

Amino Acid Profile, Total Protein and Histological Changes in Female Wistar Rats After Administration of Dichloromethane Extract of *Heterotis Rotundifolia* Leaves on Dermal Wound.

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ABSTRACT

The study investigated the amino acid and wound healing potentials of the leaves of *Heterotis rotundifolia*. Amino acid analysis was performed using HPLC. Total protein level, body weight and histology were studied. Excision wound (2cm) was induced on the dorsal skin of 75 wistar rats (176-308g) divided into five groups of fifteen animals: control, 0.1g Vaseline, 0.1g gentamicin (reference drug), 0.1g extract+0.1g Vaseline (HRE1) and 0.2g extract+0.1g Vaseline (HRE2). At the end of days 1, 7, 14 and 21, rats were sacrificed and blood was collected for wound healing parameters while wound surface was taken for histology. The result revealed a higher total protein level of HRE1 with a significant difference ($p < 0.05$) on day 21 compared to control and days 1-7 compared to gentamicin. Body weight in HRE2 significantly increased from days 1-21 with a significant difference ($p < 0.05$) on days 1-7 compared to control and 1-14 compared to gentamicin. Histological examination revealed faster collagen deposition and angiogenesis on day 14 in HRE1 compared to control and gentamicin. Eighteen amino acids were detected with aspartate, methionine and arginine, (12.94, 9.38 and 8.74mg/100g respectively) having the highest concentrations. *Heterotis rotundifolia* leaves contains essential amino acids and has wound healing potentials.

Keywords: *Heterotis rotundifolia* leaves, wound healing, amino acid and histology.

INTRODUCTION

Globally, herbal remedies derived from medicinal plant materials account for a significant portion of the medicinal market. Wound occurs when the skin is damaged anatomically or functionally. Wound healing is a complex process of tissue repair or remodeling (Ofusori *et al.*, 2023). For centuries, plants and their constituents have been extensively used for the treatment and management of wounds. However, there is currently a great deal of research being done on different types of biopolymers for developing economical, sustainable, stable, and effective delivery systems for wound treatment (Sharma *et al.*, 2021). The herbal remedies for wound healing are not only cheap and affordable but are also supposedly safe as highly sensitive reactions are barely encountered when these agents are being used. Through multiple mechanisms, these natural agents trigger healing and regeneration of the damaged tissue. However, scientific validation, standardization and safety evaluation of medicinal plants employed in traditional medicine is very imperative before they could be recommended as wound healing agents (Alam *et al.*, 2011). *Heterotis rotundifolia* (Melastomataceae) known as pink lady is a perennial decumbent herb predominant in Tropical West Africa. *H. rotundifolia* leaves are reported to possess antibacterial, antioxidative, antitrypanosomal, antiplasmodial, antidiarrhoea, antisickling, antifungal and antiulcer potentials (Yeboah and Osafo, 2017).

There is no detailed scientific study on the use of the plant in wound healing although it is used in folk medicine. This study therefore examine the effect of dichloromethane extract of *Heterotis rotundifolia*

leaves on the amino acid composition, total protein level, body weight and histology of dermal wound on female wistar albino rats.

MATERIALS AND METHODS

Samples

Heterotis rotundifolia fresh leaves were harvested from within Abuja campus, University of Port Harcourt. The voucher specimen of the same plant has been deposited at the Department of Plant Science and Biotechnology, University of Port Harcourt Herbarium (voucher number UPH/V/1323). The fresh leaves were carefully washed and air-dried at room temperature and grounded to powder using a mill.

Preparation of extract

Powdered leaves of *Heterotis rotundifolia* (477.12g) were dissolved in 1000ml of dichloromethane (70%) solvent, left for 3 days at room temperature and stirred 2-3 times a day. The mixture was filtered and the clear solution was concentrated using rotary evaporator at 45°C, it was transferred to evaporating dish placed on a water bath. The obtained extract was weighed, stored, labeled in screw-capped bottle and kept at room temperature.

Experimental animals and design

Female wistar albino rats weighing between 176-307g were purchased from the Department of Pharmacology and Toxicology animal house. The usage of the animals for this project was approved by University of Port Harcourt Ethical Committee with ethical number

UPH/CERAMAD/REC/MM60/040. Animals were housed and handled according to the appropriate guidelines principles for laboratory animal use and care. The rats were then divided into five groups of fifteen rats each, left for a week to acclimatize and they had access to normal rat chow and water ad libitum. Topical administration of animals in each group was as follows:

Group	Treatment
Control	Untreated animals (natural healing)
Vaseline	Animals treated with Vaseline (vehicle control)
Gentamicin	Animals treated with gentamicin (reference drug)
HRE1	Animals treated with 0.1g extract + 0.1g Vaseline
HRE2	Animals treated with 0.2g extract + 0.1g Vaseline

Wound creation

Partial thickness excision wound (2cm) was induced on the dorsal skin of each animal, according to Zaki *et al.*, 2011 with little modifications.

Amino acid analysis (Elkin and Griffith, 1985)

Preparation of samples and standards

Prior to derivatization, sample proteins were hydrolyzed as follows. 0.1g of lyophilized sample was weighed and put in 16×125mm screw cap pyrex (Barcelona, Spain) tube, 15ml of 6N hydrochloric acid was added and the tube was thoroughly flushed with N₂, quickly capped and put in an oven at 110°C for 24 hours.

After hydrolysis, the tube contents were vacuum filtered (Whatmann #541, Maidstone, England) to remove solids. The filtrate was filled to 25ml with water, and aliquot of this solution was further filtered in a 0.5 μ m pore size membrane (Millipore, Madrid, Spain). A standard solution having 1.25 μ mol/ml per amino acid in 0.1N HCl was created.

Derivatization procedure

The procedure used was a modification of Elkin *et al.* (1985) method. A standard solution (5,10,15, or 20 μ L) or 50 μ L of sample solution was pipetted into a 10 \times 5mm tube, dried at 65 $^{\circ}$ C in vacuo. To the residue, 30 μ L of methanol-water-phenylisothiocyanate (2:2:1 [v/v]) was added and the tube was agitated and kept for 20 minutes at room temperature. Finally, the solvents were removed under a nitrogen stream, the tube sealed and stored at 4 $^{\circ}$ C, pending analysis. Prior to injection, 150 μ L of diluents consisting of 5mM sodium phosphate with 5% acetonitrile was added to each tube.

Chromatographic procedure

Chromatography was performed at 30 $^{\circ}$ C using a gradient elution as follows. Eluent A was an aqueous buffer prepared from the addition of 0.5mL/L Triethylamine to 0.14M sodium acetate, titrating with glacial acetic acid at pH 6.20; eluent B was acetonitrile-water (60:40 [v/v]). The gradient program given below:

Gradient program employed for PTC-Amino acids separation.

Time Flow rate

(min)	(mL/min)	% Eluent A	% Eluent B
0	1.0	90	10
12.0	1.0	70	30
20.0	1.0	52	48
22.0	1.0	0	100
24	1	0	100
30	1.5	0	100
37	1	90	10

Determination of Total Protein

Total protein level was determined using Colorimetric method-Biuret reagent.

Determination of Body weight

The body weights of the animals were taken before wound infliction and on days, 7, 14 and 21 of treatment.

Histology

At the end of days 1, 7, 14 and 21, animals from each group were sacrificed to obtain skin wound tissues by a scalpel for histological observation. The skin tissues were fixed with 10 % formalin. After fixation, samples were embedded in paraffin, cut into 3 mm frozen sections with a cryostat microtome, and stained with hematoxylin-eosin reagent. Keratin, blood vessel, fibroblast and granulation tissue of the rat skin were examined under a microscope separately.

STATISTICAL ANALYSIS

Values were expressed as means \pm standard Error Mean (SEM). One way ANOVA was used to test for differences between treatment groups using statistical package for social sciences (SPSS) version 22. The results were considered significant at $p < 0.05$.

RESULTS

Amino acid composition

The result of the amino acid composition of *H. rotundifolia* leaves (Table 1) revealed the presence of 19 amino acids: nine essential amino acids and ten non essential amino acids. The leaves had a total essential amino acid composition of 40.21 and total non essential amino acid of 43.44.

Table 1: The amino acid composition of *H. rotundifolia* leaves

Parameters	Concentration (mg/100g)
Essential Amino Acids (EAA)	
Valine	3.39 \pm 0.01
Lysine	3.99 \pm 0.00
Leucine	6.56 \pm 0.03
Isoleucine	4.60 \pm 0.00
Methionine	9.38 \pm 0.01
Threonine	2.70 \pm 0.01
Tryptophan	1.16 \pm 0.04
Phenylalanine	5.23 \pm 0.04
Histidine	3.20 \pm 0.06
Nonessential amino acid(NEAA)	
Aspartate	12.94 \pm 0.01
Alanine	2.92 \pm 0.01
Glutamate	5.23 \pm 0.01
Arginine	8.74 \pm 0.02
Cysteine	1.50 \pm 0.01
Glycine	3.84 \pm 0.01
Proline	3.98 \pm 0.00
Serine	1.92 \pm 0.02
Tyrosine	2.37 \pm 0.04
Total EAA	40.21
Total NEAA	43.44
Total AA	83.65

Total Protein level

The result of the total protein level is given on Table 2. Comparison of the total protein level of control group with Vaseline, gentamicin, HRE1 and HRE2 revealed a significant difference ($p < 0.05$) only in

gentamicin group on days 1 and 7, and a significant difference ($p < 0.05$) in all groups on day 21. For comparison of vaseline, HRE1 and HRE2 with gentamicin, there was a significant difference ($p < 0.05$) in all groups on day 1, significant difference ($p < 0.05$) in Vaseline and HRE1 on day 7, no significant difference in any group on day 14 but a significant difference ($p < 0.05$) only in Vaseline on day 21.

Table 2: Effect of dichloromethane extract of *H. rotundifolia* leaves on Total protein level (g/L) of wound treated wistar rats

Group	Day 1	Day 7	Day 14	Day 21
Control	59.67±1.45 ^b	67.67±1.45 ^b	64.33±0.88	59.67±0.88 ^b
Vaseline	61.00±1.15 ^b	72.00±3.06 ^b	65.33±0.88	80.67±0.88 ^{a,b}
Gentamicin	53.67±0.88 ^a	59.67±0.67 ^a	67.33±1.20	67.00±1.15 ^a
HRE1	63.67±0.88 ^b	70.67±1.20 ^b	67.33±1.20	67.33±0.88 ^a
HRE2	63.67±0.88 ^b	58.33±3.53	66.00±0.58	68.00±1.15 ^a

Data expressed as mean ± SEM (n=5). Values with different superscripts are statistically different at $p < 0.05$. Values without superscripts are not statistically significant at $p < 0.05$. Superscript a is statistically different ($p < 0.05$) when control was compared with Vaseline, gentamicin and HRE 1 and HRE 2 on 1, 7, 14, and 21 days

Body weight

Comparison of the body weight of rats in vaseline, gentamicin, HRE 1 and HRE 2 with control indicated a significant difference ($p < 0.05$) in all groups on day 1, in vaseline and HRE2 on day 7 and in vaseline alone on day 21. There was no statistical difference in any group on day 21. For comparison of Vaseline, HRE1 and HRE2 with gentamicin, there was a significant difference ($p < 0.05$) in all groups on days 1 to 7 and significant difference ($p < 0.05$) in Vaseline and HRE2 on days 14 and in Vaseline on day 21.

Table 3: Effect of dichloromethane extract of *H. rotundifolia* leaves on body weight (g) of wound treated wistar albino rats

Group	Day 1	Day 7	Day 14	Day 21
Control	214.88±1.02 ^b	214.52±4.41 ^b	226.65±6.55 ^b	236.55±11.95
Vaseline	221.10±2.34 ^{a,b}	219.84±3.59 ^b	224.29±0.897 ^b	217.30±5.50
Gentamicin	273.74±12.12 ^a	267.73±8.08 ^a	269.85±8.66 ^a	262.25±13.25
HRE 1	236.48±1.58 ^{a,b}	230.34±5.99 ^b	249.08±8.40	242.20±16.50
HRE 2	190.72±3.86 ^{a,b}	193.37±13.36 ^{a,b}	211.26±7.396 ^b	213.40±5.10

Superscript a is statistically different ($p < 0.05$) when control was compared with Vaseline, gentamicin and HRE1 and HRE2 on 1, 7 14, and 21 days. Superscript b is statistically different ($p < 0.05$) when gentamicin was compared with Vaseline, HRE1 and HRE2 on 1, 7, 14 and 21 days.

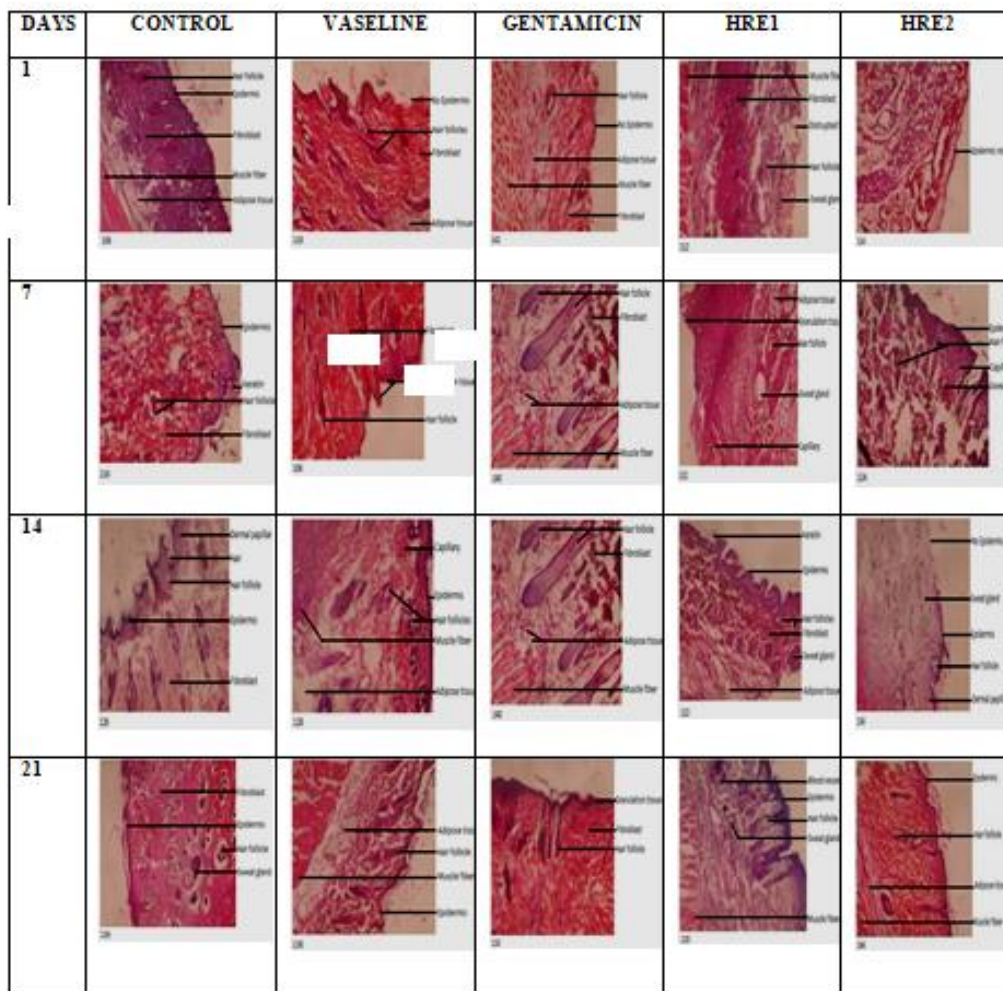
Table 4: Effect of dichloromethane extract of *H. rotundifolia* leaves body weight gain/loss (%) of wound treated wistar albino rat

Group	After 7 days	After 14 days	After 21 days
Control	-0.17	5.48	10.08

Vaseline	-0.57	1.44	-1.72
Gentamicin	-2.20	-1.42	-4.20
HRE 1	-2.60	5.33	2.42
HRE 2	1.39	10.77	11.89

HISTOLOGY

Histological study revealed that all groups were yet to reach the skin structure and morphology of collagen sequence that matches the structure of the original skin.



Photomicrograph (x100) of wound healing and epidermal/dermal from days 1 – 21 for wounds stained with H and E.

DISCUSSION

The result of the amino acid profile as presented in Table 1 revealed the presence of all the essential amino acids as well as non-essential amino acids. Aspartate (12.94mg/100g), methionine (9.38mg/100g), Arginine (8.74mg/100g) were detected in substantive amount summing up to 31.06mg/100g of the total amino acid composition. The amino acid result of *H. rotundifolia* leaves revealed that Leucine, Lysine, Alanine, Methionine, Phenylalanine, Threonine, Serine and Valine were found in reduced concentrations except Histidine in comparison with the report of Shemishere *et al.* (2018) in dried leaves of *M. cercropioides*. Aspartate is essential for purine, pyrimidine, asparagine and inositol synthesis. It also participates in the

detoxification and excretion of ammonia. Arginine is an indispensable amino acid in children because of its function in growth and development. Proteins and amino acids are relevant in the entire wound healing activity for instance methionine and cysteine participate in the biosynthesis of connective tissue and collagen while arginine aids in collagen proliferation and immune reaction.

Adequate supply of proteins is essential in wound healing. According to Candlish and Chandra (1996) the appearance of wound in animals leads to protein reduction in the blood and may change in a complex manner their protein metabolism. The significant increase in the total protein level as exhibited by the test animals (Table 2) in HRE1 as compared to control and reference drug (gentamicin) revealed the effectiveness of the plant extract in enhancing the synthesis of collagen content in excised tissue. The result also showed an enhanced nutritional condition of the rats which is vital for wound healing by improving cellular reproduction and migration to the wound tissue and fibroblast and granulation tissue formation. The observation from the histological examination of the wounded tissue also supports this result.

The percentage body weight gain recorded in HRE2 across the weeks, control and HRE1 after 2 and 3 weeks and Vaseline after 2 weeks, indicates that the extract had a positive outcome on their body weights. According to Demling and DeSanti, (2002), in spite of optimum nutrition and early wound closure, there could be notable involuntary weight loss which is majorly lean body mass. This weight loss is based on high rate of catabolic response leading to the use of body protein for energy. Also, Ma *et al.* (2015) suggestion on body weight loss in rats as seen in this work might be as a result of wound metabolic, circulatory and immunological imbalances.

Histological examination showed a normal skin for control on day 1 while other groups revealed a disrupted skin or skin without epidermis. On day 7, formation of new epithelial layer lined by thin layer of keratin, scab formation and fibroblast proliferation was revealed for control. Epithelial cells undergo mitosis to give off cells called keratinocytes, which form the superficial layers of the epidermis. Keratinized epithelium arranges a covering against abrasion, bacterial invasion and desiccation. Vaseline and gentamicin revealed proliferation of fibroblast dominated by granulation tissue formation dominating the surface of the wound, formation of epidermis. HRE1 revealed new epithelial layer formation lined by a dense layer of keratin was seen at the skin surface. According to Pastar *et al.* (2014), keratinocytes are accountable for restoring the epidermis after injury through epithelialization process. HRE2 showed an incomplete epidermis that was beginning to form from the margin and less scab formation. On the 14th day, epithelial cells began to go into the proliferation phase that is typified by granulation tissue formation and collagen formation dominated by fibroblast, less scab formation and thin epidermis formation was detected for control. For Vaseline, proliferative phase continued with collagen deposition, angiogenesis and a thick epidermis was detected also. The epidermal thickness was analogous to intact epidermis. Gentamicin revealed granulation tissues formation by the fibroblast cells and production of thin epidermis. HRE1 revealed granulation tissues formation by fibroblast proliferation, collagen deposition and angiogenesis. Collagen formation and maturation may be based on the existence of flavanoids and steroids in *Heterotis rotundifolia* leaves (Soni and Singhai, 2012; Jain *et al.*, 2018). A thin layer of epidermis can be seen formed on the outer surface, fibroblast cells and less scab formation in HRE2. Histological observation of wound tissue on the 21st day showed continuation of granulation tissue formation and deposition of collagen. This is the maturation and remodelling phase which begins day 8 through to one year. The collagen formed in granulation tissues is said to be thin. Broughton *et al.* (2006) suggests that the collagen primarily laid down is thinner than the collagen in uninjured skin (which has a basket weave pattern) and arranged parallel to the skin. He added that overtime, the initial collagen threads are reabsorbed and deposited thicker and organized along the stress lines. Vaseline and HRE1 revealed a repaired/regenerated skin showing an intact epidermis, dermis containing blood vessels, hair follicles, sweat glands and hypodermis containing muscle fibers. Gentamicin revealed continued formation of granulation tissue and thick epidermis while HRE2 revealed keratin formation, granulation tissue characterized by proliferation of fibroblast, scab formation and a thin epidermis formed on the surface.

CONCLUSION

In conclusion, the study reveals that *H. rotundifolia* leaves possess essential amino acids and wound healing potentials.

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