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Assessment of the phytoconstituents and optimal applicable concentration of aqueous extract of *Azadirachta indica* leaves for wound healing in male Wistar rats

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ABSTRACT

Background/Aim: Plant-based medicinal agents containing phytoconstituents like extracts of Azadirachta indica leaves has been reported to heal wounds. This study evaluated the phytoconstituents and determined the optimal concentration of the aqueous extract of Azadirachta indica leaves (AEAIL) affecting wound healing in male Wistar rats.
Materials and Methods: AEAIL was evaluated for phytoconstituents using GC-FID. Wounds (diameter ≈1.5cm) was excised on the dorsum of 35 rats (7 groups, n = 5). Groups 1- 6 were treated topically with 0.5, 1.5, 3.0, 5.0, 10.0 and 20.0% w/v of AEAIL respectively. Group 7 was treated with distilled water as the control. Wound contractions were measured daily. Assay of tissue hydroxyproline levels was evaluated (Bergman and Loxley method) using a hydroxyproline assay kit.
Results: The phytoconstituents identified were under alkaloids, flavonoids, phenol and polyphenols. The highest and lowest mean tissue hydroxyproline levels were obtained with 1.5 and 3.0% w/v of AEAIL exhibiting the respective differences of 54.75 and 2.82% against the control, depicting the optimal concentration of AEAIL for wound healing as 1.5% w/v.
Conclusion: AEAIL contains abundant useful phytoconstituents and possesses potent wound healing properties. A 1.5% w/v of it is the optimal effective concentration for wound healing.

Keywords: Azadirachta indica, aqueous-extract, wound-healing, phytoconstituents

INTRODUCTION

A wound can be understood as a disorder of cellular, anatomical, and functional stability of bodily tissue which may be triggered by physical, chemical, thermal, microbial, or immunological abuse to the tissue. It is described as an open wound once the skin is torn, cut, or pierced and it is known as a closed wound if a blunt force trauma causes a bump. Burn wounds originate through fire, heat, radiation, chemicals, electricity, or sunlight.[1,2] Restoration of an injured part of a body is a natural recuperative reaction to tissue damage whereby multifaceted cellular activities take place to bring about the rebuilding and re-establishment of the tensile strength of a wounded skin.[3] If the outer cover of the body is injured, an involuntary initiation of the sequence of actions toward healing is established by the body. These occur to restore and rebuild the wounded tissues.[4] This sequence of healing procedures is categorized into four coinciding stages noted as hemostasis, inflammatory, proliferative, and maturation.[5] The first segment of wound healing is described as the coagulation phase. In the beginning of damage to the skin, hemostasis is naturally initiated as the body triggers

its emergency repair mechanism to begin the restoration process and the essence of this is to halt the hemorrhage as platelets and collagen come in contact to cause activation and aggregation. At this point, thrombin begins the development of fibrin network thereby adding strength to the platelet clumps to form a stable clot.[6,7] The inflammatory stage is the second step of wound healing and is the defensive phase.[7] It centers on the destruction of bacteria and the removal of debris. This is achieved through the activities of neutrophils which penetrate the injured site to prepare the wound bed for the development of new tissue. When the activities of the neutrophils are exhaustive, macrophages emerge to clear the debris further, thereby secreting growth factors and proteins which cause the immune system cells to appear to expedite tissue repair. This phase is characterized by edema, erythema, heat, and pain.[7-9] The third segment of restoration of a wounded body part is the proliferative stage. This involves the filling and covering of the injured site. This aspect accomplishes three basic roles such as filling the wounded site, its contraction and covering up of the wound, a process described as epithelialization. In the early period of the proliferative phase, a glistening deep red granulation tissue plugs the wound base with connective tissue, and fresh plasma vessels appear. In the course of contraction, the borders of the wound diminish, pulling in the direction of the center of the injury. The culmination of the proliferative phase witnesses the rise of epithelial cells from the base of the wound or its boundaries and commences drifting through the bed of the wound until the injured site is enclosed with epithelium.[7,10,11] In the maturation and strengthening phase, designated as the fourth stage of wound restoration, fresh tissue gradually advances and collagen fibers rearrange with tissue remodeling, maturity, and general proliferation in tensile strength. At this stage, there is cell apoptosis since such cells are not useful anymore.[7,12,13]

The progression of wound healing can be significant and multifaceted.[14] The processes could equally be prone to disruption due to some localized factors, including moisture, infection, as well as systemic factors like age and nutritional status. With the existence of the proper healing setting, the body performs optimally to heal and substitute devitalized tissue.[15,16] Wound healing may well be enhanced by covering their surface and this could be accomplished if the injured site is cleaned and dressed up regularly.[17]

A delay in wound healing could equally occur due to poor blood supply to the site of the wound as this is a very vital factor in wound healing.[18] Adequate blood circulation is key since the restoration of an injured body may take a protracted time since healing may not take place if oxygen and essential nutritional supplies are not sufficient.[19,20] Other factors that could deter a wound from healing quickly include obesity, repeated trauma, medication, patient behavior or lifestyle, skin moisture or level of skin hydration and chronic disorders such as diabetes, hypertension or related vascular diseases, and immunodeficiency states.[21,22]

The collagen comprising an amino acid, hydroxyproline is the major component of extracellular tissue that offers strength and support. Fragmentation of the collagen liberates hydroxyproline and its peptides. The analysis of hydroxyproline could, therefore, serve as a biochemical indicator for tissue collagen and depicts the degree of collagen turnover.[23]

Crude plant extracts or their preparations have been employed in the management of body injuries over the years as these extracts can restore injured body, support plasma coagulation, combat contamination due to germs, and fast-track the wound healing process.[23-25] Plant phytoconstituents include the alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, and phenolic compounds.[26,27] The medicinal worth of plant extracts depends on the bioactive phytochemical components which offer positive physiological action on the human body.[27,28] Wound healing is a multifaceted procedure comprising hemostasis, inflammation, proliferation, and remodeling. In addition to other cellular interactions, it is similarly subjective to the effect of proteins and glycoproteins, such as cytokines, chemokines, growth factors, inhibitors, and their receptors. These facts position the use of plant extracts for wound management at advantage compared to the conventional therapies. The plant extracts are very rich in multiple phytochemicals with multiple benefits and mechanisms of actions which matches the multifaceted processes involved in wound healing. There are insufficient conventional drugs for wound treatment and they are not matching with the multiple mechanisms involved in wound healing. The few that are commercially available are not affordable by the economic class of people who experience body injuries; hence, chronic wounds are on the rise and constitute a socio-economic burden to the medical community and patients. The *in vitro* assessment of these herbal extracts for wound healing will be beneficial as they are swift in action and moderately cheap.[23]

The World Health Organization (WHO) promotes traditional medicine as a source of cheap, broad medical care, particularly in emerging economies. About 8% of the world's populace depends on plant-based medical products for their primary healthcare. The WHO has also documented the treatment approaches, strategies, and standard for plant-based medicinal substances.[29,30]

Neem (*Azadirachta indica)* is a traditional medicinal plant used in India for curing wounds, cuts, and other skin diseases. It has also been widely used by various tribes. Medicinal properties of its leaves such as antioxidant and antimicrobial activity were contributed by its phytoconstituents. The flavonoids present in them act as antioxidants which protect against free radicals that damage cells and tissues and also the tannins promote wound healing.[31-33] It has been prominently used in Ayurveda, Unani, and Chinese medicines in the inhibition and management of a diversity of ailments.[34,35] Due to the rich nature of each part of the plant in in phytochemicals, it has remained a valued base for natural medicinal substances.[36] Extracts from neem possess free-radical scavenging actions due to its rich basis of antioxidant.[37] Other phytoconstituents found in it include the nimbin, nimbidin, nimbolide, and limonoids which are useful in managing various illnesses through the modulation of several genetic pathways and other actions. Earlier, polyphenolic flavonoids like the quercetin and ß-sitosterol were separated from the neem and they were recognized to possess antifungal and antibacterial properties.[38] Abundant organic and pharmacological characteristics have been

documented concerning these phytochemicals which include antibacterial,[39] antifungal,[40] and anti-inflammatory, anti-arthritic, antipyretic, hypoglycemic, anti-gastric ulcer, and anti-tumor properties.[41-44]

Several studies have been carried out and documented on the wound healing properties of the ethanolic or methanolic extracts of *Azadirachta indica* leaves using several modes of assessments.[45-47]

The purpose of this study was to evaluate the composition of some phytochemical constituents of the aqueous extract of *Azadirachta indica* leaves and to determine its optimal effective concentration affecting wound healing in male Wistar rats using hydroxyproline as a biomarker.

MATERIALS AND METHODS

Materials

The materials used for the studies were ethanol (96%), anhydrous sodium sulfate, potassium hydroxide (Sigma-Aldrich, USA), *n*-hexane (BDH, England), and hydroxyproline assay kit (Elabscience, China).

Methods

Fresh neem leaves were identified by a Taxonomist and deposited in the University of Port Harcourt (voucher no. EH/P/070). A 100 g of the air-dried leaves were pulverized and macerated in 1 L of distilled water at ambient temperature with occasional agitation for 48 h. Its filtrate was concentrated under a reduced temperature to obtain the extract.

Qualitative phytochemical screening

Qualitative phytochemical screening of secondary metabolites was carried out as follows.[48-50]

Test for alkaloids

This was executed out using Wagner's reagent. A 1.27g of iodine and 2 g of potassium iodide were added to 100 ml of distilled water and agitated. A 2 ml of the Wagner's reagent was introduced to a solution of the extract in a test tube. Formation of reddish-brown color precipitate was detected.

Test for tannins

A 3 ml of 10 % alcoholic ferric chloride solution was introduced to a solution of the extract in a test tube. Formation of dark-blue color compound was observed.

Test for saponins

A little amount of the extract was briskly shaken with 5 ml of distilled water. There was frothing. About three drops of olive oil were blended with the froth which resulted to formation of an emulsion.

Test Glycosides

A 2 ml of glacial acetic acid mixed with a drop of ferric chloride solution was introduced to a few drops of the extract solution in a test tube. The blend was cautiously added to 1 ml of concentrated sulfuric acid in a different test tube such that the concentrated sulfuric acid was right underneath the mix. There changes in color were noted.

Test for flavonoids

Little drops of 1% dilute ammonia solution were introduced into a small portion of the solution of the extract in a test tube, and later, little drops of concentrated sulfuric acid solution were added. A yellow coloration was noted.

Test for phenols

Few drops of 5% ferric chloride solution were introduced into a little amount of the solution of the extract in a test tube and a greenish color was seen.

Evaluation of quantitative phytochemical composition

Extraction of phytochemicals

A 1 g quantity of the extract was transferred into a test tube. A 15 ml of ethanol and 10 ml of 50% w/v potassium hydroxide were added. The content of the test tube was at first left to react in a water bath at 60°C for 1 h. The outcome of the reaction was emptied into a separatory funnel. The tube was washed successively with 20 ml of ethanol, 10 ml of cold water, 10 ml of hot water, and 3 ml of *n*-hexane, which was all transferred to the funnel. These extracts were combined and washed 3 times with 10 ml of 10% v/v ethanol aqueous solution. The solution was dried with anhydrous sodium sulfate and the solvent was evaporated. The sample was solubilized in 1000 µl of *n*-hexane, of which 200 µl was transferred to a vial for analysis.[51,52]

Quantification of phytochemicals by Gas chromatography–Flame ionization detector (GC-FID)

The analysis of phytochemicals was performed on a BUCK M910 Gas chromatography equipped with a flame ionization detector (FID). A RESTEK 15 meter MXT-1 column (15 m × 250 um × 0.15 um) was used. The injector temperature was 280 with a splitless injection of 2 *u*l of sample and a linear velocity of 30 cms^{-1}, Helium 5.0 was the carrier gas with a flow rate of 40 ml min^{-1}. The oven was operated initially at 200. It was heated to 330 at a rate of 3 min^{-1} and was kept at this temperature for 5 min. The detector was operated at a temperature of 320.

Phytochemical content was determined by the ratio between the area and mass of internal standard and the area of the identified phytochemicals. The concentration of the different phytochemicals presents in the extract was expressed as *u*g/g of the extract.[51,52]

Table 1: Qualitative phytochemical screening of an aqueous leaf extract of *Azadirachta indica*

Phytochemical constituent	Results
Alkaloids	+
Flavonoids	+
Glycosides	+
Tannins	+
Saponins	+
Phenols	+

Assessment of the optimal applicable concentration of aqueous extract of Azadirachta indica leaves affecting wound healing in male Wistar rats

Thirty-five male adult Wistar rats weighing 200-250g, sourced from the animal house of the Faculty of Pharmaceutical Sciences, University of Port Harcourt, were kept in separate cages for

Table 2: GC-FID phytochemical analysis of an aqueous extract of *Azadirachta indica* leaves

Phytochemical	Retention	Area	Height	Concentration
Proanthocyanin	0.210	5426.7124	354.142	5.61124 ppm
Lunamarin	2.390	12419.2996	701.553	41.0861μg/ml
Ephedrine	4.120	6543.2930	371.126	5.0277 μg/ml
Anthocyanin	6.016	18234.4938	1020.754	14.8194 μg/ml
Ribalinidine	7.470	8483.3000	480.619	4.8759 μg/ml
Naringenin	10.366	19625.3075	1096.775	17.2940 μg/ml
Spartein	12.970	6252.9987	354.931	1.8262 μg/ml
Tannin	15.460	4978.2621	282.655	3.5503 μg/ml
Sapogenin	17.963	11351.0066	641.436	30.3656 μg/ml
Phenol	20.313	12766.1384	680.813	10.9647 ppm
Flavonones	22.730	9583.1170	539.390	5.3880 ppm
Steroids	25.650	10090.1032	570.418	7.4725 ppm
Epicatechin	27.536	11538.7025	649.857	10.1802 μg/ml
Kaempferol	29.860	5484.7925	311.586	2.6799 μg/ml
Phytate	32.993	14337.0773	803.990	1.6503 μg/ml
Flavone	34.600	6059.4052	344.109	4.2680 μg/ml
Oxalate	36.876	6996.1836	394.001	13.6654 μg/ml
Catechin	39.200	10239.6298	576.278	6.3456 μg/ml
Resveratrol	42.276	3510.2092	199.554	2.5842 ppm
Rutin	44.170	10547.7802	596.529	7.7370 μg/ml

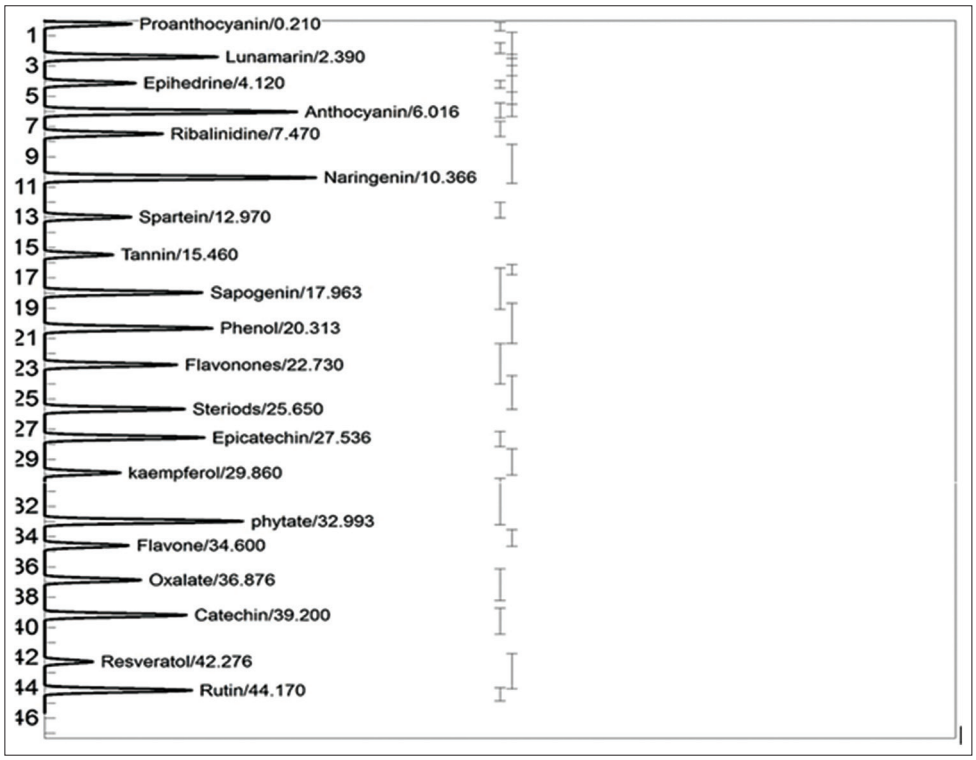

Figure 1: Chromatogram showing the phytochemical constituents of the aqueous extract of *Azadirachta indica* leaves

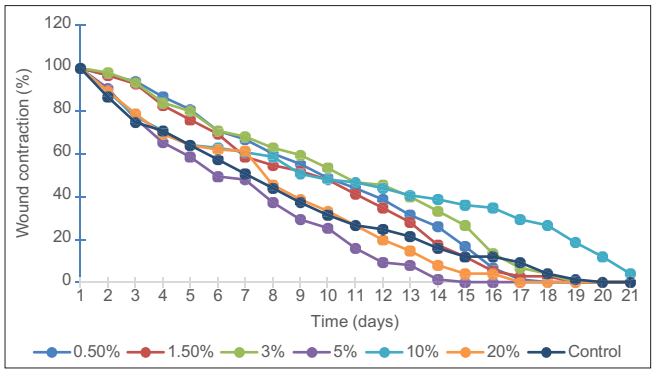

Figure 2: Percentage of wound contraction following treatment with the various concentrations of an aqueous extract of *Azadirachta indica* leaves

two weeks for acclimatization with free access to standard feed and water under standard conditions of temperature (25–29°C), relative humidity (55–66%), and natural dark/light cycle. Each rat was anesthetized by 50 mg/kg ketamine intramuscularly. The dorsal area of the rats was shaved and cleaned. A 1.5 cm × 1 cm full-thickness open excision wound was made.[53] Other animal care and handling were conducted in strict adherence to the ethical provisions of the University of Port Harcourt. The investigation was conducted in line with the procedures for ethical conduct in the care and use of non-human animals in research.[54]

All experiments were examined and approved by the Research Ethics Committee of the University of Port Harcourt with approval reference no.: UPH/CEREMAD/REC/MM71/043.

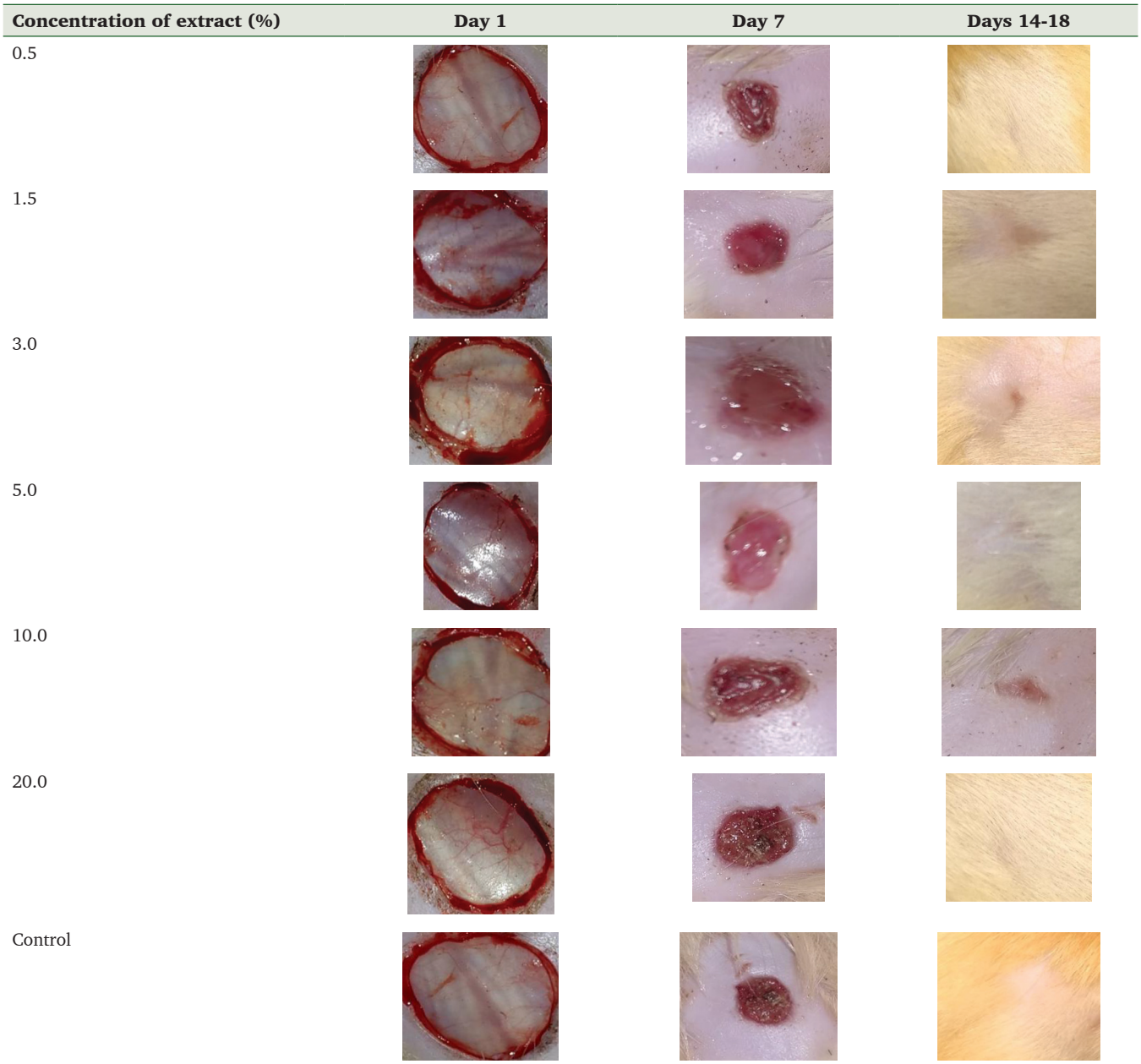

Figure 3: Wound dimensions on day 1, day 7, and day of maximum wound closure (within 21 days)

Table 3: The differences in the mean tissue hydroxyproline levels of treated groups compared to the control group

Conc. AEAIL (%)	Tissue hydroxyproline levels (μg/g)	P-value	Difference in mean hydroxyproline levels compared to the control group (%)
0.5	1.5254±0.24	0.000	47.54
1.5	1.600±0.08	0.000	54.75
3.0	1.0631±0.01	0.697	2.82
5.0	1.4878±0.05	0.000	43.90
10.0	1.4565±0.17	0.000	40.87
20.0	1.5595±0.05	0.000	50.84
Control	1.0339±0.02	-	-

Evaluation of wound healing

A total of 35 male Wistar rats were divided into seven groups of five rats per group. Groups 1-6 received topical treatment of 0.5, 1.5, 3.0, 5.0, 10.0, and 20.0% w/v of aqueous extract of *Azadirachta indica* leaves in water, respectively. Group 7 was treated with distilled water as a control. The wounds were photographed. The treatment was carried out daily and wound contraction was recorded each day before cleaning and treatment until the closure of the wounds. The percentage of wound contraction[55] was calculated using equation 1.

$$Percentage\ of\ wound\ contraction = \frac{Initial\ wound\ size\ \ specific\ day\ wound\ size}{Initial\ wound\ size} X\,100$$

Determination of tissue hydroxyproline

At the complete closure of the wounds, the rats were sacrificed. Tissue bioassay was conducted with 100 mg of the tissues collected from the site of the healed wound of the respective rats. This was added to 1 ml of 6M hydrochloric acid and boiled for 6 h and cooled. The pH was adjusted to 6.8 and the volume was made up to 10 ml using distilled water. The respective samples were centrifuged and 1ml supernatant was used for the hydroxyproline assay using kits obtained from Elabscience (China) and conducting the experiment in line with the protocols in the manufacturer's manual which based on the technique described by Bergman and Loxley[56] which is in line with the principle that the oxidation product produced by hydroxyproline under the action of an oxidant reacts with dimethylaminobenzaldehyde (DMAB; Ehrlich's reagent) and shows a purplish red color, the hydroxyproline was calculated by measuring the absorbance at 550 *n*m using a UV-VIS spectrophotometer (Jenway 6405, UK). The values were reported as μg/g dry weight of tissue.

Statistical Analysis

The figures were presented as a mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed, followed by Fisher's Least Significant Difference (LSD) *post hoc* test to determine the level of significance.

RESULTS AND DISCUSSION

The qualitative phytochemical components of the aqueous extract of *Azadirachta indica* leaves are shown in Table 1 while the result of GC-FID quantification of the phytochemicals is presented in Table 2 and Figure 1. The result of this study indicated that the aqueous extract of *Azadirachta indica* leaves possesses relevant phytochemical constituents including the alkaloids, flavonoids, glycosides, tannins, saponins, and phenols, most of which are very useful for the maintenance of good health.

The pattern of wound contraction is presented graphically in Figure 2 and pictorially in Figure 3, depicting a consistent wound closure which was observed to be accomplished within 14–18th days following dorsal excision. By the aid of the biomarker employed in the study, it was recorded that the cutaneous wound healing in rats treated with various concentrations of aqueous extract of *Azadirachta indica* leaves was better than those in the control group which was managed with distilled water. The presence of the phytochemicals in the extract could have contributed to its potency for the wound healing action. Flavonoids are indispensable assemblage of polyphenols widely distributed among the plant flora. Their occurrence possibly suggests the basis for the ethnomedicinal use of aqueous extract of *Azadirachta indica* leaves for the treatment of wounds.[46] Many flavonoids have both antimicrobial and antioxidant actions as well as nourishing supplement.[57,58]

The result of the tissue assay of hydroxyproline indicated that the rats treated with 0.5, 1.5, 5.0, 10.0, and 20.0 % w/v of the aqueous extract of *Azadirachta indica* leaves, respectively, exhibited significantly elevated mean tissue hydroxyproline levels compared to the animals treated with distilled water (control) ($p < 0.05$), as shown in Table 3.

The highest and lowest mean tissue hydroxyproline levels were recorded in the rats treated with 1.5 and 3.0% extracts, respectively, having corresponding percentage differences of 54.75 and 2.82% compared to the control group ($p < 0.05$), depicting that the optimal and the best concentration of aqueous extract of *Azadirachta indica* leaves for wound healing is 1.5%w/v. The mechanisms involved in wound healing is complex comprising hemostasis, inflammation, proliferation, and remodeling as well as inclining to the actions of proteins and glycoproteins, such as cytokines and chemokines.[59] The aqueous extract of neem is considered effective to bring about wound healing considering its potent antioxidant, antibacterial and, especially, the anti-inflammatory actions based on its rich flavonoid contents as over 60% of the phytochemicals are flavonoids.[38-44]

CONCLUSIONS

The aqueous extract of *Azadirachta indica* leaves contains relevant phytochemical constituents such as the alkaloids, flavonoids, glycosides, tannins, saponins, and phenols. Most of these are very useful for the maintenance of good health. The study showed that the cutaneous wound healing in rats treated with the various concentrations of the aqueous extract of *Azadirachta indica* leaves exhibited better results than those in the control group that was managed with distilled water. This is based on their hydroxyproline levels. It is suspected that the presence of the phytochemicals contributed to the potency of the extract for wound healing. The optimal effective and the best concentration of the aqueous extract of *Azadirachta indica* leaves for wound healing were determined to be 1.5%w/v. It may be necessary to carry out further studies to confirm the effectiveness of this concentration of the extract for wound healing in a dosage form such as an ointment or a cream.

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