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Healing Potentials of Oral *Moringa Oleifera* Leaves Extract and Tetracycline on Methicillin Resistant *Staphylococcus Aureus* Infected Wounds of Wistar rats

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Summary: The effects of oral dose of aqueous extract of Moringa oleifera and tetracycline antibiotics on cutaneous wounds infected with Staphylococcus aureus were studied in eighteen adult wistar rats (159±31.5g) randomized into three groups: Group A, n = 6, Moringa oleifera-(300 mg/kg). Group B, n = 6, tetracycline (9.4 mg/kg) and Group C, n = 6, Sterile water (control). Six millimetres diameter nape wound, created on each rat under 2% xylazine (5 mg/kg) and 5% ketamine (35 mg/kg), was contaminated with Staphylococcus aureus (108 Colony Forming Unit (CFU). Following infection, treatment was commenced with daily oral dose of test preparations and the wounds were evaluated every other day i.e., day 3, 5, 7, 9, 11, 13 and 15 for wetness (wound exudation), wound edge oedema, hyperaemia, granulation tissues and contraction (diameter). Severe wound exudation existed in all the groups between days 0-3 ($p = 1.00$). A significantly less ($p<0.05$) wound exudation was observed at days 3-5 ($p = 0.000$) and 5-9 ($p = 0.003$) (Control< Tetracycline <Moringa). Wound edge oedema was significantly less ($p<0.05$) on days 5-9 ($p = 0.000$) and 9-15 ($p = 0.001$) (Control<Moringa<Tetracycline). Hyperaemia was pronounced in all the groups from days 0-3, but became significantly less ($p<0.05$) at days 5-7 ($p = 0.002$) and 9-15 ($p = 0.001$) (Control<Moringa<Tetracycline). A significantly ($p<0.05$) more wound granulation tissue was observed among the groups at days 5-9 ($p = 0.002$) and 9-15 ($p = 0.001$) (Control> Moringa> Tetracycline). Differences in wound diameter was not significant except at days 5-9 ($p = 0.013$) (Control> Moringa >Tetracycline). Oral doses of Moringa oleifera extract (300mg/kg) and tetracycline (9.4mg/kg) are not effective as antimicrobial or immune-boosting agents to enhance healing of wounds infected with Staphylococcus aureus and hence not recommended for rapid clearance of Staphylococcus aureus infected wounds.

Keywords*:* Infected wound, Moringa oleifera, Staphylococcus aureus, Tetracycline.

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INTRODUCTION

Success in infected wound management depends, to a large extent, on an understanding of wound healing process, properties of wound healing agents and the ability to decide on the most appropriate and cost effective method for wound management. This is most important in poor resource settings where modern and proven wound management materials are rarely available and affordable by only a few (Eyarefe et al., 2014).

A wound is a disruption of normal anatomic structure and function and may be with, or without loss of tissue (Cooper et al., 2001). Wound healing as a biologic process involves complex interactions of an array of mediators and the immune system and geared at restoration of lost tissues and tissue integrity (Singer and Clark, 1999, Fossum et al.,2007). The rate at which wound heals is a measurable index of interest in wound research (Eyarefe et al., 2014). Infection has been known to complicate and prolong healing by interrupting the biologic process of wound repair (Heggers, 2003). Local and systemic signs of wound infection, such as, erythema, oedema, changes in character and rate of drainage, increased odour, fever and increased white blood cell count constitute practical indices for prediction of rate of wound healing (Doughty, 1992, Paul and Sharma, 2004). Success in wound management therefore depends on understanding of the healing processes combined with the knowledge of the properties of wound healing agents.

Antibiotics are generally indicated in the management of contaminated and infected wounds in animals (Verwilghen and Singh, 2015; Fossum et al., 2007). The tetracyclines are the commonest drugs used in treatment of varieties of infections (Griffin et al., 2010). The rapid emergence of bacteria resistance to tetracyclines has, however, heightened resurgence of interests in the study of mechanism of drug resistance and development of herbal alternatives (Palaniappan and Holley, 2010).

Moringa oleifera is a small, fast-growing evergreen deciduous tree that usually grows as high as 9 m, with a soft and white wood and gummy bark (Mishra et al., 2011). The tree is native to India, but has been cultivated in north-eastern Pakistan, north-eastern Bangladesh, Sri Lanka, West Asia, the Arabian Peninsula, East and West Africa, throughout the West Indies and southern Florida, in Central and South America from Mexico to Peru, as well as in Brazil and Paraguay (Roloff et al., 2009; Mishra et al., 2011). Traditionally, the plant is used as antispasmodic, stimulant, expectorant, diuretic, cardiac circulatory tonic and antiseptic, antipyretic, anthelmintic and antiepileptic (Nadkarni, 2009).

Several phytochemicals have been isolated from various parts of the Moringa plant with claims of efficacies against a wide array of diseases. Renitta et al., (2009) investigated the phytochemicals present in the leaves, seeds and flowers using ethanolic extract of Moringa oleifera. The leaves contain fifteen complex components. The major compounds were hexadecanoic acid, Ethyl palmitate, Palmitic acid ethyl ester, 2, 6- 0.5Dimethyl-I, 7 -octadiene-3-ol, 4-Hexadecen-6-yne, 2-hexanone, 3-cyclohexyliden-4-ethyl - E2- Dodecenylacetate, Hi-oleic safflower oil. The seeds also contain Moringyne, 4-(0.- L-rhamnosyloxy) benzyl isothiocyanate and several amino acids. From the flowers, 9- Octadecen - 1- 01, cis - 9 - Octadecen - 1 -01, Oleol, Satol, Ocenol, Sipo, Decanoic acid, Dodecanal were identified as major compounds.

Moringa oleifera is widely consumed by a large proportion of rural and city dwellers in developing countries of Africa and Asia for its medicinal and nutritional values. The leaf contains aspartic acid, glutamic acid, glycine, threonine, alanine, valine, leucine, isoleucine, histidine, lysine, phenylalanine, tryptophan, cysteine and methionine (Dubey et al., 2004; Rastogi and Mehrotra, 2006). Some acclaimed medicinal properties (Mishra et al., 2011) include immune-boosting, antimicrobial and wound healing potentials (Walter et al., 2011, Ghebremichael et al., 2005, Lockett et al., 2000, Rathi et al., 2006, Vijay and Kumar, 2012). Caceres et aI., (1991) reported antimicrobial activities of Moringa oleifera leaves, roots, bark and seeds in an in-vitro study against bacteria, yeast, dermatophytes and helminths by a disk-diffusion method. The fresh leaf juice and aqueous extracts from the seeds inhibited the growth of Pseudomonas aeruginosa and Staphylococcus aureus. However, in another of an in-vitro study (Alam et al., 2009), Moringa oleifera fresh leaves exhibited inhibitory effect against all the tested Gram-negative bacteria and Gram-positive bacteria except against S. aureus and Streptococcus-B- haemoytica. Vijay and Kumar, (2012) investigated the wound healing potentials of stem bark Moringa oleifera in some wistar rats, while Rathi et al., (2006) reported an increase in wound healing rate following oral doses (300mg/kg) aqueous extract of Moringa oleifera leaves in un-infected excision wounds of male Swiss albino mice.

The reports on the effects of Moringa oleifera leaf extract on Staphylococcus aureus are conflicting (Alam et al., 2009; Caceres et aI., 1991) .Besides, there is paucity of in-vivo studies on oral dose antibacterial and wound healing effects of Moringa oleifera on wounds infected with Staphylococcus aureaus; since Staphylococcus aureaus is the most commonly isolated organism from wounds of man and animals. This study therefore investigated the effects of oral dose of aqueous extract of Moringa oleifera on Staphylococcus aureus infected nape wounds of wistar rats.

MATERIALS AND METHODS

Experimental animals: Eighteen adult male rats (mean body weight 159±31.5g) were bred and housed in well ventilated cages and exposed to 12 h light and 12hour dark period. They were fed on rodent chow and had access to water ad libitum. Animal vital parameters including temperature, body weight and faecal examination were assessed and found to be consistent with good health status before the commencement of the study (Eyarefe and Amid, 2010).

All of the animals received humane care according to the criteria outlined in the Guide for the Care and the Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institute of Health. The ethics regulations were followed in accordance with national and institutional guidelines for the protection of the animals' welfare during experiments (Welfare, 2002)

Study design: A 6mm full thickness biopsy punch wound was created on the nape of each rat under anaesthesia and the wounds were contaminated with Staphylococcus aureus (10^8 Colony Forming Unit (CFU) as previously described (Shaikh, 1994, Eyarefe et al., 2014). The rats were then randomized into three groups: Group A, n = 6, *Moringa oleifera* (300 mg/kg). Group B, n = 6, Tetracycline (9.4 mg/kg) and Group C, n = 6, Sterile water (control).

Anaesthesia and wound creation: Each rat was anaesthetized with an intramuscular injection of 5% Ketamine (Rottexmedica, Germany) (35.0 mg/kg) and 2% Xylazine (Arendonik, Germany) (5.0 mg/kg) via the quadriceps group of muscles as earlier described (Eyarefe and Amid, 2010). Following anaesthesia, the dorsum [nape] of each rat was prepared for aseptic surgery by shaving and sterilization with chlorhexidine and alcohol. A full-thickness punch biopsy wound was created using a 6mm skin biopsy punch (Kai Industries Co. ltd, Germany) on the dorsum [nape] of each rat. Each wound was inoculated

with 108 Colony Forming Unit (CFU) of MRSA Staphylococcus aureus earlier isolated and prepared for the purpose as described by Shaikh, 1994. Rats were returned to their cages following recovery from anaesthesia for further monitoring of wounds.

Confirmation of infection: The wounds were left untreated for 48 hours (Khoo et al., 2010), following which infection was confirmed using Gram stain technique, catalase and coagulase tests, as earlier described (Shaikh, 1994).

Preparation of Moringa oleifera aqueous extract: Leaves of Moringa oleifera were obtained from moringa plantation in Ibadan, Nigeria and confirmed at the Herbarium of Botany department, University of Ibadan, Ibadan, Nigeria. The plant leaf derived aqueous extract was prepared in our laboratory by dissolving 30g of air dried and blended into powder of Moringa Oleifera in 100 ml of sterile water to form (300 mg/ml) aqueous extract.

Administration of Oral preparations: Following the confirmation of wound infection, each rat in Group A received 0.5 mL (150 mg) by oral gavage through a pipette twice daily for 7 days based on Moringa Oleifera effective and safety dose evaluation in previous studies rats (Adedapo et al.,2009; Rathi et al., 2006). Rats in group B received 0.5 ml of the tetracycline (0.7%) antibiotics, while rats in group C served as control (no treatment). The oral preparations were administered with minimal discomfort to the animals.

Gross evaluation of wound: The wound of each animal was evaluated every other day (i.e., day 3, 5, 7, 11, 13, 15) for 15 days and scored as earlier described (Khoo et al., 2010; Eyarefe et al., 2014), using wetness/dryness, wound edge oedema, colour, granulation tissues and contraction as vital wound infection and healing parameters. Wound was either wet or dry. Wound edge edema was either present or absent. Wound colour (hyperaemia) was severe, moderate or absent (0). Granulation tissue was graded as high, low or absent (0). Wound size (mm) was measured by taking the dimensions of wound with a digital veneer caliper (Globetronics and Co. ltd, Germany).

RESULTS

Wound wetness (exudation) evaluation: A severe wound wetness was observed in all the groups between days 0-3 ($p = 1.00$). Wounds of animals in the Control group showed significantly less ($p>0.05$) wetness at days 3-5 ($p = 0.000$) and 5-9 ($p = 0.003$) compared with those of moringa and tetracycline. The trend of wound wetness was control< tetracycline<moringa Table 1.

Wound edge oedema: Wounds of rats in the control group had significantly less ($p>0.05$) wound edge oedema compared with those of moringa and tetracycline on days 5-9 ($p = 0.000$) and 9-15 ($p = 0.001$). The trend of wound edge oedema was control <moringa <tetracycline). On the fifth day post-infection, 33.3% of rats in control group had moderate wound edge oedema, while 66% of rats in the moringa and 83.3 % of rats in the tetracycline group had wound edge oedema Table 2.

Hyperaemia evaluation: Hyperaemia was pronounced in all the groups from days 0 - 3, but became significantly less ($p<0.05$) at days 5-7 ($p = 0.002$) and 9-15 ($p = 0.001$) with trend being (Control <Moringa <Tetracycline) Table 3.

Granulation tissue: A significantly ($p<0.05$) more wound granulation tissue was observed among the groups at days 5-9 ($p = 0.002$) and 9-15 ($p = 0.001$) with trend being (Control>Moringa> Tetracycline). Granulation tissue formation was protracted in the moringa and tetracycline groups compared with the control Table 4.

Wound contraction: Differences in wound diameter (a reflection of wound contraction) was not significantly notable in all the groups at various days of measurement except at days 5-9 ($p = 0.013$) with the control group showing greater contraction than moringa and tetracycline groups (Control> Moringa> Tetracycline) Table 5.

Table 1: Evaluation of wetness/dryness of wounds in moringa, tetracycline and control rats at different postoperative days

Day	Moringa		Tetracycline		Control	
	n	%	n	%	n	%
0	0	0.00	0	0.00	0	0.00
3	6	100	6	100	6	100
5	3	50.0	4	66.7	0	0.00
7	1	16.7	0	0.00	2	33.3
9	2	33.3	1	16.7	0	0.00
11	0	0.00	0	0.00	0	0.00
13	0	0.00	0	0.00	0	0.00
15	0	0.00	0	0.00	0	0.00

n = Number of animals. % = percentage of animals with wet wounds

Table 2: Evaluation of wound edge edema in moringa, tetracycline and control groups at different postoperative days

Day	Moringa		Tetracycline		Control	
	n	%	n	%	n	%
0	0	0.00	0	0.00	0	0.00
3	6	100	6	100	5	83.3
5	4	66.7	5	83.3	2	33.3
7	2	33.3	3	50.0	0	0.00
9	1	16.7	2	33.3	0	0.00
11	0	0.00	2	33.3	0	0.00
13	0	0.00	0	0.00	0	0.00
15	0	0.00	0	0.00	0	0.00

n = Number of animals. % = percentage of animals with edema of wound edge

Table 3: Evaluation of degree of wound hyperemia in Moringa, tetracycline and control groups at different postoperative days.

Day	**Moringa**				**Tetracycline**				**Control**			
	S. Hyp		M. Hyp		S. Hyp		M. Hyp		S. Hyp		M. Hyp	
	n	%	n	%	n	%	n	%	n	%	n	%
0	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
3	5	83.3	1	16.7	5	83.3	1	16.7	4	66.7	2	33.3
5	4	66.7	2	33.3	2	33.3	4	66.7	2	33.3	4	66.7
7	1	16.7	5	83.3	3	50.0	3	50.0	2	33.3	4	66.7
9	0	0.00	5	83.3	0	0.00	4	66.7	0	0.00	6	100
11	0	0.00	1	16.7	0	0.00	1	16.7	0	0.00	0	0.00
13	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
15	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00

n = Number of animals with moderate hyperaemia (M. hyp) and severe hyperaemia (S. Hyp), %= percentage of animals with S. hyp or M. hyp.

Table 4: Granulation tissue evaluations in moringa, tetracycline and control groups at different postoperative days

Day	Moringa				Tetracycline				Control			
	H.Gra		M.Gra		H.Gra		M.Gra		H.Gra		M.Gra	
	n	%	n	%	n	%	n	%	n	%	n	%
0	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
3	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
5	0	0.00	6	100	0	0.00	6	100	0	0.00	4	66.7
7	4	66.7	2	33.3	3	50.0	3	50.0	1	16.7	5	83.3
9	2	33.3	0	0.00	2	33.3	2	33.3	5	83.3	1	16.7
11	0	0.00	2	33.3	0	0.00	1	16.7	0	0.00	1	16.7
13	0	0.00	2	33.3	0	0.00	0	0.00	0	0.00	0	0.00
15	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00

n = Number of animals with high granulation (H.gra) and low granulation (L. gra). % = percentage of animals with M.gra and H. gra

Table 5: Mean (±Standard Deviation) of wound diameter (Mm) in Moringa, tetracyclineand control groups at different Post-operative days.

Day	***Moringa***	**Tetracycline**	**Control**
0	9.217±0.61	9.400±0.49	8.567±0.79
3	6.700±0.95	6.983±0.55	6.833±1.27
5	5.600±0.84	6.600±1.01	5.150±1.05
7	4.767±1.39	5.850±2.46	3.100±1.30
9	3.660±1.70	3.750±2.53	1.400±0.64
11	2.800±2.55	2.800±2.16	0.000±0.00
13	3.600±0.57	4.300±0.00	0.000±0.00
15	0.000±0.00	0.000±0.00	0.000±0.00

DISCUSSION

The result of this study shows that oral aqueous extract of Moringa oleifera and tetracycline are ineffective in aiding debridement and repair process of wounds with MRSA infected wounds. The pathogenic strain of Staphylococcus aureus used in this study was selected through careful laboratory bacteriological process as in previous study (Eyarefe et al., 2014). Staphylococcus aureus was used in this study for its established prominence in skin wound infections (Franklin and Lowly, 1998). The dose of M. oleifera used was calculated based on previously established safety and effective dose derivation for rats (Adedapo et al., 2009, Rathi et al., 2006). Oral route of administration of study preparations was adopted to simulate the commonest route of consumption of these products. Wound wetness, edge oedema, cplour (hyperaemia), granulation tissue and contraction were wound healing indices as in previous studies (Khoo et al., 2010, Eyarefe et al., 2014). The severity of wound wetness within days 0-3 in all the groups (Table 1) was a reflection of tissue response to injury and a local sign of wound infection (Paul and Sharma, 2004, Eyarefe et al., 2014). The improved wound dryness in the

control group as compared with M. oleifera and tetracycline groups shows their better immune response for wound debridement at the acute period of tissue response to injury and infection. Wound edge oedema, also a local sign of wound infection and associated with tissue response to Staphylococcus aureus cytoxins (Franklin and Lowly, 1998), was very prominent in all the groups between days 3-7. It however resolved more quickly in the control group than in M. oleifera and tetracycline groups (Table 2). Hyperaemia was pronounced in all the groups from days 0-3, but became significantly less ($p<0.05$) at days 5-7 ($p = 0.002$) and 9-15 ($p = 0.001$) with trend being (Control <Moringa < Tetracycline). Wound hyperaemia which was concurrent with wound edge edema and tissue exudation are evidence of wound infection, associated with endothelial responses necessary to mobilize cellular immune mediators to combat infection and initiate wound repair process (Slatter et al., 2003, Brem and Tomic-canic, 2007). Wound repair phase is characterized by stimulation of Deoxyribonucleic Acid (DNA) and fibroblast proliferation by macrophages, coupled with a complex interaction of arrays of wound repair bio-factors including new capillaries, fibroblast and fibrous tissues that form bright red and fleshy granulation tissue lay down often observed 3-5 days after injury (Fossum et al., 2007).

The delay in the initiation of granulation tissue lay down among the groups, as observed in this study, may be associated with the presence of wound infection which prolonged the initiation of the repair phase (Fossum et al., 2007, Eyarefe et al , 2014). The low granulation tissue in tetracycline group may be associated with development of resistance to the drug by the infected organism (Franklin and Lowly, 1998). The lower granulation tissue lay down of the moringa as compared with control group at days: 5-9 ($p = 0.002$) and 9-15 ($p = 0.001$) (Table 4) may be associated with the challenge of detoxification and biotransformation induced stress associated with the administered agents (Siwik et al., 2001). Stress on the immune system alters cellular physiological activities making healing more difficult (Siwik et al., 2001, Soneja et al., 2005). This has also reflected in this study by the slow pace of wound contraction which was not significant in all the groups at various days of measurement except at days 5-9 ($p = 0.013$) with the control group showing greater contraction than moringa and tetracycline groups (Table 5).

We therefore hypothesized that, in the absence of immune suppression prior to injury, the body will adequately initiate wound healing process with or without external intervention. Also, in the event of Staphylococcus aureus wound infection, the oral dose of moringa extract and tetracycline used are not recommended as this study has not found their wound healing benefits in MRSA infected wounds.

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