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Original Article

Evaluation of Wound Healing Potential of *Chrysophyllum albidum* Hexane Seed Extract in Wistar Rat

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ABSTRACT

Phytochemical, antioxidant and antimicrobial activities of the seeds of *Chrysophyllum albidum* have been established. However, scientific evaluation of its wound healing activity is lacking. Thus, this study was aimed at evaluating the wound healing potential of *Chrysophyllum albidum* hexane seed extract using wistar rats. The seed cotyledon was collected, air-dried, blended and extracted with n-hexane by continuous cold extraction. Phytochemicals, antioxidant and antimicrobial activities of the extracts were assayed by standard methods. Three groups of male wistar rats consisting of seven animals each were used for the animal studies.Wound excision was induced on all animals in the sterilized shaved dorsal fur region.Group1 served as the negative control (untreated), groups 2 and 3 were treated with n-hexane extract (test group) and Povidone-iodine ointment (positive control) respectively. Wound contraction rate and epithelialization time were used to assess the efficiency of the treatment. Group 3 rats had best wound contraction rate with epithelialization time of 16 post-wounding day followed by group 2 (20 days) and group 1 (28 days). The wound healing efficiency of the extract was less than that of the standard ointment but it healed the wound inflicted on the rats faster than the untreated ones. The low wound activity of the seed extract may be due to low phytochemical, antioxidant and antimicrobial activity of the extract. *Chrysophyllum albidum* hexane seed extract have some wound healing activity and therefore have potential for being used as wound healing ointment.

Keywords: *Chrysophyllum albidum, Epithelialisation, Hexane-seed, Wound.*

INTRODUCTION

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Healing of wound is a complicated and expensive process. It is a natural, fragile and complex phenomenon that induces an important biological process involving tissue repair and regeneration.[1] Wound care and maintenance involves a number of measures including dressing and administration of analgesics, use of anti-inflammatory and anti-oxidative agents, topical and systemic antimicrobial agents and healing promoting drugs. The process involves three different phases including haemostasis, inflammation and regeneration. The haemostasis stage leads to the formation of thrombus by blood coagulation, platelets adhesion/accumulation. The inflammatory phase involves the appearance of granulocytes to clear the wound area by destroying invading microorganisms. The regeneration or repair phase is a complex process characterized by endothelial budding in the nearby blood vessels forming new capillaries (neovascularization) that penetrate and nourish the injured tissues (re-epithelization). All these phases are enhanced, fastened and improved by using ointment that supplies one or more requirements needed for the stages functioning, thereby making the wound to heal faster.[2]

C. albidum (Family: Sapotaceae) is a popular tropical fruit tree that is widely distributed in the low land rain forest zones. Among the African regions where it can be found are Nigeria, Uganda, Niger, Cameroun and Cote d' Ivoire.[3] Its rich sources of natural antioxidants have been established to promote health by acting against oxidative stress related disease such as diabetes, wound, cancer and coronary heart diseases.[4]

The seed cotyledon is used for easy separation of cord of newly born baby whereby the cotyledon is grinded, mixed with shea butter oil and added to nerve cord of baby.[5] However, no systematic study on its wound healing potential has been reported in the literature. Therefore in the present investigation, we screened the n-hexane - seed extracts of *C. albidum* for wound healing potential.

MATERIALS AND METHODS

Seed collection and preparation

The seeds of *Chrysophyllum albidum* was collected from Oyo town environs and deshelled. Seeds were shade-dried and blended. It was then subjected to extraction with n-hexane by continuous cold extraction and the extract was concentrated using rotary evaporator under reduced pressure.

In-vitro Analysis

Qualitative phytochemical analysis of the extract was done by the following methods.

Flavonoids

About 5 mL of dilute ammonia solution were added to a portion of the aqueous filtrate of test extracts followed by addition of concentrated H_2SO_4. A yellow colouration observed in each extract indicated the presence of flavonoids. The yellow colouration disappeared on standing. Flavonoid was further confirmed by adding few drops of dilute NaOH solution to 1 mL of stock solution. An intense yellow colour appeared which turned colourless on addition of a few drop of dilute acid.[6-7]

Tannins

Few drops of 1 % lead acetate were added to the test extract (2 mL). A yellowish precipitate indicating the presence of tannins was observed.

Saponins (frothing test)

The test extract (5 mL) was mixed with 20 mL of distilled water and then agitated in a graduated cylinder for 15 minutes. Formation of foam indicates the presence of saponins.[8]

Cardiac glycoside

The test extract (0.5 g) was dissolved in 2 mL of acetic anhydride and cooled in ice after which conc. H_2SO_4 acid was carefully added. The colour change from violet to blue or green indicates the presence of a steroidal nucleus i.e. aglycone portion of the cardiac glycoside.[7,9]

Alkaloids

About 0.5 g test extract was stirred with 5 mL of 1% dilute hydrochloric acid on a water bath. The resulting solution was then filtered and 1 mL of the filtrate was then treated with a few drops of Mayer's reagent and a second 1 mL portion with Dragendorff's reagent. Turbidity or precipitation with either of these reagents was taken as evidence for the presence of alkaloids in the extract. Alkaloid was further confirmed by Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow coloured precipitate.[7,9]

Steroids (Salakowsti test)

Five (5) drops of concentrated H_2SO_4 was added to 1 cm^3 of the extracts, red colouration indicated the presence of steroids.

Phlobatannins

1 cm^3 of the extracts was added to 1 % HCl. A red precipitate indicates the presence of phlobatannins.[10]

Triterpenes

5 drops of acetic anhydride was added to 1 cm^3 of the extracts. A drop of concentrated H_2SO_4 was then added and the mixture was steamed for 1 hour and neutralized with NaOH followed by the addition of chloroform. A blue green colour indicates the presence of triterpenes.[10]

Terpenoids

5 ml of aqueous extract of the sample was mixed with 2 ml of $CHCl_3$ in a test tube, 3 ml of conc. H_2SO_4 was then carefully added to the mixture to form a layer. An interface with a reddish brown coloration is formed if terpenoids constituent is present.

Amino acid Two drops of ninhydrin solution (10 mg of ninhydrin in 200 ml of acetone) was added to 2 ml of aqueous filtrate. A characteristic purple colour indicates the presence of amino acids.

Quantitative phytochemical analysis

Triterpenes

0.50 g of sample was weighed into a 50 ml conical flask and 20 ml of 2:1 chloroform-methanol mixture was added, shaken thoroughly and allowed to stand for 15 minutes. The supernatant obtained was discarded and the precipitate was re-washed with another 20 ml chloroform-methanol mixture for re-centrifugation.

The resultant precipitate was dissolved in 40 ml of 10 % sodium dodecyl sulphate (SDS) solution.1 ml of 0.01 M ferric chloride solution was added to the above at 30 seconds intervals; shaken well, and allowed to stand for 30 minutes. Standard triterpenes of concentration range 0-5 mg/ml were prepared from 100 mg/l stock triterpenes solution from sigma-Aldrich chemicals, U.S.A. The absorbances of sample as well as that of standard concentrations of triterpenes were read on a digital spectrophotometer at a wavelength of 510nm.[11]

The percentage of triterpenes was calculated using the formula:

$$= \frac{\text{Absorbance of sample} \times \text{Average gradient} \times \text{Dilution factor}}{\text{Weight of sample} \times 10{,}000}$$

Total tannin content

500µl of the sample was weighed into a 50 ml plastic bottle. 50 ml of distilled water was added and shaken for 1 h in amechanical shaker. This was filtered into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtrate was pipetted out into a test tube and mixed with 2 ml of 0.1 M $FeCl_3$ in 0.IM HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 min.[12]

Alkaloid determination

1 ml of the sample was taken and 5 ml 60 % H_2SO_4 was added and allowed to stand for five minutes and 2 ml of 0.5 % formaldehyde was added. The solution was incubated for 3 hours and absorbance was taken at 565 nm.

Antioxidant analysis

DPPH free radical-scavenging assay

This assay has been used to investigate the scavenging activity of antioxidant compounds. In fact, DPPH is a stable free radical that can be reduced by a proton-donating substrate like an antioxidant, causing the discoloration of DPPH and reduction of the absorbance at 517 nm. The DPPH free radical scavenging activity of the n-hexane seed extract was determined according to the method reported by Brand-Williams *et.al.* with slight modification. The stock solution of the radical, prepared by dissolving 24 mg DPPH in 100 mL methanol, was kept in a refrigerator until further use. The working solution of the radical was prepared by diluting the DPPH stock solution with methanol to obtain an absorbance of about 0.98 (±0.02) at 517 nm . In a test tube, 3 mL DPPH working solution was mixed with 100 µL seed extract (1 mg/mL) of five different concentrations (0.2-1mg/ml) of the extract. The absorbance was measured at 517 nm for a period of 30 min. Radical-scavenging activity was expressed as the inhibition percentage and was calculated using the equation of DPPH radical scavenging activity.[13]

DPPH radical-scavenging activity (%)

$$= \frac{A\text{control} - A\text{sample}}{A\text{control}} \times 100$$

*A*control is the absorbance of the control reaction and *A*sample is the absorbance of extract/standard BHT samples. The test was carried out in triplicate.

Total antioxidant capacity assay

Total antioxidant capacity assay is a spectroscopic method for the quantitative determination of antioxidant capacity, through the formation of phosphomolybdenum complex. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green phosphate Mo (V) complex at acidic pH. Total antioxidant capacity can be calculated by the method described by Prieto *et. al.*[14] 0.1 mL of sample (100 µg) solution is combined with 1 mL of reagent (0.6 M H_2SO_4 acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tube is capped and incubated in a boiling water bath at 95^{o}C for 90 min. After cooling the sample to room temperature, the absorbance of the aqueous solution is measured at 695 nm against blank in UV spectrophotometer. A typical blank solution contained 1 mL of reagent solution and the appropriate volume of the same solvent was used for the

sample and it was incubated under same conditions as rest of the sample. For samples of unknown composition, antioxidant capacity was expressed as equivalents of ascorbic acid.

OH radical scavenging activity assay

The radical was generated in a mixture of 1.0 ml of 1,10 – phenanthroline, 2.0 ml of sodium phosphate buffer (pH 7.4), 1.0 ml of $FeSO_4$ and 1.0 ml of H_2O_2. After addition of 1.0 ml sample solution, the mixture was incubated at 37˚C for 30min. Then, the absorbance of the mixture at 536 nm was measured. The hydroxyl radical (˙OH) scavenging activity was calculated by the following equation:

$$\text{Scavenging activity (\%)} = \frac{(\text{Abs sample} - \text{Abs blank})}{(\text{Abs0} - \text{Abs blank})} \times 100$$

Where Abs0 is the absorbance of the deionized water instead of OH- and sample in the assay system.

Antimicrobial activity

Minimum inhibitory concentration (MIC) of the extract

The micro dilution method was used for the determination of the minimal inhibition concentration (MIC) against five gram negative bacteria species. This was conducted on the five extracts all prepared into eight different fractions each using 2 fold serial dilution. Samples were dissolved in distilled water to yield a final concentration of 50 mg/ml-0.3906 mg/ml. The microtiter plate, microtiter tips and silica foils was sterilized using the UV light for 30 minutes in the inoculating chamber. A cell suspension of the test organisms was prepared and 50µL of the suspension likewise various concentrations of the extract was dispensed into each of the wells from A to H. Well F was the negative control containing only the test organism while well G was the positive control containing the organism and 50 µL Ciprofloxacine USP. After 24 h of incubation at 30°C, the bacterial growth was observed by pipetting 50 µL of chloronitrotetrazolium salt solution which served as an indicator and reacts with the acid produced by the bacteria to produce a pink or red coloration indicating the microbial growth in that well.

Experimental animals

Twenty-one male Wistar rats of weights between 100-130 g were selected and divided into three groups having seven animals each comprising of the control group, standard group and the test group. They were housed in polypropylene cages and well-ventilated rooms in the animal house of Ajayi Crowther University under controlled conditions of 22–25 °C, 60–70 % relative humidity, and 12 hours of dark-light cycle throughout the study. The animals were left for a week to acclimatize to the animal room conditions. Animals were allowed access to standard pelletized food and tap water. Group I was untreated and served as a negative control (the wounds were cleaned with distilled water only). Groups II animals were treated with n-hexane seed extract and served as the test group while group III was treated with standard ointment (Povidone-iodine ointment) and served as a standard reference (positive control). The povidone-iodine ointment was manufactured by Stedman pharmaceuticals private LTD., Tamilnadu, India and it is obtainable from local pharmacy. The animals were anaesthetized prior to and during the creation of experimental wounds with ketamine hydrochloride (100 mg/kg bodyweight.). A circular excision wound model of 200 mm^2 surface area was induced on the depilated ethanol sterilized shaved rats' dorsal fur region.

The day the wound was created is considered as day 0 and all the wounds were covered with a gauze dressing, treatment was applied on the induced wound of animals once a day and treated until they were completely healed while adequately observing the healing pattern of the wounds.

Wound healing rate

The healing of wound was assessed by tracing the wound using transparent paper and a marker and the recorded wound areas were measured graphically. Pictures of the wounds from the first day of the wound induction until the day of complete wound closure (CWC), i.e., complete healing were taken every fourth day to measure the rate of wound healing and comparative wound healing efficiency of the control, test and standard groups were observed. A digital camera was used for taking the wound pictures and a graph sheet was used as a scale to measure the size of the wound. Percent relative healing efficiency (RHE) of the extract was calculated to measure how fast the extracts completely healed the wound using the following formula:

$$\text{\% Relative wound healing efficiency} = \frac{T_N - T_E}{T_N} \times 100$$

T_N=Time required for natural wound healing i.e., CWC without any drug/extract. T_E=Time taken for wound healing i.e., CWC with drug/extract.[15]

Statistical analysis

Statistical analysis was carried out on each group, followed by ANOVA test (graph padprism software) which was used to compare the mean value of each treatment. Significant differences were determined between the means of parameters using Dunnian T test ($P< 0.05$).

RESULTS

Formulations yield

Table 1: %Yield of n-hexane extract of *C. Albidum* seed

Extract	Total weight of sample (g)	Total volume of solvent (ml)	Total yield (g)	Yield (%)
N-hexane	800	2100	11.18	1.40

- The extract yield was 1.40% and was found to be oil.

Qualitative and quantitative phytochemical analysis

Table 2: Results for qualitative phytochemical screening

Phytochemical	n-Hexane extract
Saponins	-
Tannins	+
Phenols	-
Phlobatannin	-
Steroid	+
Amino acid	-
Terpenoid	+
Flavonoid	-
Alkaloid	+
Glycoside	+
Triterpene	+

- Key: Presence of constituent = +;Absence of constituent = -

Table 3: Results for quantitative phytochemical analysis

Phytochemical mg/100g	n-hexane extract
Tanin	6.05
Steroid	116.35
Alkaloid	10.59
Glycoside	0.04
Triterpene	37.85

Antioxidant analysis

Table 4: DPPH % scavenging activity

Concentration (mg/ml)	n-Hexane extract	Standard (Galic acid)
0.2	13.10	37.70
0.4	16.60	
0.6	17.70	51.44
0.8	36.30	
1	46.30	85.73

Table 5: Hydroxyl scavenging radical results

Extract	OH^- radical scavenging activity (50mg/100g)
N-hexane	11.05

Table 6: Total antioxidant capacity (TAC) result

Extract (1mg/ml)	% TAC
N-hexane	73.02

Antimicrobial activity

Table 7: Activity level of extract using MIC technique

Test Organism	n-Hexane
Serratia fonticola	25
Serratia liquefaciens	12.5
Pseudomonas	6.25
Klebsielle pneumonia	6.25
Aeromonas veronii	25

*values are concentration in mg/ml of the extracts

Wound healing experiment results

Table 8: Average animal weight (g) increase per week after wound induction

Week	Untreated	n-Hexane	Standard
1	0	2	10
2	2	5	16
3	6	8	20

- The negative control (untreated) animals had the least average weight increase for the weeks studied after wound induction.

Table 9: Feed weight (g) per week after wound induction

Week	Untreated	n-Hexane	Standard
1	93	103	66
2	121	160	96
3	207	175	124
4	246	224	145

- The feed intake increased gradually through the weeks for all groups.

Surface area of wound

Table 10: Percent wound healing rate

Day	Untreated (%)	n-Hexane (%)	Standard (%)
4	22	32	47
8	41	45	87.5
12	66	52	98
16	66	72	100
20	86	86	
24	95	100	
28	100		

- The decreasing order of wound healing capacity of the groups was obtained to be standard > n-hexane >untreated respectively.

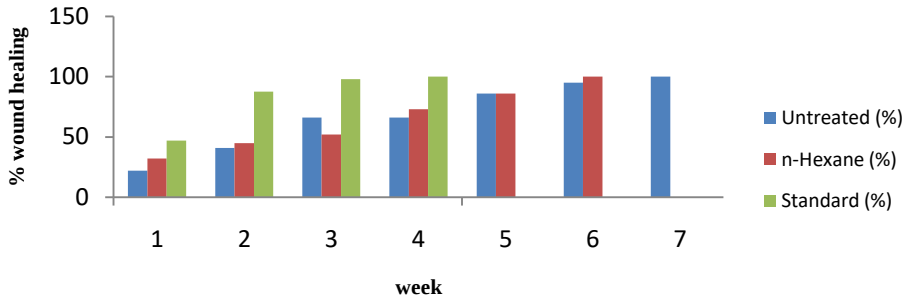

Fig. 1: percent wound healing rate for the extracts and standard

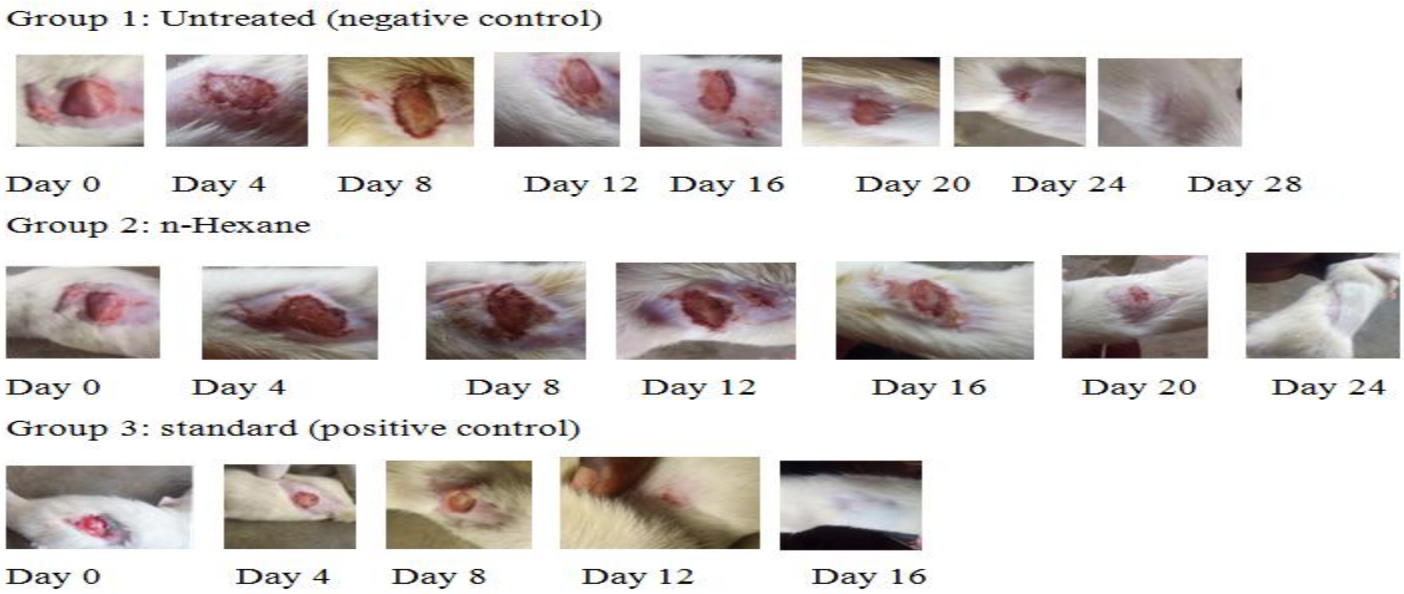

Fig. 2: Pictures of wound every 4th day after induction

DISCUSSION

The present study evaluated the wound healing potential of *C.albidum*n-hexane seed extract in Wistar rats. The extract yield was 1.40% which is lower compared to that obtained for chloroform extract of *Allium cepa* which is 2.00%.[16]It was found to be oil in nature and yellow in color. The phytochemicals that are found to be present agrees with the findings of previous researches.[17-23]These phytochemicals provides better tissue remodeling when applied on wounds and also act as proangiogenic agents during wound healing.[24] The aqueous extract of *C.albidum* was found to contain saponin and flavonoid [17] which is not observed in its n-hexane extract from this study.

Antioxidant property obtained for the extract is in accordance with those observed by Okoli & Okere, Osuntokun *et. al.*[17,18]The extract showed a minimum inhibitory concentration (MIC) of 25, 12.5, 6.25, 6.25, and 25 against *Serratia fonticola, Serratia Liquefaciens, Pseudomonas, Klebsielle Pneumoniae and Aeromonas Veronii* organisms respectively which agrees with the findings of David *et. al.*[25]

In all animal groups, the feed intake increased gradually through the weeks studied. The decreasing order of wound healing capacity of the groups was obtained to be standard > n-hexane > untreated respectively. This is expected as the phytochemical components of the *C. albidum* seed might have aided in the regeneration of lost tissue.[26,27] The granulation tissue section of negative control animals showed decreased epithelialization, fibrosis and more aggregation of macrophages with less collagen fibers indicating incomplete healing. The tissue section from animals treated with povidone–iodine ointment (standard) and the extract showed complete healing with prominent epithelialization and increased collagenation and fibroblast. The lack of mortality of the experimental rats throughout the study implies that the n-hexane extracts of this seed have low toxicity.[28]

CONCLUSION

This study demonstrated the topical application of the n-hexane extracts of *Chrysophyllumalbidum* seed for the acceleration of wound healing in Wistar rat. According to this finding, extract showed accelerated wound healing activity a little better than the negative control (untreated). This may be due to high percentage of triterpene present in the extract. The results of this study strongly document the beneficial effect of the n-hexane seed extract for the acceleration of wound healing.

RECOMMENDATION

Based on the findings of this study, the following recommendations are therefore put forward:
The active components of the extract should be carefully isolated.
Further studies should be carried out using polar, appreciable polar and another non-polar solvent extraction of the seed in comparative analysis to determine the most potent.
Further studies should be done to carry out the acute toxicology studies of the aqueous extract which will be most easily accessible and producible locally.

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