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Investigation into the Wound Healing Activity of *Monodora myristica* and *Monodora tenuifolia* Seed Extracts in Albino Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Authors IAA and AAR designed the study, carried out the research, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author ARU took part in carrying out the research. All authors read and approved the final manuscript.

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ABSTRACT

Ethnopharmacological Relevance: *Monodora myristica* and *Monodora tenuifolia* are among the plants that are been used by traditional medicine practitioners in Nigeria. They have been used extensively for the treatment of various ailments. The parts of these plants have some medicinal values and are being used in different regions of Nigeria for wound healing, but the scientific proof of wound healing activity of these plant parts and plant seeds are lacking, hence, this is a necessary to have a validate record of the medicinal uses of *M. myristica* and *M. tenuifolia* seeds to expand their use to include integration into modern medical healthcare systems. In this study an attempt was made to validate the ethno-medicinal uses of *M. myristica* and *M. tenuifolia* seeds through botanical identification and biological assessment of their value as complementary medicine for treatment of wound in rats.
Materials and Methods: Phytochemical analysis was carried out to known the various phytoconstituents in the extracts of *M. myritica* and *M. tenuifolia* seeds. The antimicrobial activities

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of the extracts were examined on multiple drug resistant bacteria viz: *S. aureus*, *E. coli, B. subtilis, P. aeroginosa* and fungi viz: *C. albicans, A. niger* using agar the technique of well diffusion and method of broth dilution. The wound healing effect of seeds of *M. myristica* and *M. tenuifolia* were studied by incorporating the extracts into paraffin in concentrations of 5% and 10% w/w. Wound healing activities of the extracts were studied by determining the wound area (mm^2), percentage of wound closure, period of epithelialisation and histological analysis of the control and test groups.

Results: Phytochemical screening revealed the presence of tannins, reducing sugar, terpenoids, alkaloids, tannins and flavonoids in *M. myristica* seed extract while tannins, saponins, flavonoids, alkaloids and terpenoids were detected in that of *M. tenuifolia*. The extract of *M. myristica* seeds showed significant activity against *S. aureus* and *B. subtilis* having the same MIC value of 50 mg/ml, *P. aeroginosa* and *A. niger* with MIC value of 100 mg/ml, *E. coli* and *C. albican* having an MIC value of 200 mg/ml while MIC value of 50 mg/ml was recorded for *E. coli, A. niger* and *P. aeroginosa*, 100 mg/ml for *S. aureus* and 200 mg/ml for *B. subtilis* and *C. albican* using *M. tenuifolia seed extract*. A profound wound healing effect was noticed for *M. tenuifolia* seed extract. This is confirmed that the seed extract of *M. tenuifolia* has a better wound healing capacity than that of *M. myristica* as it was revealed from the experimental values of the wound closure area, improved tissue regeneration at the wound site observed through the daily monitoring and histopathological parameters related to the healing of the wound.

Conclusion: The results affirmed the ethnomedicinal application of *M. myristica and M. tenuifolia* seeds for wound healing.

Keywords: M. myristica and M. tenuifolia; phytochemical; antimicrobial; wound healing.

1. INTRODUCTION

Wound is defined as the disruption or opening in the epithelial tissue of the skin that could be caused by physical, chemical, thermal or mechanical injury. Wound repair is a natural process of reconstructing dermal and epidermal tissue. Injury to the tissue may result in cell death and tissue destruction and also involves continuous cell-cell and cell-substrate interactions that allow the process to progress in three overlapping phases viz: Inflammation (0-3 days), cellular proliferation (3-12 days) and remodelling phases (3-6 months), respectively [1-3]. Plants are the store house of chemicals and act as potential healers [4]. Many researchers have reported wound healing activity of indigenous medicinal plants in recent years. Kumar et al. [5] reported the wound healing activity of *Portulaca oleracea* L., The wound healing effect of *Tephrosia purpurea* was also reported by Rashed et al. [6]. Marwah et al. (2006) and Csupor et al. (2009) also gave details of wound healing activity of *Centaureas adleriana*.

Monodora myristica (annonaceae) with a local name 'Ariwo' being called in South Western part of Nigeria is a tropical tree that grows wild in different countries in African including Nigeria. Nutritional important of *M. myristica* is as result of its usefulness as a seasoning because it has aromatic flavor. The kernel removed from the seeds is a popular condiment used as spicing agent in both African and Continental cuisines in Nigeria. The aromatic nature of the seeds make it serves as an additional value in using the plant as a stimulant to snuff and medicine. Many of the medicinal uses of this plant have been reported; the bark of the plant is being used for the treatments of stomach-aches, febrile pains, eye diseases and haemorrhoids. It was reported by Koudou et al. (2007). The Central African Republic people are using the seeds as condiment and drug for the treatment of headache and hypertension. The antisickling effect of *M. myristica* was reported by Uwakwe and Nwaoguikpe [7]. The determination of the chemical constituents of the seeds reveals the presence of fiberro-latic oils, resins, terpene, lactose, arocine, saponins, flavonoids and tannins [8].

Monodora tenuifolia has nomenclature variations including English; African nutmeg, Igbo; Ehuru ofia, Yoruba; Abo-lakoshin. The anti-diarrheal properties of seed extract of *Monodora tenuifolia* was investigated by Ezenwali et al. [9] using rodent models of diarrhea. They discovered an oral LD_{50} value greater than 5000 mg/kg in mice revealed by acute toxicity and lethality studies carried out on the methanolic seed extract. They concluded that the seeds of *M. tenuifolia* possess anti-diarrheal properties mediated through inhibition of hyper secretion and gastrointestinal motility which substantiate the use in the treatment of diarrhea in traditional medicine.

In the same way, Ekeanyanwu et al. [10] reported the toxicity studies on the *Monodora tenuifolia* fraction rich of flavonoid using albino rats. Their studies revealed that the LD_{50} value of the flavonoid rich fraction was above 5000 mg/kg body weight in rats observed for 48 hours. They also observed that, after Day 14, biochemical markers of liver injury such as serum alanine aminotransferase, and aspartate aminotransferase decreased significantly. They concluded that the flavonoid rich fraction of the seed extract is potentially safe. The seed oil of *M. tenuifolia* was also analysed for fatty acid composition and was studied on albino rats by feeding them to evaluate it nutritional value as edible oil [11]. They found out after the experiment that the haematological analysis of the blood sample of the rats indicated that they were not anaemic and no mortality was recorded. It seemed *M. tenuifolia* seed oil could find application as edible oil or as potential industrial raw material. This work reports the wound healing effect of the extracts of the seeds of two varieties of *Monodora* species grown in Nigeria namely *M. myristica* and *M. tenuifolia*.

2. MATERIALS AND METHODS

2.1 Plant Material

The seeds of *M. myristica* were collected from a local market in Ibadan, Oyo state, Nigeria while *M. tenuifolia* seeds were collected from the Botanical Garden of the University of Ibadan and authenticated at the Herbarium Unit, Department of Botany, University of Ibadan, Ibadan, Nigeria where voucher specimen has been deposited.

2.2 Preparation of Seed Extracts

The collected seeds were dried and reduced to coarse powder with an electronic blender. Approximately 500 g of dried powdered sample was put in an aspirator bottle, 1 L of methanol was added to it and left at room temperature (30±2℃) for 4 days with continuous stirring to allow it to macerate [12] after which it was filtered. The filtrate was then concentrated with rotary evaporator (Model Zirbus 302®, Italy) at 35℃. The extract was stored in universal bottle and refrigerated at 4℃ prior to use.

2.3 Phytochemical Screening

The extracts were subjected to various chemical tests in order to detect the presence of different phytoconstituents. Qualitative tests for the presence of plant secondary metabolites such as carbohydrates, alkaloids, tannins, flavonoids, saponins and glycosides were carried out using standard procedures described by Yadav et al. [13].

2.4 Test Microorganisms

Mult Drug Resistant (multi) clinical isolates of *Aspergillus niger* and *Candida albicans* were used as the fungal tested organisms. *Pseudomonas aerugonisa, Escherichia coli, Bacillus subtilis, Bacillus cerus* were used as the bacterial tested organisms. The pure bacterial and fungal strains were obtained from the Department of Pharmacy, University of Ibadan, Ibadan, Nigeria. The bacterial strains were cultured overnight at 37℃ in nutrient agar (Oxoid, Hampshire, UK) while fungal strains were cultured overnight at 28℃ using potato dextrose agar (Oxoid).

2.4.1 Antimicrobial activity assay

The antimicrobial activity of the seed extracts were evaluated against a few pathogenic bacteria such as *Staphylococcus aureus*, *Escherichia coli, Bacillus subtilis, Pseudomonas aeroginosa*, and the fungal isolates like *Candida albicans* and *Aspergillus niger.* Bacteria were cultured over night at 37℃ in Mueller Hinton (MH) broth and fungi in Potato Dextrose Broth (PDB) at 28℃ for 72 h. The overnight culture was tested to determine the visible growth on agar plates or microplate wells containing different concentrations of the extracts (0-200 mg/ml) and standard antibiotics by dilution method [14]. For disk diffusion test sterile filter paper disks (6 mm diameter containing 6.25-200 mg/disk of the extracts) were placed on the agar surface and incubated overnight at 37℃ for evaluation of growth inhibition.

2.4.2 Minimum Inhibitory Concentrations (MIC) of seed extract

Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of the extracts that inhibit the visible growth on agar surface or turbidity in microwell broth. Minimum inhibitory concentrations both for bacterial and fungal strains were measured as reported in literature by Sarker et al. [15].

2.5 Experimental Animals

Wistar albino rats of either sex, weighing 101-120 g were selected for the study. Animals were

maintained in polypropylene cages with free access to food and water *ad libitum.* All experimental protocols were in compliance with University of Ibadan Ethics Committee on Research in Animals (14/0059/UIECRA) as well as international accepted principles for laboratory animal use and care.

2.6 Evaluation of Wound Healing Activity

The excision wound model was used to evaluate the wound healing activity of the *M. myristica* and *M. tenuifolia* seeds extracts. The rats were divided into five groups, each containing six animals and the 50 mg ointments formulated were applied topically once a day. The animals of group1 received ointment base (control), group 2 and 3 animals were treated with 10% w/w of *M. myristica* and *M. tenuifolia* extract ointments while groups 4 and 5 were treated with 5% w/w of *M. myristica* and *M. tenuifolia* extract ointments. The animals were anaesthetized with ketamine hydrochloride (100 mg/kg, i.p.) prior to and during infliction of the wound [12]. All animals were closely observed for any infection, so that the infected animals can be excluded from the study.

2.6.1 Crude extract formulation

5% and 10% (w/w) extracts of the *M. myristica* and *M. tenuifolia* seeds were prepared by mixing the extracts (2.50 g) and (5.00 g) in yellow paraffin (50.00 g) obtained from Chemistry Department, University of Ibadan store unit [16].

2.6.2 Excision wound model

The animals were anaesthetized prior to and during the creation of experimental wounds with ketamine hydrochloride (100 mg/kg, i.p.) [12]. Rats were then inflicted with excision wound according to the method described by Morton and Malone [17]. The dorsal fur of the dorsolateral flank area was shaved with an electrical clipper. After wound area preparation with 70% alcohol, the skin from the predetermined shaved area was excised to its full thickness to obtain a wound area of about 200 mm^2 using forceps, a surgical blade and scissor. Excision wounds were created on the dorsal thoracic region 1.5 cm from the vertebral column on either side. Haemostasis was achieved by blotting the wound with a cotton swab soaked in normal saline. The wound was left open and all the animals were treated using the formulated ointments and the healing of wound was monitored by tracing the wound on the first, 4^{th}, 8^{th}, 12^{th}, 16^{th} and 20^{th} post wounding days. The wound closure was measured at regular intervals to calculate the percentage wound closure and epithelialisation time that indicates the formation of new epithelial tissue to cover the wound [18].

2.6.3 Wound healing activity study

To evaluate the wound healing ability of the prepared formulations, the parameters like: (1) wound area (mm^2) of the excision wound, (2) rate of wound contraction and epithelialisation time (excision wound), and (3) histopathological studies of healed tissues were measured.

2.6.4 Rate of wound contraction

The rate of wound contraction was measured as percentage reduction of size at every 4 day interval. Progressive decrease in the wound size was measured periodically using transparency paper and a maker, and the wound area was measured graphically to monitor the percentage of wound closure which indicates new epithelial tissue to cover the wound. The percentage wound contraction was measured according to Srivastava and Durgaprasad [19] formula:

$$\text{Percentage wound contraction} = \frac{A_o - A_t}{A_t} \times 100\ \%$$

Where

A_0 = Initial area of wound at day "0'' of experiment

A_t = Area of wound at day "t'' of experiment.

The number of days required for filling of the scar without any residual of the raw wound gave the period of epithelialisation.

2.7 Histological Study

The skin tissues were collected on the 21^{st} day of the experiment from all the five groups of animals and processed for histological study to determine the pattern of lay-down for collagen. The skin specimens from the treated animals were collected in 10% buffered formalin and were subjected to sectioning and 6 µm thickness sections were stained with hematoxylin and eosin. The stained slides were visualized for histological changes under a light microscope.

2.8 Statistical Analysis

Statistical analysis was performed on each group n=7 and ANOVA test (IBM SPSS statistical 20.0 software) was used to compare the mean value of each treatment. Significant differences between the means of parameters were determined by using the Duncan T test ($P < 0.05$). The results represented means and standard deviation of 7 replicated determinations.

3. RESULTS

3.1 Phytochemical Analysis

The phytochemical analysis of the two extracts of *M. myristica* and *M. tenuifolia* seed are shown in Table 1. The result of phytochemical analysis showed the presence of various chemical constituents such as tannins, alkaloids, terpenoids, flavonoids and reducing sugars in the extract of *M. myristica* seed while saponins, tannins, flavonoids, alkaloids, steroids and terpenoids were detected in *M. tenuifolia* seed extracts.

3.2 Antimicrobial Activity of *M myristica* and *M. tenuifolia* Seed Extracts Against Pathogenic Microorganisms

The results of the antimicrobial activity of the extracts against the tested bacterial and fungal are presented in Table 2. The two seed extracts were found to effectively inhibit the growth of all organisms at different concentrations as compared to both negative and positive controls. Gram (+) organisms (*S. aureus* and *B. subtilis*) were found to be more susceptible than Gram (-) organisms (*E. coli* and *P. aeroginosa*) and fungi (*C. albicans* and *A. niger*) for *M. myristica* seed extract while *A. niger* and *P. aeroginosa* organisms were found to be more susceptible for *M. tenuifolia* seed extract of all the tested organisms. *M. myristica* extract was observed to prevent the growth of *S. aureus* at all the concentration ranges and the remaining organisms were noticed to grown at the applied concentration of 12.5 mg/l and 6.25 mg/ml. *M. tenuifolia* seed extract was noticed to be more effective than *M. myristica* and inhibited the growth of all the tested organisms. *M. myristica* was found to have MIC of 6.25 mg/ml against *S. aureus* and 25 mg/ml against all other tested organisms while the extract of *M. tenuifolia* inhibited the growth of the organisms with minimum concentration of 12.5 mg/ml (Table 2). Among the bacteria, *M. myristica* seed extract showed maximum activity against *S. aureus* producing the maximum zone of inhibition of 21.50±0.71 mm followed by *B. subtilis* (18.0±0.71 mm). The zones of inhibition recorded for *E. coli* and *P. aeroginosa* were 15.50±0.71 mm and 14.00±0.00 mm while the values of 18.0±0.00 mm and 12.50±0.00 mm were observed for *A. niger* and *C. albican* respectively. *M. tenuifolia* recorded significant activity with the following values of zone of inhibition recorded for each of the organism; *E coli* with the highest value 20.0±0.00 mm followed by *A. niger* and *P. aeroginosa* (18.00±0.00 mm), *S. aureus* (17.0±0.00 mm) and *B. subtilis* and *C. albicans* having least zone of inhibition (14.00±0.00 mm).

3.3 Minimum Inhibitory Concentration (MIC) Values of *M. myristica* and *M. tenuifolia* Seed Extracts Against Pathogenic Microorganisms

The MIC of *M. myristica* and *M. tenuifolia* seed extracts presented in Table 3 indicated that the extracts have moderate antibacterial and antifungal activities. Further assay of the extract with different concentrations of the seed extracts against test organisms showed that *S. aureus and B. subtilis* were found to be most sensitive among tested organisms and were found inhibited by *M. myristica* extract at minimum concentration of 50 mg/ml. *E. coli, C. albicans* and *A. niger* were found inhibited by the extract at 100 mg/ml. MIC of 200 mg/ml was observed for *P. aeroginosa* as the least sensitive organism to the seed extract while *M. tenuifolia* was found to effectively inhibit the growth of *P. aeroginosa, A. niger* and *E. coli with* MIC of 50 mg/ml followed by *S. aureus* with MIC of 100 mg/ml. *B. subtili* and *C. albicans* were found to be most resistant to the extract with MIC of 200 mg/ml.

3.4 Effects of Wounds and Seed Extracts of *M. myristica* and *M. tenuifolia* on Weight of the Model Rats

Table 4 showed the weight gain of the rats throughout the experiment. It was observed that there was a gradual increase in the mean body weight of the rats throughout out the experiment. This is a good indication that the extracts of *M. myristica and M. tenuifolia* seeds had no negative effect on the general well being of the animals and effectively healed the wounds as reflected by their weekly weight gain.

3.5 Wound Healing Activity Study

3.5.1 Wound contraction and epithelialisation time

Wound contraction indicates the rate of reduction of unhealed area during the healing process. Thus, the fast rate of wound closure indicates the better efficacy of medication. The progressive reduction in wound area of different groups of animals over 18 days by extracts of *M. myristica* and *M. tenuifolia* seeds are presented on Table 5 and (Fig. 1 in all the groups). The fastest healing of wound was observed in group 3 which was treated with 10% (w/w) extract ointment of *M. tenuifolia*, along with complete healing (100% wound contraction) within 16 day, as compared with the group 1 treated with pure paraffin ointment, group 2 treated with 10% w/w extract ointment of *M. tenuifolia* and groups 4 and 5 treated with 5% (w/w) extract ointment of *M. myristica* and *M. tenuifolia*. On 16^{th} post-wounding day, the rate at which the wound was closing in group 3 was much faster than the rest of the groups. Ointment treated animals (group 1) showed the same total reduction in the wound areas (1.46±1.14) with group 4 (1.13±0.89) with almost epithelialisation time (18.43±0.54) and (17.14±0.38) respectively while groups 2, 3, and 5 were almost healed. Within 16^{th}-18^{th} post wounding day complete epithelialisation was noticed in all the treated animals. The lowest rate of wound healing with highest epithelialisation time 18.43±0.43 day (calculated time of epithelialization or formation of scars on the wound which indicates the total wound healing calculated for all the animals in each of the group) was observed in paraffin ointment base group. The group 3 10% w/w extract ointment formulation of *M. tenuifolia* healed the wound faster (epithelialisation time of 16.14±0.38) than the group 2 10% w/w extract ointment formulation of *M. myristica* treated group (epithelialisation time of 16.57±0.54). Those of groups 4 and 5 with the same epithelialisation time of (17.14±0.38) were also found to heal the wound better than the ointment base treatment group 1 (18.43±0.43).

3.6 Histopathology Analysis

The histological examination of the skin samples collected from the wound areas that were treated for 21 days with ointment base, *M. myristica* and *M. tenuifolia* seed extract ointments revealed the presence of a mature granulation tissue in almost all the depth of the dermis for excision wound model. Epidermal skin layer, myofibroblasts and fibroblasts, collagen deposition, angiogenesis and cell infiltration examined on the wound healed revealed the tissue regeneration in both the experimental and control groups as shown on (Table 7) in all the groups.

4. DISCUSSION

According to the definition of Chattopaddhya et al. (2012), healing of wound is complex in nature and dynamic process which involves tissue structure restoring back to its normal state. Healing depends upon the repairing ability of the tissue, type and extent of damage, and general state of the host's health [20]. It is characterised by haemostasis, re-epithelialisation, granulation, remodelling of the extracellular matrix and scar formation [21,22]. The present investigation described some distinct characteristics of *M. myristica* and *M. tenuifolia* seeds with regards to potency of their wound healing capacity in excision wound model rats. The aim of this study is to verify the traditional use of M. myristica and *M. tenuifolia* seeds for wound healing.

Table 1. Phytoconstituents analysis of *M. myristica and M. tenuifolia* seed extracts

Phytochemical	*Monodora myristica*	*Monodora tenuifolia*
Tannins	+	+
Saponins	-	+
Alkaloids	+	+
Terpenoids	+	+
Steroids	_	+
Flavonoids	+	+
Reducing sugars	+	_

Key; - indicates absence of phytochemical and + presence of phytochemical

Table 2. Antimicrobial activity of *M. myristica* and *M. tenuifolia* seed extracts against pathogenic microorganisms

*Test organisms	Zone of inhibition diameter (mm)							Zone of inhibition diameter (mm)					
	Concentration of *M. myristica* seed extract (mg/ml)							Concentration of *M. tenuifolia* seed extract (mg/ml)					
	-ve control	200.0	100.0	50.0	25.0	12.5	6.25	200.0	100.0	50.0	25.0	12.5	6.25	+ve control
S. aureus	-	21.00±1.00	18.33±0.58	16.0±0.00[c]	14.33±0.58	12.0±0.00[e]	10.0±0.00	17.0±1.00[a]	14.0±0.00	12.0±0.00	10.00±0.0	-	-	38.0±0.00
E. coli	-	15.00±1.00	12.0±0.00	10.0±0.00	-	-	-	20.0±0.00	18..0±0.0	15.0±1.0	13.0±1.0	10.0±0.0	-	38.0±0.00
B. subtilis	-	18.33±0.88	16.0±1.00	13.0±1.00	10.00±0.00	-	-	14.0±0.0	12.0±0.0	10.0±0.0	-	-	-	38.0±0.00
P. aeroginosa	-	14.0±0.00	11.67±0.58	10.0±0.00	-	-	-	18.0±0.0	16.0±0.0	12.0±0.0	10.0±0.0	-	-	40.0±0.00
C. albicans	-	13.00±1.13	12.00±0.00	10.0±0.00	-	-	-	14.0±0.0	12.0±0.0	10.0±0.0	-	-	-	28.0±0.00
A. niger	-	17.67±0.58	15.33±1.16	12.33±2.08	10.00±0.00	-	-	18.0±0.0	16.0±0.0	14.0±0.0	12.0±0.0	10.0±0.0	-	28.0± 0.00

*Values are expressed as mean ± SD of three experiments

Natural products from plant sources have ability to heal wound due to their wound healing effects and serves as agent to the healing of wounds and this is more reliable due to their availability in almost everywhere, they are not toxic, the side of effects of these natural products are minimal and very efficient by preparing them as crude in formulations [23]. Suguna et al. [24,25] also reported that *Centella asciatia* and *Terminala chebula* have high efficient in healing wound based on studies carried out on rats using extracts of the two plants.

Table 3. MIC of *M. myristica* and *M. tenuifolia* seed extracts against pathogenic microorganisms

Test organisms	Minimal inhibitory concentration (MIC) in mg/ml							
	Concentration of *M. myristica* seed extract				Concentration of *M. tenuifolia* seed extract			
	200	100	50	25	200	100	50	25
S. aureus	+	+	+	-	+	+	-	-
E. coli	+	-	-	-	+	+	+	-
B. subtilis	+	+	+	-	+	-	+	-
P. aeroginosa	+	-	_	-	+	+	-	-
C. albicans	+	+	-	-	+	-	+	-
A. niger	+	+	-	-	+	-	+	-

Key: (+): indicates inhibition (no growth of organisms) and (–): no inhibition (growth of organism)

Table 4. Weekly weight gain of rats

*Animal groups	Weight gain of rats (g)			
	Week 0	Week 1	Week 2	Week 3
1	121.86±37.58^{bc}	126.86±36.87^{bc}	138.29±20.29^{b}	145.29± 8.96^{b}
2	101.86±16.76^{c}	113.57±15.46^{c}	122.29±20.08^{b}	138.00±16.46^{b}
3	100.14±16.13^{c}	106.43±17.59^{c}	123.29±14.67^{b}	131.00±11.63^{b}
4	153.29±19.62^{ab}	163.57±18.81^{ab}	178.14±20.73^{a}	188.86±21.69^{a}
5	142.86±12.86^{a}	150.57±16.50^{a}	166.29±22.44^{a}	172.86±25.99^{a}

Values are expressed as mean ± SD for groups of seven animals each. Data with different superscript letters along the same column are significantly different (p<0.05) using one ANOVA followed by Duncan's test

Table 5. Effect of topical application of *M. myristica* and *M. tenuifolia* seed extracts on wound healing area (mm^2) and period of epithelialisation

*Animal groups	Wound healing area (mm^2)					Epithelialisation time
	Day 0	Day 4	Day 8	Day 12	Day 16	
1	176.79±0.00^{a}	97.06±3.43^{a}	30.98±19.37^{a}	4.26±1.92^{a}	1.46±1.14^{a}	18.43±0.54^{a}
2	176.79±0.00^{a}	84.73±16.07^{a}	24.55±7.13^{a}	3.14±0.00^{a}	0.79±0.00^{a}	16.57±0.54^{c}
3	176.79±0.00^{a}	55.68±40.07^{a}	13.56±8.17^{a}	0.79±0.00^{a}	0.39±0.00^{a}	16.14±0.38^{c}
4	176.79±0.00^{a}	88.67±18.33^{a}	30.19±23.72^{a}	3.88±1.58^{a}	1.13±0.89^{a}	17.14±0.38^{b}
5	176.79±0.00^{a}	88.67±18.33^{a}	24.55±7.13^{a}	3.14±0.00^{a}	0.79±0.00^{a}	17.14±0.38^{b}

Values are expressed as mean ± SD for groups of seven animals each. Data with different superscript letters along the same column are significantly different (p<0.05) using one ANOVA followed by Duncan's test)

Table 6. Effect of topical application of *M. myristica* and *M. tenuifolia* seed extracts as percentage wound contraction (%)

Groups	*Percentage (%) wound healing			
	Day 4	Day 8	Day 12	Day 16
1	45.15±1.98^{b}	82.34±10.81^{b}	97.59±1.08^{b}	99.17±0.65^{b}
2	52.03±9.14^{ab}	85.94±10.55^{b}	98.12±0.00^{b}	99.85±0.00^{a}
3	68.04±0.51^{a}	92.18±0.17^{a}	99.31±0.29^{a}	100.00±0.0^{a}
4	49.84±10.37^{b}	82.92±13.05^{b}	97.59±1.08^{b}	99.36±0.50^{b}
5	49.24±0.41^{b}	86.03±0.53^{ab}	98.09±0.11^{b}	99.46±0.20^{ab}

Values are expressed as mean ± SD for groups of seven animals each. Data with different superscript letters along the same column are significantly different (p<0.05) using one ANOVA followed by Duncan's test)

Table 7. Histopathology of the skin wound area

Group	Skin layer	Myofibroblasts and fibroblasts	Collagen deposition	Angiogenesis	Cell infiltration
1	Numerous endocrine glands with deep pink eosinophilic materials	Abundant myofibroblasts and fibroblasts present	Abundant loose collagen deposition	Numerous endocrine glands present in the subcutis	Moderate cells include predominantly lymphocytes and a few macrophages and neutrophils
2	Complete thin layer of epidermis with keratin	Moderate amount of fibroblasts and myofibroblasts	Moderate dense collagen deposit. Presence of moderate loose collagen in subcutis	Not apparent	Few lymphocytes and macrophages
3	Abundant adipose tissue, very sebaceous glands and endocrine ducts present with numerous hair follicles	Numerous fibroblasts and myofibroblasts	Moderate dense collagen deposit	Moderate angiogenesis	Not apparent
4	Complete epidermal layer with keratin	Numerous fibroblasts and myofibroblasts	Abundant dense collagen in the dermis	Moderate angiogenesis	Moderate predominantly macrophages and lymphocytes
5	Complete epidermal layer with hair follicles	Abundant dense collagen (granulation tissue)	Moderate dense collagen deposit	Few lymphocytes in the subcutis	Abundant fibroblasts and myofibroblasts

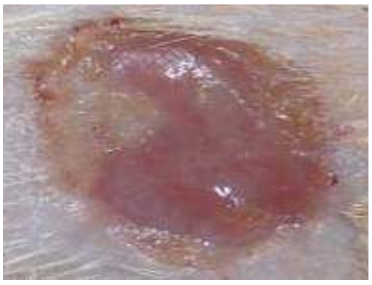
Different chemical analyses were performed in this study to review the wound healing activity of both *M. myristica* and *M. tenuifolia* extracts from their seeds as potential wound healing agents. Among these analyses is the phytoconstituents analysis. The result of the phytoconstituents analysis showed that the following constituents are present Viz: Reducing, sugars, terpenoids tannins, alkaloids, and flavonoids in *M. myristica* seed extract while tannins, saponins, alkaloids, terpenoids steroids and flavonoids were detected in *M. tenuifolia* seed extract. The chemical components present in the extracts of the two plant seeds, mostly terpenoids and flavonoids might be the ones responsible for the role of wound healing effect exerted as it was observed in this work however, further research work on this studies are essential to isolate all these active compound (s) that exerted significant pharmacological activities to this studies [26,12]. As reported by Scortichini [27] and Sasidhran et al. [16], terpenoids are believed to increase the efficient of wound healing process, which is due to their astringent and high antimicrobial activities assumed to be the one playing crucial role in wound contraction and which also rapid the period of epithelisation (Terpenoids or isoprenoids have high antifungal or antimicrobial properties as a result of the non-mevalonate pathway effect. This pathway is very crucial for fungi, protozoans, gram negative bacteria and other micro-organisms to synthesis components of their cell membranes, protein prenylation and as the means of getting carbon [28]. There are studies which have been carried out on some plants that have the same phytoconstituents which are believed to be the ones playing the role of the wound healing activity in rats [29].

In this work, it shows that the extracts from the two seeds exhibited favourable antimicrobial activity against all the tested microorganisms. Hence, *S. aureus, E. coli, A. niger, C. albican* and *B. subtilus* infections could be treated favourably with the extracts of *M. myristica* and *M .tenuifolia*. Extract of *M. myristica* was noticed to prevent the growth of *S. aureus* and *B. subtilus* with the same MIC value of 50 mg/ml, *P. aeroginosa* and *A. niger* also have the same MIC value 100 mg/ml concentration of the extract and *E. coli* have the lowest MIC value at 200 mg/ml of the extract. *M. tenuifolia* was noticed to have higher antimicrobial property more than *M. myristica* seed extract because it inhibited the growth of *E. coli, A. niger, C. albican* and *B. subtilus* at MIC value of 50 mg/ml. *P. aeroginosa* and *S. aureus* were found to be more resistant than the rest of tested organisms with MIC value of 100 mg/ml. Fabry et al. [30] reported that any extract having activity with MIC value below 8 mg/ml can act as an effective antimicrobial agent; it therefore indicated that the extracts of the two species of *Mondora* possess higher effective antimicrobial activity.

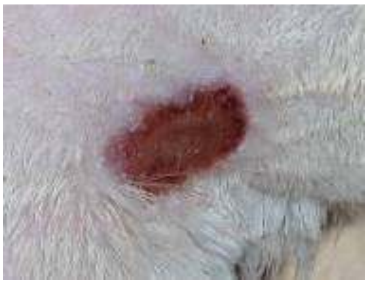
Herbal ointment containing different medicinal plants are used in the folk medicine and have been reported to be beneficial in wound care, promoting wound healing, minimizing pain, discomfort and scarring of the patient [31]. Ointment is the obvious choice of dosage form due to its convenience of topical application; however no single method is adequate to represent the various components of wound healing process [4].

The process of shrinkage of wound area depends on the repairing abilities of the tissue type, extents of the damage and states of tissue health [32]. *In vivo* studies revealed the enhanced rate of wound contraction in animals treated with ointment containing the extracts of *M. myristica* and *M. tenuifolia* seeds as compared to control group. This might be due to enhanced epithelialisation in shorter time because the two species promoted epithelialisation either by facilitating the proliferation or by increasing the viability of epithelial cells.

Day 0



Day 4 (Group 1)



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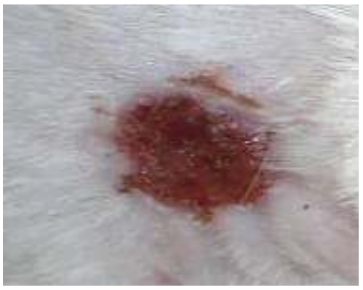

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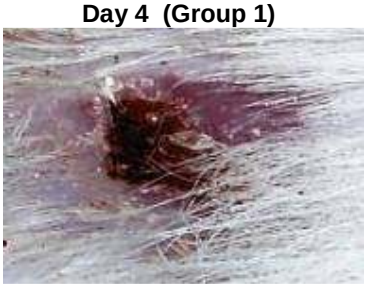
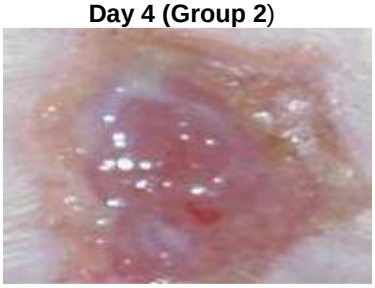
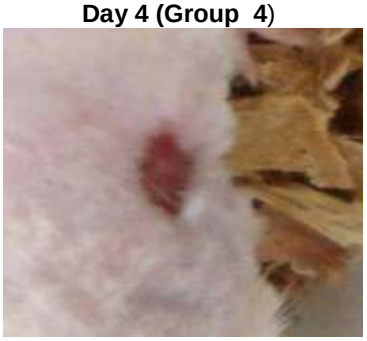


Day 4 (Group 4)



Day 4 (Group 5)

Day 8 (Group 1)



Day 8 (Group 2)

Day 8 (Group 3)

Day 8 (Group 4)

Day 8 (Group 5)

Day 12 (Group 1)

Day 12 (Group 2)

Day 12 (Group 3)

Day 12 (Group 4)

Day 12 (Group 5)

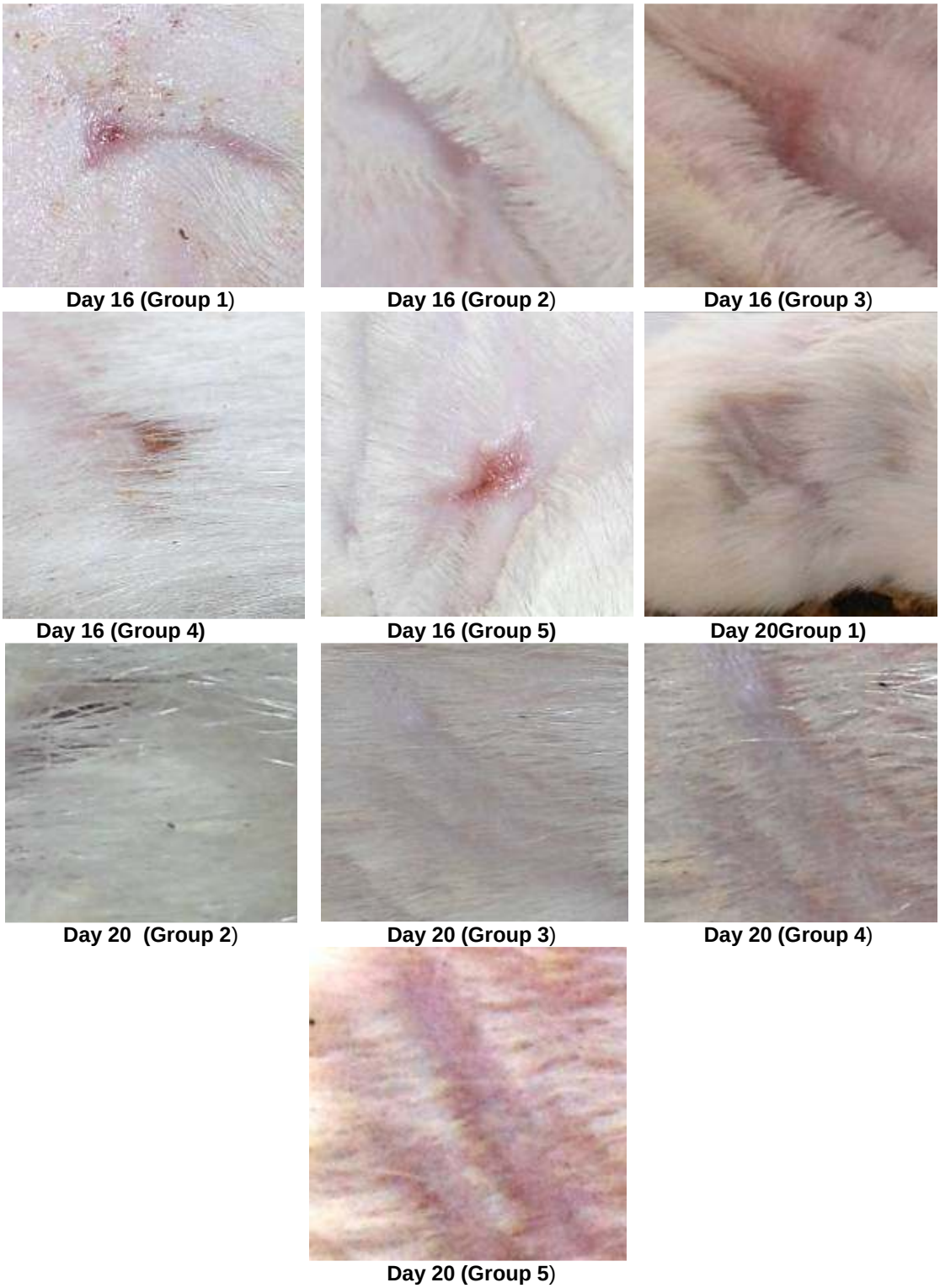
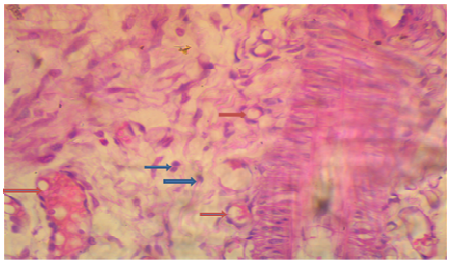
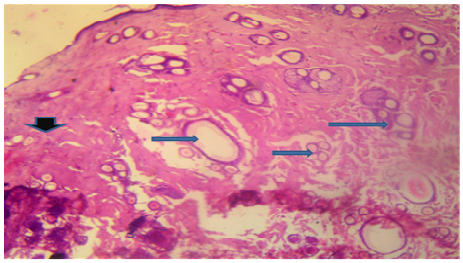


Fig. 1. Photograph showing various stages of wound healing activity of methanolic extract of *Mondora myristica* and *Monodora tenuifolia* seeds (Excision wound model)

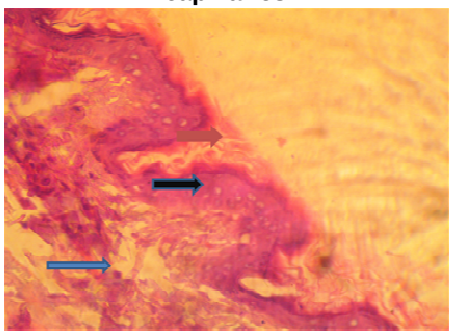
Wound contraction, is one of the stages of the proliferative phase of healing wound, it occurs via the centripetal movement of the tissues surrounding the wound and is controlled by myofibroblasts [33]. The wound contraction increased observed in all the groups treated with the extracts may be as a result to the enhanced activity of fibroblast by the extracts of *M. myristica* and *M. tenuifolia.* The increase in collagen content noticed significantly may be enhancement of migration of fibroblasts and epithelial cells to the wound site as it was observed during the wound healing process in the treated groups. The regeneration of dermal and epidermal layers that was noticed very quickly in the treated group affirmed that the ointment-extracts impacted a positive effect toward cellular proliferation, granulation tissue formation, and epithelialisation. The formation of collagen bundles in the treated groups as shown in haematoxylin and eosin staining for histological studies support the efficacy of *M. myristica* and *M. tenuifolia* on fibroblast proliferation and synthesis of extracellular matrix during healing (Table 7), (Fig. 1, days 8-20) and (Fig. 2 in all the groups). Therefore, a treatment could influence the healing process by intervening one or more phases of healing. Here, the animals treated with the extract of *M. tenuifolia* seeds showed a significant increased amount of granulation tissue, indicating increase collagen turnover more than *M. myristica* and Chithra et al. [34] reported that collagen is the predominant extracellular protein in the granulation tissue of wounds. The present study, between the five tested groups, showed that the wound healing activity of the groups 2, 3, 4 and 5 of the extracts ointment base treatment are higher than the ointment base alone. This is probably because the extracts contain higher amount of phytoconstituents which are responsible for the wound healing activity in rats than the paraffin ointment.



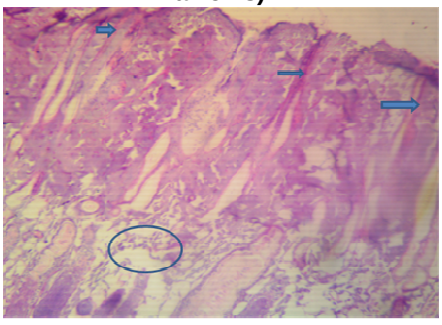
H&E x100 for group (1) control animals treated with ointment: Section showing an area with active angiogenesis just below the epidermis. Few can be seen in the section. Red arrows show newly formed capillaries



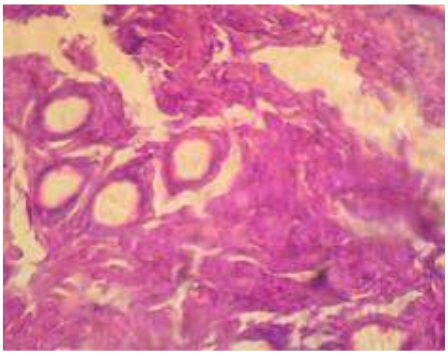
H&E x40 for rat in group (2) experimental animals treated with 10 % *M. myristica extract*: Section of skin with abundant dense collageneous tissue (mature) (black arrow) and numerous hair follicles (blue arrows)



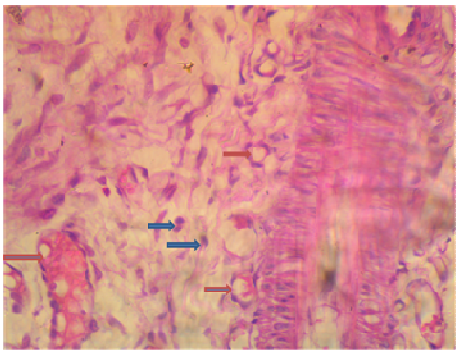
H&E x100 for rat in group (1) control animal treated with ointment: Red arrow indicates the keratin layer. The black represents the epidermis and the blue shows the dermis with predominantly loose connective tissue



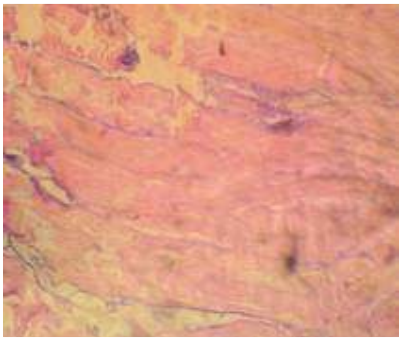
H&E x40 for rat in group (2) experimental animal treated with 10 % M. myristica extract ointment: Section of healing skin with numerous hair follicles (blue arrow), the dermis has a marked amount of dense collageneous tissue. Few infiltrating cells are present in the dermis (blue circle)



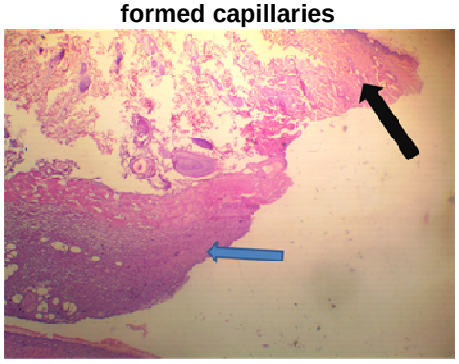
H&E x40 for rat in group (3) experimental animal treated with 10 % *M. tenuifolia* extract: Section showing a locally extensive area of dense collageneous tissue



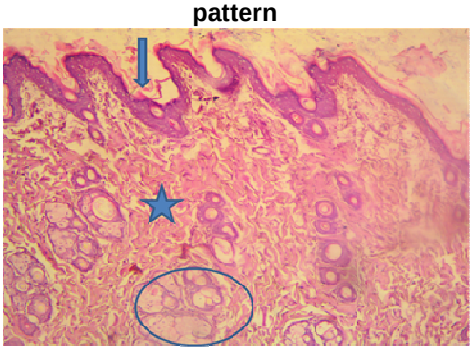
H&E x100 for rat in group (3) experimental animal treated with 10 % *M. tenuifolia* extract: Section showing an area with active angiogenesis just below the epidermis. Few lymphocytes can be seen in the section. Red arrows show newly formed capillaries



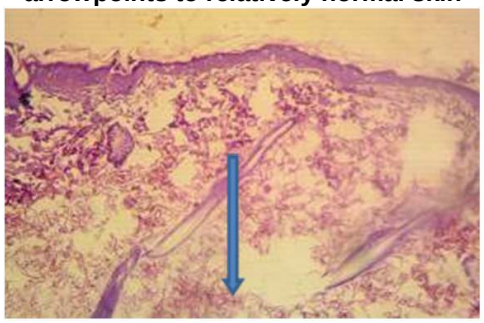
H&E x40 for rat in group (4) experimental animal treated with 5 % M. myristica extract: Section of the dermis with organized collageneous tissue arranged in layer wavy pattern



H&E x40 for rat in group (4) experimental animal treated with 5 % M. myristica extract: Blue arrow pointing to dense fibro-adipose tissue (scar tissue) while the black arrowpoints to relatively normal skin



H&E X100 for rat in group (5) experimental animal treated with 5% *M. tenuifolia* extract: Complete skin with keratin layer (arrow) dense connective tissue (star) and glands (circle)



H&E x100 for rat in group (5) experimental animal treated with 10% *M. tenuifolia* extract: Section showing complete skin with predominantly loose connective tissue in the sub cutis. Few hair follicles are also evident (arrow)

Fig. 2. Haematoxylin and eosin stained sections of the granulation tissues in control and experimental groups

5. CONCLUSION

The present study demonstrated that the topical application of extracts of *M. myristica* and *M. tenuifolia* seeds promote wound healing activity in rats, probably due to their ability to scavenge free radicals, inhibition of some mediators of inflammatory pathway, and inhibition of some bacteria that might have been harboured on the wound [35]. The histopathological results, along with the wound contraction rate and period of epithelialisation also demonstrate the wound healing effect of the two species of the plants. Thus, the results of the present study offer pharmacological evidence of the folk use of *M. myristica* and *M. tenuifolia* plants for wound healing.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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