. calabura* with varying concentrations has potential in aid in woundhealing process. Varying concentration of *M. calabura* ethanolic extract (5% and 10%)is also compared with each other having significance indicating that both concentrations have efficacy in accelerating wound healing. This significance is attributed to the presence of collagen which is a major protein of the extracellular matrix and is the component that ultimately contributes to wound strength. Whencollagen breaks down, it liberates free hydroxyproline and its peptide. Hence, measurement of hydroxyproline was used as an index for determining collagen turnover (Ilango & Chitra, 2010).

Table 12
Multiple Comparison of the Effect of different Concentration of Ethanolic Extractof *M. calabura*

	Concentration	p-value	Interpretation
Group I:10% Povidone Iodine	Group II (5%Povidone iodine)	0.000	Significant
	Group III (Negative Control)	0.000	Significant
	Group IV (5% *M. calabura*)	0.000	Significant
	Group V(10% *M. calabura*)	0.012	Significant
Group II:5% Povidone Iodine	Group I (10%Povidone iodine)	0.000	Significant
	Group III (Negative Control)	0.000	Significant
	Group IV (5% *M. calabura*)	0.000	Significant
	Group V(10% *M. calabura*)	0.000	Significant
Group III: Negative Control	Group IV (5% *M. calabura)*	0.000	Significant
	Group V(10% *M. calabura*)	0.000	Significant
Group IV:5% *M.calabura*	Group V(10% *M.calabura*)	0.000	Significant

**significant level at p value <0.05*

The groups treated with extract showed significant increases in the level of hydroxyproline,which reflects the increase in collagen content. This was supported by the histopathological examination of wound granulation tissue which showed re-epithelialization, extensive fibrous tissue proliferation, neovascularization and inflammatory cellinfiltrates.

CONCLUSION

The result of the study indicates that *M. calabura* ethanolic leaf extract possesses wound healing properties inSprague Dawley

rats. The groups thatreceived *M. calabura* ethanolic extract (5% and 10%) had a significant increase in the wound contraction rate, epithelialization period and the production of fibroblasts and collagen fibers (hydroxyproline level) that maybe attributed to the phytochemical constituents found the ethanolic leaf extract.Based on the statistical data and histopathological findings, it was proven that *M. calabura* leaves have wound healing properties depending on dosage. The optimum concentration that facilitated the wound healing is 5% *M. calabura* ethanolic extract since lower concentration produces same efficacy with lesser possibility of toxicity.

RECOMMENDATION

The researchers would like to recommend the use of other concentrations of the extract. Furthermore, purification and isolation of extract would also be desirable along with broader phytochemical testing for other active components that could be synergistic with the astringent properties of saponins and flavonoids which promotes the wound healing. Lastly, the researchers would like to suggest the use of actual participants to correlate the findings of this study.

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