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Wound Healing Properties of Root Extract of *Harungana madagascariensis* on Wistar Rat Model

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Abstract:- The wound healing properties of root extract of Harungana madasgascariensis on Wistar rat was studied. The excision wound was created on rats by cutting away a 4.9cm^2 full thickness of skin from a determined area on the back of selected rat. The ethanol extract of roots of *H.Madagascariensis* given orally at the doses of 150mg/kg/day, 200mg/kg/day and 250mg/kg/day for 14 consecutive days resulted in a fast wound epithelization, which was more significant in the ones that received a high dose of H.M. The result of wound epithelization of the ones treated when compared to control was statistically significant ($p<0.05$). These results were also supported by an increased Neutrophils and Monocytes in the blood which was statistically significant when compared to negative control. This fast wound epithelization was as measured at days 1, 5 and 10 wound epithelization days until wound healing was completed. These results suggested that H.madagascariensiss extract was potent in management of wound though the active ingredient/s responsible for this action was yet to be known. Hence, suggests that H.M contains products that aid the wound healing process as described by folkloric medicine.

Keywords;- *Harungana madagascariensis, Wound Healing, Excision, Neutrophils, Monocytes.*

I. INTRODUCTION

In this study, we assessed the wound healing properties of root extracts of *H.madagascariensis* after establishing the toxicity levels. The world we live in is highly endowed with lots of natural resources including medicinal plants. This further explains the role played by most of these medicinal plants before the advent of orthodox medicine in the management of disease conditions.

Wound healing remarkably has been categorized into three phases, the early stage or the inflammatory, the proliferative and then remodelling. This inflammatory phase began immediately homeostasis was reached; the primary aim of this stage is to aid the washout of pathogens and other foreign materials from the wound site. However, to restore tissue homeostasis at some point of irritation, there can be a discount or cessation of tissue infiltration by neutrophils and apoptosis of used neutrophils, leading to cascade of events that brought about a counter-regulated mechanism that would transform macrophages from a greater classical cells to a greater activated opportunity cells thereby starting up healing (Reville *et al*, 2006).

White blood cells such as neutrophils and monocytes were mobilized to the wound site; this was attributed to increased permeability of the vascular system together with vasodilation. Cytokines have been suggested as the main inflammatory mediator responsible for this phase (Wynn and Barron, 2010). The production of lots of collagen, which cycle around the fibroblasts now led to the release of substances which formed the foundation for tissue scaffold around the wound area. Normally within 3 days, the proliferative stage was completed. The fast growth phase and angiogenesis that was initiated by the endothelial cells led to the granulation of the tissues creating a good blood anastomosis which supplied areas considered to be very active for the healing. Interestingly, within 2-3 weeks the remodelling stage of wound had occurred, in this very phase collagen type I rather than type III is seen in the new epithelium [Haukipuro *et al.*, 1991].

H.madagascariensis is well associated with a high folkloric claim which wound healing model is a vital part of, this plant has also been used in procuring abortion(Nwobodo and Ezeigbo, 1992). *H. madagascariensis* has been shown to be an excellent analgesic and anti-inflammatory model (Nwodo, 1989). Traditionally, this plant is also used as an antiseptic, in the treatment of anaemia, asthma, tuberculosis, fever, angina, diarrhoea, dysentery, syphilis, gonorrhoea, malaria, parasitic skin diseases, and wounds, as a natural source of dermatological agents and cosmetics (*Tona et al.*, 1998; EMEA, 1999; Lukwa *et al.*, 2001; Erah *et al.*, 2003). The aim of this work is to ascertain the anti-inflammatory activities of root extract of *Harungana madasgascariensis* on Wistar rat.

II. MATERIALS AND METHOD

Plant materials/Prep/ Extraction: The plant was sourced locally from Nenwe in Aninri Local Government area of Enugu state, Nigeria and taken for authentication in Nnamdi Azikiwe University herbarium where voucher specimens No:NAUH 195A were deposited. The root tuber of H. madagascariensis was chopped off and sun dried for 14 days and turned into coarse powder using a pulverizer. The extraction was through maceration in 99% ethanol for 48 h. The brownish filtrate became targeted to dryness in vacuo (yield 5.0% w/w). The phytochemicals of the extract showed that it includes alkaloids, tannins, saponins, flavonoids and glycosides.

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Ethical approval

Ethical approval was obtained from the Faculty of Basic Medical Science, College of Health Science, Nnamdi Azikiwe University, Nnewi campus with ref: NAU/CHS/NC/FMBS/413. Rats managing and remedies conformed to pointers of the Nnamdi Azikiwe University Animal Research Ethics Committee (NAU-AREC) for laboratory animal care and use.

Animals:30 adult male Wistar rat weighing 180±20g were used. It was selected from the Animal house of Nnamdi Azikiwe University; Housed under standard conditions of temperature, humidity and light /dark cycle and fed with rat pellets (Pfizer) and water ad libitum.

Toxicity test: The median deadly dose (LD50) of Harungana Madasgascariensis root extract was anticipated by the use of Albino Wistar rat using oral management of various doses of 100mg/kg/100ml, 150mg/kg/100ml, 200mg/kg/100ml, 250mg/kg/100ml, 300mg/kg/100ml. The animals were examined for manifestation of symptoms of toxicity which includes reduced motor activity, reduced body/limb activity, reduced respiration and subsequently loss of life. Record of death was determined as soon as a loss of life occurred, dosage was then adjusted downwards. Testing was terminated when the top limit (2000-5000mg/kg) was reached without mortality. The LD50 was determined by the highest dose producing (a) 100% and minimal dose producing (b) 0% mortality (Bruce RD,1985)

Assessment of wound healing; excision wound model

Wound excision model: The excision wound was created on rats as described by Marton and Malon (1972) by cutting away a 4.9cm^2 full thickness of skin from a determined area on the back of selected rat. The excised wound was left open. The animals were kept in five groups; 1. Positive control (water and feed only), 2. Negative control (wound excision without treatment), 3. Wound excision with 150mg/kg of H.m , 4. Wound excision with 200mg/kg of H.m and, 5. Wound excision with 250mg/kg of H.m) of 6 animals each, Thirty animals were used for this study. Wound healing potential was determined by wound contraction and wound closure time (Period of epithelization). Wound area was measured using a digital Venier caliper after wound excision and during treatment on alternative days until the wound is completely healed. Blood samples of the Wistar rat was collected using Edta and plain bottles and haematological parameters such as total white blood cell count, Erythrocyte sedimentation rate and white blood cell differential counts were also assessed.

Histological Assessment:

Liver samples were excised and washed with ordinary saline and put in 10% formalin solution. The tissue biopsies were processed with automatic tissue processor and embedded in paraffin. Sections 5µm thickness were sliced with rotary microtome (Leica RM 212 RT). Cut sections were floated on a water tub at 45ºC and left on a warm plate at 65ºC to permit the sections stick firmly to the slide. The tissue sections were then tainted with haematoxylin and eosin (HE) for morphology assessment (Preece, 1972). Histological sections were tested using Leica Light microscope (Leica DM 750) and then photographed.

Total and Differential WBC Counts:

Whole blood specimens were received in EDTA vacutainer tubes. Complete blood cell (CBC) counts were measured inside 6 hours after series and saved at room temperature, or inside 24 hours after series and saved at 4 degrees Centigrade. Any specimens that were clotted or crammed much less than 1/2 of of the tube were rejected. The general and differential WBC counts were assessed via the the Technicon H-1 automated hematology analyzer (Technicon Instruments Corp, Terrytown, NY, USA). (Bruilas C.D *et al*.,1987; Tanner C.M *et al*.,2012)

Analysis of data

Data were expressed as mean ± S.E.M. A One-way Anova was used for the comparison between the test groups and the control. Analysed data was presented using tables. A P scale less than 0.05 was considered statistically significant.

III. RESULTS

Table I. Study on the effect of *H.madagascariensis* on Neutrophils, Lymphocytes, Monocytes and Total white blood cell count when compared with Negative control

GROUP	**NEUTROPHILS MEAN±SEM**	**P-VALUE**	**LYMPHOCYTE MEAN±SEM**	**P-VALUE**	MONOCYTE MEAN±SEM	P-VALUE	TWBCC MEAN±SEM	P-VALUE
NEGATIVE	23.4±0.82		75.8±1.50		0.2±0.2		13.42±1.06	
150MG/KG OF HM	37.8±0.58	0.00	64.0±0.45	0.59	2.2±0.58	1.12	19.25±0.24	0.00
200MG/KG OF HM	32.2±1.02	0.03	72.2±1.62	0.99	2.6±0.81	0.04	17.76±0.05	0.00
250MG/KG OF HM	50.8±2.89	0.00	53.0±4.09	0.07	3.0±0.71	0.02	18.58±0.38	0.00

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Result showed that Neutrophils, Total white blood cell count in the group that received 150mg/kg, 200mg/kg and 250mg/kg of H.m when compared to the Negative control were statistically significant ($p<0.05$). However, the monocytes were statistically significant for the group that received 200mg/kg and 250mg/kg when compared to Negative control.

Table II. Study on the Effect of *H.madagascariensis* on Neutrophils, Lymphocytes, Monocytes and Total white blood cell count when compared with control (water/feed only).

GROUP	NEUTROPHILS MEAN±SEM	P-VALUE	LYMPHOCYTE MEAN±SEM	P-VALUE	MONOCYTE MEAN±SEM	P-VALUE	TWBCC MEAN±SEM	P-VALUE
CONTROL	17.4±0.51		61.8±11.8		0.00±0.00		24.38±0.6	
NEGATIVE	23.4±0.82	0.59	75.8±1.50	0.429	0.2±0.2	0.99	13.42±1.06	0.00
150MG/KG OF HM	37.8±0.58	0.00	64.0±0.45	0.99	2.2±0.58	0.073	19.25±0.24	0.00
200MG/KG OF HM	32.2±1.02	0.00	72.2±1.62	0.695	2.6±0.81	0.026	17.76±0.05	0.00
250MG/KG OF HM	50.8±2.89	0.00	53.0±4.09	0.805	3.0±0.71	0.008	18.58±0.38	0.00

Result showed that Neutrophils, Total white blood cell count in the group that received 150mg/kg, 200mg/kg and 250mg/kg of H.m when compared to the control were statistically significant ($p<0.05$). However, the monocytes were statistically significant for the group that received 200mg/kg and 250mg/kg when compared to control. When comparing Negative control to control(water/feed) only total white blood cell count result is significant.

Table III. The study on the Effect of *H.madagascariensis* on wound epithelization,ALT,AST and ALP when compared to control(water/feed only)

GROUP	WOUND HEALINGLENGHT MEAN±SEM	P-VALUE	ALT MEAN±SEM	P-VALUE	AST MEAN±SEM	P-VALUE	ALP MEAN±SEM	P-VALUE
CONTROL	0.00±0.00		16.4±0.5		53.0±2.5		58.2±0.8	
NEGATIVE	3.73±0.16	0.00	32.8±0.9	0.00	105.4±1.2	0.00	118.0±2.8	0.00
150MG/KG OF HM	2.88±0.16	0.00	23.8±1.02	0.004	105.0±3.5	0.00	77.4±2.7	0.002
200MG/KG OF HM	3.05±0.08	0.00	29.8±2.4	0.00	103.6±6.7	0.00	98.4±5.6	0.00
250MG/KG OF HM	3.0±0.12	0.00	25.72±0.4	0.00	107.8±0.7	0.00	96.6±0.9	0.00

Result showed that Wound epithelization length, ALT, AST and ALP in the group that received 150mg/kg, 200mg/kg, 250mg/kg of H.m and Negative control when compared to the control were statistically significant ($p<0.05$).

Table IV. Study on the effect of *H.madagascariensis* on wound epithelization, ALT, AST and ALP when compared to negative control.

GROUP	WOUND HEALINGLENGHT MEAN±SEM	P-VALUE	ALT MEAN±SEM	P-VALUE	AST MEAN±SEM	P-VALUE	ALP MEAN±SEM	P-VALUE
NEGATIVE C	3.73±0.16		32.8±0.9		105.4±1.2		118.0±2.8	
150MG/KG OF HM	2.88±0.16	0.000	23.8±1.02	0.000	105.0±3.5	1.000	77.4±2.7	0.000
200MG/KG OF HM	3.05±0.08	0.005	29.8±2.4	0.450	103.6±6.7	0.991	98.4±5.6	0.002
250MG/KG OF HM	3.0±0.12	0.003	25.72±0.4	0.006	107.8±0.7	0.989	96.6±0.9	0.001

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Result showed that Wound epithelization length and ALP in the group that received 150mg/kg, 200mg/kg, 250mg/kg of H.m and when compared to the Negative control were statistically significant ($p<0.05$). However, ALT was statistically significant in the group that received 150mg/kg and 250mg/kg of H.m when compared to Negative control.

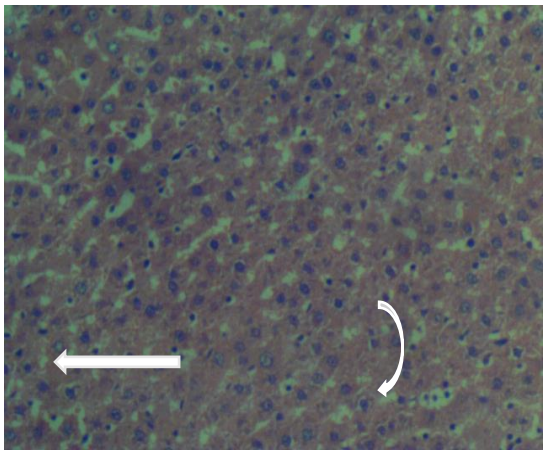

Fig. I (Control): Photomicrograph of liver tissue shows morphology consistent with liver histology. The sinusoids (curved arrow) and hepatocytes (arrow) are normal with no obvious sign of injury (H&Ex400).

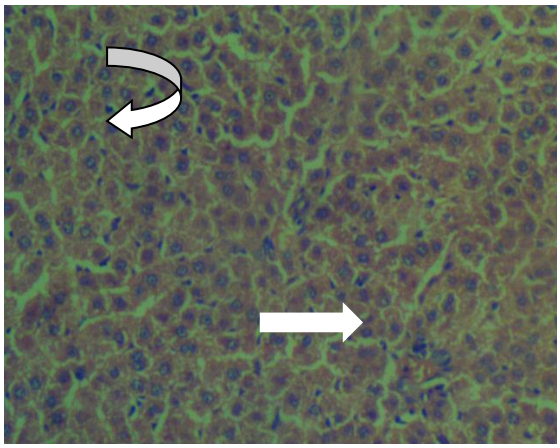

Fig. II: Received 250mg/kg of HM: Photomicrograph of liver tissue shows morphology consistent with liver histology. The sinusoids (curved arrow) and hepatocytes (arrow) are normal with no obvious sign of injury (H&Ex400).

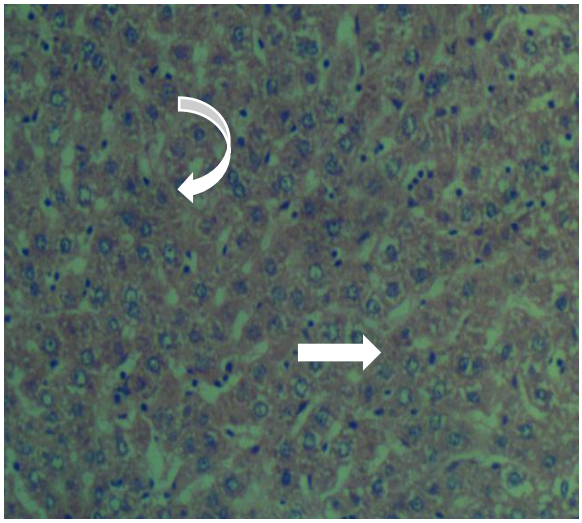

Fig III: Received 150mg/kg of Hm: Photomicrograph of liver tissue shows morphology consistent with liver histology. The sinusoids (curved arrow) and hepatocytes (arrow) are normal with no obvious sign of injury (H&Ex400).

IV. DISCUSSION

The everyday wound recovery system was divided into four overlapping phases: coagulation, inflammation, formation of granulation tissue (proliferative phase), and remodelling or scar formation. During the coagulation phase, blood-clotting occasions save the immoderate bleeding and offered meantime safety of the wounded area. Progression of the inflammatory phase resulted in the recruitment of leukocytes, neutrophils, and macrophages; the production of increase factors; and the activation of dermal and epidermal cells. Completion of the proliferative phase of wound recovery results in formation of ECM-rich, vascularized granulation tissue. Finally, ECM transformation and cell apoptosis cause the formation of scar tissue with bodily residences which are similar with unwounded skin (Singer and Clark,1999; Falanga,2005; Humer *et al*.,2002; Folkman and Klagsbrun,1987 and Sullivan A, *et al*.,1997).

Oral administration of ethanol extracts of roots of *H.madagascariensis* showed a significant increase in Neutrophils and Monocytes in the animal models that received 150mg/kg of H.m, 200mg/kg of H.M and much active in 250mg/kg of H.M when compared to control (one that received water/fed only). Further comparison showed that there was also a significant difference in the ones treated when compared to the negative control (wound healing without treatment). P-value<0.05 was considered as statistically significant. This change has indicated that Proliferative stage of wound healing has started and was more active in the ones treated with the extract which further validated the claim that wound healing properties are present in the extract.

Physiologically, once a wound occurred, this tear of tissue would result in inflammation that would bring about a cascade of events that led to mobilisation of white blood cells to the wound site (Neutrophils and Monocytes) to respond to the damaged tissue in order to help fight off any unwanted pathogens thereby initiating healing process. The result did show that Neutrophils and monocytes were mobilised at a high rate in the ones treated with the extract. The mobilisation of neutrophils and monocytes to the wound site was also as a result of increased vascular permeability together with vasodilation. Since cytokines helped in regulating this phase, a complex interplay that allows monocyte conversion to macrophages which was seen by some as a master regulator with respect to inflammatory phase of wound healing [Wynn and Barron, 2010]. This extract has shown to be more potent in initiating this cascade of events that led to healing proper thereby confirming its role as a wound healing model. These macrophages in turn started up this mechanism; Production of lots collagen, which cycles around the fibroblast, which now resulted in the release of substances which led to the formation of tissue scaffold at the wound site. This occurred within 3 days and formed part of the proliferative stage. It's pertinent to note that the endothelial cells progressed to a fast growth phase and angiogenesis occurred especially during the granulation of tissue, thereby forming very rich blood anastomosis that supplied areas considered as the active site for the healing. These Typically occurred within 2 weeks, wound was seen to be modified or completed, this phase was where collagen type was restored type I rather than type III seen in a new wound [Haukipuro *et al*.,1991] as seen in the study.

When comparing the wound epithelization of the negative control to the ones treated with extract, results showed that it's statistically significant. This helped to broaden the belief by folkloric medicine that this extract was used in wound healing process. The increased neutrophils and monocytes in the ones treated when compared to the untreated and its faster wound epithelization are all closely associated and hence was linked to the action of the extract.

The liver transaminases such as AST (aspartate transaminase), ALT (alanine transaminase) and Alkaline phosphatase(ALP) have long been known to be used in the assessment of liver injury, hence used as a biomarker of choice for centuries (Howell *et al.*,2014). However, ALT was most abundantly found in the liver hence making ALT the more specific indicator of liver inflammation than AST which was found in other organs like cardiac muscle and kidney. The result showed that the ones treated when compared to the untreated, ALT and ALP elevations were statistically significant ($p<0.05$). Inflammation that occurred in the liver was for the protection of organ from infection and injury, however excessive inflammation in the hepatocytes would lead to its loss, Ischemia-reperfusion injury, and metabolic alterations and finally causes permanent damage on the liver (Brenner *et al.*, 2013). The inflammation that occurred was neither excessive nor chronic. This could be given as the reason for slight increase in ALT and ALP in the ones treated. However, the liver histology did not show any distorted morphology in the ones treated when compared to the ones not treated. This again confirmed that the extract was safe at 250mg/kg while being used in management of disease conditions in animal model.

Our study indicates that the root extract of *H.madagascariensis* has proven to be potent in control of wound recovery due to its decreased wound epithelization consequently the interest to extract the active substances found in this extract for better pharmacological uses in future. This study showed that safety level was high in rats and it has a wide usage among human subjects in many conditions.

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