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Research Article

Evaluation of the Antioxidant and Healing Properties of the Hydroethanolic Extract of *Kalanchoe pinnata* (Lam) Pers of the Togolese Flora

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Abstract

Natural herbal remedies have always been important in the management of skin disorders and the treatment of skin infections due to limited access to pharmaceuticals and the affordable cost of herbal products. *K. Pinnata* is a plant used as a medicinal herb by almost all traditional medicines in tropical Africa. The aim of the present work is to evaluate the antioxidant and healing properties of *K. pinnata* (Lam) pers extract from Togolese flora. The hydroethanol extract of *K. pinnata* (Lam) pers was subjected to in vitro antioxidant activity screening models, such as molybdate reduction, iron reduction assay (FRAP) and DPPH radical scavenging assay. A 1.5 cm diameter excision wound model was also used to assess the plant's healing activity. Wistar rats were treated after excision by topical application of vaseline, vaseline supplemented with 3% (EHKP 3%) and 10% (EHKP 10%) hydroethanol extract of *K. pinnata* (Lam) pers leaves, sulfadiazine (reference), or no treatment (negative control). Total antioxidant activity by the molybdate reduction method was 0.12 ± 0.01 µg EAA/mg EL, while the iron reduction assay (FRAP) showed an antioxidant capacity of 4.21 ± 0.04 mol Eq FeS04/ g extract and 10.17 ± 0.03 mol Eq FeS04/ g extract respectively for extract concentrations of 100 µg/ml and 200 µg/ml. In addition, the extract showed a dose-dependent ability to trap the DPPH radical. Results showed a significant reduction in wound surface area in animals treated with 10% EHKP. The hydroxyproline assay showed a significant difference in collagen production in animals treated with 10% EHKP, compared with the control group ($P < 0.001$) and the vaseline group ($P < 0.01$) on the last day. Histological analysis showed rapid re-epithelialization in animals treated with the extracts.

Keywords: *K. pinnata*, Antioxidant activity, Healing activity, Hydroxyproline

INTRODUCTION

A wound is a disruption or break in the anatomical or cellular continuity of living tissue[1]. The skin plays an essential role in protecting the body from the external environment. Damage to skin integrity caused by incisions, burns, scalds and human injuries (diabetic foot, venous ulcers, bedsores, etc.) are considered wounds[2]. Effective wound management is a major challenge for human health[3]. Lack of healing and prolonged healing times increase economic and social costs for healthcare institutions and professionals, patients and their families[4]. To compensate for tissue loss, the body initiates a healing process. This dynamic process unfolds through physiologically distinct stages of coagulation, inflammation, re-epithelialization and tissue remodeling[5,6]. Despite the existence of several wound-healing products, wounds often become chronic, which remains a public health problem to this day[7]. Scientists and clinicians are therefore actively seeking alternative, more effective products to treat and manage chronic wounds. In developing countries, wounds have a considerable impact on economic, physical and social health[8].

The World Health Organization (WHO) estimates that around 80% of the world's population relies on traditional practices for primary healthcare, and that 85% of them use plants. Given the difficulty these populations have in accessing conventional treatments, the WHO suggests the adoption of traditional practices as a tool for maintaining health, and encourages the development of public policies aimed at incorporating them into the official health systems of its 191 member countries. According to WHO statistics, around 5 million people could die every year because their wounds do not heal properly[9]. Today, despite advances in modern medicine, a large proportion of the world's population uses and relies on traditional medicine for their health needs. *K. pinnata* is used as a medicinal plant by almost all traditional medicines in tropical Africa[10,11,12]. In Togo, *K. pinnata* leaves are used as an antioxidant, antibacterial

and healing agent in various forms[11]. The aim of the present work is therefore to evaluate the antioxidant and healing properties of the hydroethanolic extract of *K. pinnata* (Lam) pers from Togolese flora.

MATERIALS AND METHODS

Plant Material

The plant material consists of young leaves of *K. pinnata* (Lam) Pers. (Family: Crassulaceae). The fresh leaves of the plant were harvested in the early morning of October to November 2021 at Togblecopé-Akoin, Lomé. The plant was then identified at the Botany and Plant Ecology Laboratory of the University of Lomé and registered under number TOGO15833.

Extraction Procedure

The leaves were then cut into very small pieces and dried at room temperature in the shade, before being ground into a coarse powder using a suitable mill. The powder was then stored in an airtight container in a cool, dark, dry place until analysis. The powders obtained from grinding the plants were delipidated with petroleum ether. To 400 g of ground plant material, we added 2 L of petroleum ether for 24 hours. After delipidation, we proceeded with extraction by maceration in 2000 ml of a hydroethanolic solution composed of 70% alcohol 95° and 30% distilled water in a clean, flat-bottomed plastic container. The whole was stirred continuously for 72 hours at laboratory temperature, then filtered through Whatman N°.1 paper, then evaporated under vacuum at 50°C at 125 rpm using a Heidolph-type electric rotavator. The resulting concentrated hydroethanol extract was lyophilized at low temperature and stored at 4°c protected from light in a dry bottle until use.

Animal Material

The animal material consisted of albino rats of the Wistar strain. These animals were bred in the animal house of the Faculty of Sciences of the University of Lomé. The animals were fed a standard diet consisting of proteins, carbohydrates and lipids, and water ad libitum. They were thus acclimatized to laboratory conditions.

Evaluation of the Antioxidant Activity of the Extract

Molybdate Reduction Method

The principle is based on the reduction of molybdate MoVI to molybdate MoV by antioxidant compounds, resulting in the formation of a green phosphomolybdate MoV complex with an absorption maximum at 695 nm. The antioxidant capacity of the extract was assessed by the phosphomolybdate reduction method, performed as described by Porter et al.[13]. The phosphomolybdate reagent is prepared from a mixture of 90 ml 0.6 M sulfuric acid, 5 ml sodium hydrogen phosphate (0.1%) and 5 ml ammonium molybdate (1%). For the test, 1 ml of extract at a concentration of 28.6 mg/ml is added to 9 ml of reagent. The solution is heated in a water bath at 95°C for 90 minutes, then cooled to room temperature. Absorbances were read at 695 nm on a UNICO Model 1200E spectrophotometer. Ascorbic acid was used as the standard. The test was performed in duplicate. The equation of the calibration curve obtained from the assay of successive dilutions of ascorbic acid will give the concentration of antioxidant compounds expressed in ug Ascorbic Acid Equivalent per mg freeze-dried extract.

Iron Reduction Assay (FRAP)

The FRAP method developed by Benzie and Stains[14] corresponds to the reduction of a ferric tripyridyltriazine complex $[(Fe(III)TPTZ)_2]^{3+}$ (orange-yellow) to a ferrous tripyridyltriazine complex $[(Fe(II)TPTZ)_2]^{2+}$ (dark blue) by an electron-donating antioxidant, at a pH of 3.6 to maintain iron solubility. The protocol used is that described by Benzie and Strain[14] and repeated by Agbodan et al.[15].

Reagent Preparation: To prepare the FRAP assay reagent, three solutions were mixed: acid buffer pH = 3.5 (50 ml), 2,4,6- tripyridyl-s-triazine (TPTZ) solution (5 ml) and iron III chloride solution (5 ml).

Acid Buffer (pH = 3.5): Dissolve 0.310 g CH_3COONa, $3H_2O$ in a 100 ml flask, then add 1.6 ml pure acetic acid and make up to the mark with distilled water.

TPTZ solution: Dissolve 156 mg of TPTZ in 50 ml of 40 mmol/L HCl.

Iron III chloride solution (20 mmol/L) was prepared by dissolving 27 mg $FeCl_3$,$6H_2O$ in a 50-mL flask and topping up with distilled water. The sulfate solution of iron II for the FRAP assay was prepared by dissolving 27 mg $FeSO_4$,$7H_2O$ in 50 ml methanol. A solution of molar concentration C = 2000 µmol. L-1 was obtained. This solution was successively diluted with methanol to obtain several solutions of different concentrations of iron II sulfate.

Protocol: To 3 ml of freshly prepared FRAP reagent in a test tube, 100 µl of the different iron II sulfate solutions at different concentrations (1000 ug/ml; 500 ug/ml; 250 ug/ml; 125 ug/ml) were added. The mixture was vigorously vortexed and the optical density was read after 5 min with a spectrophotometer at 593 nm (METASH UV-5200PC UV/VIS Spectrophotometer). The absorbance of the TPTZ-Fe^{2+} complex was used to plot a calibration curve based on the concentration range of the iron sulfate solution ($FeSO_4$, $7H_2O$) dissolved in methanol. For test extract samples, FRAP reagent (3ml) and test extract solution (100 µl) at different concentrations (200 ug/ml and 100 ug/ml) were mixed in the same proportions as for standard curve plotting. Optical density was read after 5 minutes at 593 nm. The antioxidant capacity of the extracts was measured using the calibration curve obtained from the concentration range of the iron sulfate solution ($FeSO_4$, $7H_2O$) and expressed in mol Eq FeS04/ g freeze-dried extract. The tests were repeated three times.

DPPH Radical Reduction Assay

Antioxidant activity was assessed according to the method described by Sahin16 and repeated by Ait-Mimoune17. 0.5 ml hydroethanolic extract of Kalanchoe pinnata at different concentrations (500 ug/ml, 400 ug/ml, 200 ug/ml, 100 ug/ml, 50 ug/ml, 25 ug/ml) in ethanol were mixed with 1.5 ml ethanolic solutions of DPPH (0.004%). Mixtures were vortexed and kept in the

dark for 30 minutes. Absorbance was measured at 517 nm using a spectrophotometer against a blank (DPPH) and compared with a standard (ascorbic acid). Percentage inhibition was calculated according to the following formula: % free radical scavenging = (A. blank - A. sample) / A Blank × 100 Where A. Sample and A. Blank are the absorbances of the DPPH solution after addition of the extract and the absorbance of the DPPH solution with ethanol, respectively. The IC50 value (extract concentration required to scavenge 50% of the initial DPPH radicals) was obtained from the graph by plotting the percentage inhibition as a function of extract concentration. Trials were repeated twice.

Evaluation of the Healing Activity of *Kalanchoe pinnata* Leaves

Ointment Formulation

From the crude extract of *Kalanchoe pinnata* leaves, two ointments at 3% and 10% were prepared from pure petroleum jelly (Qsp 50 g). Sodium benzoate was used as a reference preservative at a rate of 0.075 g per 50 g of ointment. Sodium benzoate and crude extract powder were crushed in a mortar using a pestle. Vaseline was added gradually, with gentle stirring, until the mixture was homogenized. The ointments were packaged in hermetically sealed jars and stored at room temperature out of direct sunlight[18].

Table 1: Composition of the Different Ointments

	Placebo (Vaseline)	Ointment (KPE 3%)	Ointment (KPE 10%)
KPE	0	1.5	5
Vaseline (g)	49.925	48.425	44.925
Sodium Benzoate (g)	0.075	0.075	0.075
Total (g)	50	50	50

KPE: *K. pinnata* Extract

Study Animals and Batches

Wistar rats aged 8-10 weeks and weighing on average 150-192 g were used for this study. The animals were bred in the animal house of the Faculty of Science at the University of Lomé (Togo). The rats were acclimatized to laboratory conditions for two weeks before starting the test. The animals had free access to food and water. The principles of laboratory animal care as described in the institutional guidelines and ethics of the Physiology and Pharmacology Laboratory of the University of Lomé-Togo (ref: 001/2012/CA-FDS-UL) were followed.

Chemicals and Reagents

Pure vaseline and sodium benzoate were sourced from Sigma (Merck KGaA, Darmstadt, Germany) while sulfadiazine was supplied by Thermo Fisher Scientific (Waltham, USA).

Wound Induction

Thirty rats were divided into 5 groups of 6 rats each as follows: Untreated rat group; control group (pure petroleum jelly, used as excipient); 3% extract ointment group (EHKP-3%); 10% extract ointment group (EHKP-10%); and sulfadiazine group, reference drug. The excision wounds were created on the skin in the dorsal region of each animal; the choice of this region was motivated by ease of access, both for performing the excision and for monitoring the evolution of the wound. Locating wounds at this level also prevents the animals from self-arrowing the wounds. The slightly modified method was used for wound induction[18]. Rats were anesthetized by exposure to diethyl ether-soaked absorbent cotton in a vial. The depth of anesthesia was determined by reflex responses such as voluntary movements from stimuli (leg extension). To facilitate wound creation, the rats were shaved beforehand with a scissors and Bic razor, taking care to avoid skin lesions. Using a chisel, a 1.5 cm diameter area previously delimited with an ink pad was excised[18].

Wound Treatments

Treatments were administered daily for 15 days by topical application. Photographs of the wound were taken on the first day (D0) immediately after induction, and then every 48 h for 15 days, using a high-resolution Android phone (Google Pixel 4XL; 12.2 megapixels) at the same fixed distance from the wound camera. Wound diameters were taken using a graduated ruler every day prior to treatment application. Wound areas were calculated using the free Image J 1.48v software (National Institutes of Heath, USA). The following formula was used to determine the contraction rate of each wound[5].

$$\text{Percentage of contraction} = \frac{(\text{Surface J0} - \text{surface Jn})}{\text{Surface J0}} \text{ X } 100$$

Where J0 is the initial wound area on day 1, and Jn is the wound area on day x after wound induction.

On the last day, the remaining rats were sacrificed, again by overdosing the anesthetic, and biopsies were taken for hydroxyproline assay[19]. Biopsies for histological tests were preserved in 10% formalin, while those for hydroxyproline assays were stored in the freezer.

Hydroxyproline Assay

Hydroxyproline is a major component (amino acid) of the collagen protein produced by hydroxylation of the amino acid proline. It plays a key role in the stability of collagen,

the protein involved in the formation of granulation tissue during wound healing. Its measurement enables an indirect determination of collagen quantity. The colorimetric technique of Bergman and Loxley[20] adopted by Zouari Bouassida et al.[21] was used with slight modifications.

Briefly, the principle is based on hot hydrolysis of dried wound biopsies (60°C oven drying for 12 h) in a concentrated acid medium (HCl 6 N) for 4 h at 130°C, with the aim of releasing amino acids including hydroxyproline, which is then assayed. Hydrolysates in sealed glass tubes were recovered with 10 ml distilled water, homogenized; mixed successively with 50 µl CuSO4 (0.01 M), 50 µl NaOH (2.5 N) and 50 µl H202 (6%); incubated at 80°C in the oven for 5 min. After cooling the tubes to room temperature, 2 ml H2SO4 (3N) and 1 ml P-dimethylaminobenzaldehyde 5% were added before reading with a spectrophotometer at 540 nm. Standard solutions with hydroxyproline concentrations of 1.0, 2.0, 4.0 and 8.0 mg/mL were also prepared in triplicate to establish a calibration curve. Results were expressed as µg hydroxyproline/mg dried tissue.

Histological Studies

On the last day, rats were anesthetized with diethyl ether and sacrificed by dislocation for histological examination. Skin wound samples for histological examination were embedded in 10% neutral buffered formalin, processed and embedded in kerosene. Five-micrometer sections of skin were cut with a microtome and stained with hematoxylin-eosin (H&E). Tissues were qualitatively assessed under a light microscope (Olympus BX 51) at 100x magnification. Parameters such as granulation, epithelialization, vascularization and inflammatory cells were highlighted.

Data Analysis

Antioxidant test results are presented and expressed as mean values and standard deviations. Healing test data were entered and processed using the free Image J 1.48v software (National Institutes of Heath, USA). Graph Pad Prism 8.0.2 (Boston, USA) was used for two-way analysis of variance (ANOVA) followed by Bonferoni's test at the significant threshold of $P < 0.05$. Results were presented as percentages and means with standard error on the mean (M ± SEM).

RESULTS

Antioxidant Activity Assessment

Total Antioxidant Activity by the Molybdate Reduction Method

The equation of the calibration curve y = 4.864x - 0.3144 obtained from the assay of successive dilutions of ascorbic acid enabled us to evaluate the total antioxidant activity of the extract. The antioxidant capacity obtained was 0.12 ±0.01 µg EAA/mg EL (ug Ascorbic Acid Equivalent per mg freeze-dried extract).

Iron Reduction Test (FRAP)

The antioxidant potential of *Kalanchoe pinnata* leaf extracts was determined by the FRAP method, based on the ability of antioxidants present in the extracts to reduce TPTZ-Fe (III) to TPTZ-Fe (II). The antioxidant capacity of the extracts was measured using the calibration curve y = 0.0007x + 0.001 obtained from the concentration range of the iron sulfate solution (FeSO4, 7H2O) and expressed in mol Eq $FeSO_4$/ g freeze-dried extract. The result of this test showed that the antioxidant potential of *Kalanchoe pinnata* hydroethanol extract is dose-dependent. We found that the higher the concentration, the higher the antioxidant potential. Thus, the antioxidant capacity of our extract is 4.21 ± 0.04 mol Eq $FeSO_4$/ g extract and 10.17 ± 0.03 mol Eq $FeSO_4$/ g extract respectively for extract concentrations of 100 µg/ml and 200 µg/ml. These results demonstrate that *Kalanchoe pinnata* extracts have a marked ability to reduce ferric ions (Fe^{3+}) and thus neutralize free radicals by donating electrons.

DPPH Reduction Assay

The antioxidant activity of the hydroalcoholic extract of *Kalanchoe pinnata* leaves towards the DPPH radical was evaluated spectrophotometrically at 517 nm, by monitoring the reduction of this radical, which is accompanied by a change from violet to yellow. Percentage inhibition was calculated according to the following formula:

% free radical scavenging= (A. blank - A. sample) / A Blank × 100

Where A. Sample and A. Blank are the absorbances of the DPPH solution. The following table shows the evolution of the DPPH radical inhibition percentage of ascorbic acid and extract as a function of concentration.

Table 2: Percentage DPPH Radical Inhibition as a Function of Extract Concentration

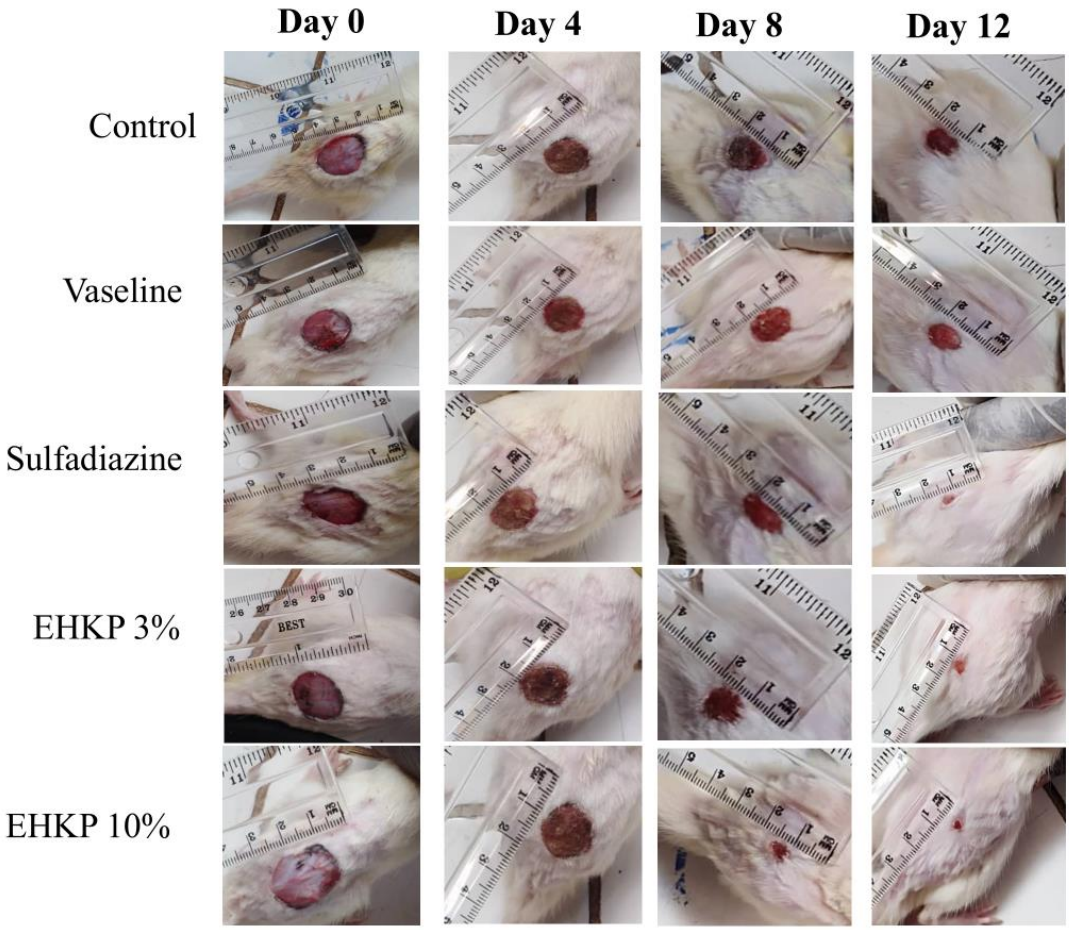
Concentration (µg/ml)	Percentage DPPH Radical Inhibition (%)	
	HEKP	AA
25	27.42	70.72
50	30.11	89.59
100	38.38	94.14
200	49.35	-
400	71.56	-
500	80.02	-

AA: Ascorbic acid; EHKP: Hydroethanolic extract of *K. pinnata*

Healing Activity of *K. pinnata* Leaves

Assessment of Wound Surface

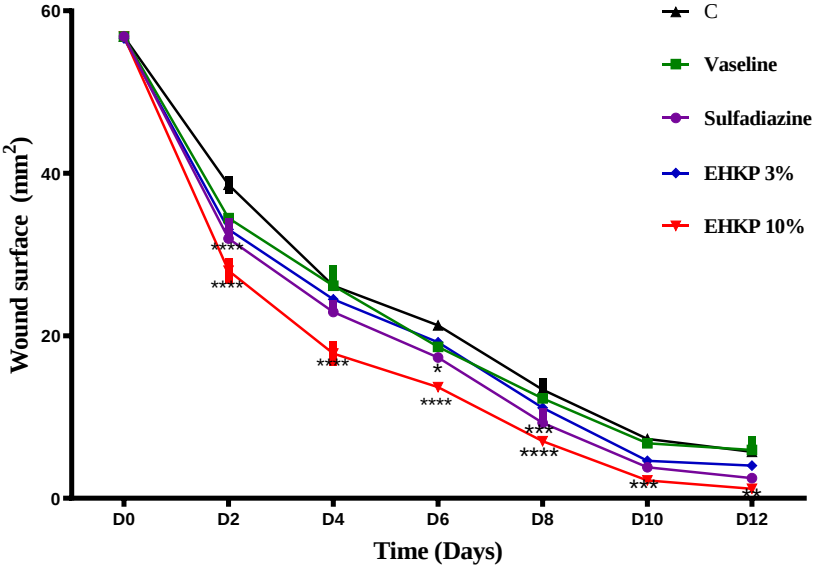
Macroscopic observations and photographs were taken to monitor the evolution of wound healing over time for the different treatment groups (Figure14). The images in this figure 14 show that wound surface was significantly reduced, with a significant reduction observed in the groups treated with AHKP-3%, EHKP-10%, and Sulfadiazine.



EHKP 3% = Vaseline with 3% hydroethanol extract of *K. pinnata* leaves;
EHKP 10% = Vaseline with 10% hydroethanol extract of *K. pinnata* leaves.

Figure 1: Macroscopic Observation of Healing Progress in the different Groups

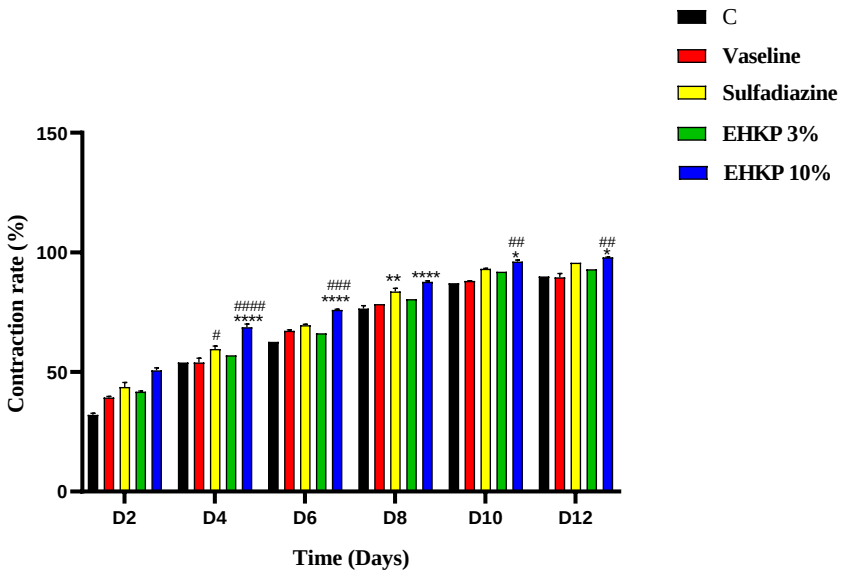
Wound surface measurements were also taken and the data used to plot the evolution of wound surface as a function of time (Figure15). On day one, the figure shows that all groups have similar wound surfaces after wound induction. A reduction in wound area was observed in all five groups after treatments, with a significant reduction ($p<0.05$) for groups treated with sulfadiazine, EHKP 10% from day 2 observed in groups treated with sulfadiazine, EHKP 10%.



Values are expressed as mean ± SEM, n= 6; Units: mm2. D= Day; EHKP 3% = Vaseline with 3% Hydroethanol Extract of *K. pinnata* leaves; EHKP 10% = Vaseline with 10% Hydroethanol Extract of *K. pinnata* leaves. *P<0.05; ** P<0.01; *** P<0.001; *** P<0.0001: compared with Control Group.

Figure 2: Evolution of Wound Surface Area

The calculation of contraction rates presented in the figure corroborates the results observed previously and reveals that from day 4 onwards, there was a significant difference in the wound contraction process in the groups treated with sulfadiazine, EHKP 10% compared with the control group and the vehicle-vaseline group. Significant differences in contraction rates were observed on day four in the sulfadiazine-treated groups (p<0.5 vs. vehicle-vaseline group) and EHKP 10% (P < 0, 0001 versus control and vaseline), on day eight in the EHKP 10% and sulfadiazine groups, respectively 87.65 ± 0.54% and 83.66 ± 2.28% (P < 0.0001, P < 0.001 versus control); then on days ten and twelve, only in the EHKP 10% treatment groups (p<0.5; p<0.01 vs. control and vaseline groups respectively).

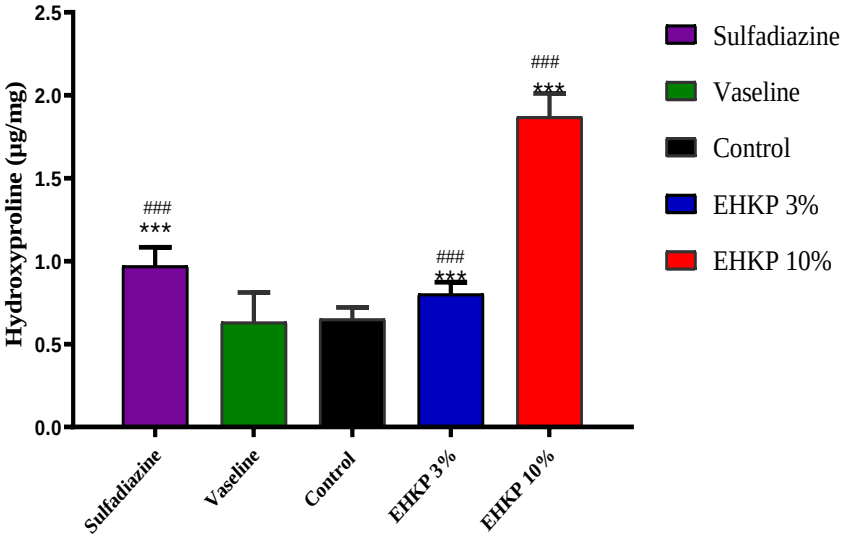


Values are expressed as mean ± SEM, n = 6. Units: %. D= Day ; ; EHKP 3% = Vaseline with 3% Hydroethanol Extract of *K. pinnata* leaves ; EHKP 10% = Vaseline with 10% Hydroethanol Extract of *K. pinnata* leaves. *P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001: Compared with Control Group. #P<0.05; ##P<0.01; ###P<0.001; #### P< 0.0001: Compared with Vehicle Vaseline Group.

Figure 3: Wound Contraction Rate

Effect of Extract on Hydroxyproline Levels

The measurement of hydroxyproline is generally used as an indicator to determine collagen levels. According to the figure, hydroethanol extract of *Kalanckoe pinnata* leaves in vaseline promoted collagen production, as shown by hydroxyproline content. A significant difference was observed at day 12 between the hydroxyproline content of the control group and the groups treated with EHKP 3%, EHKP 10% and sulfadiazine (*** P<0.0002). Significant differences were also observed in collagen production in groups treated with EHKP 3%, EHKP 10% and sulfadiazine compared with the group treated with vaseline (### P<0.0002).

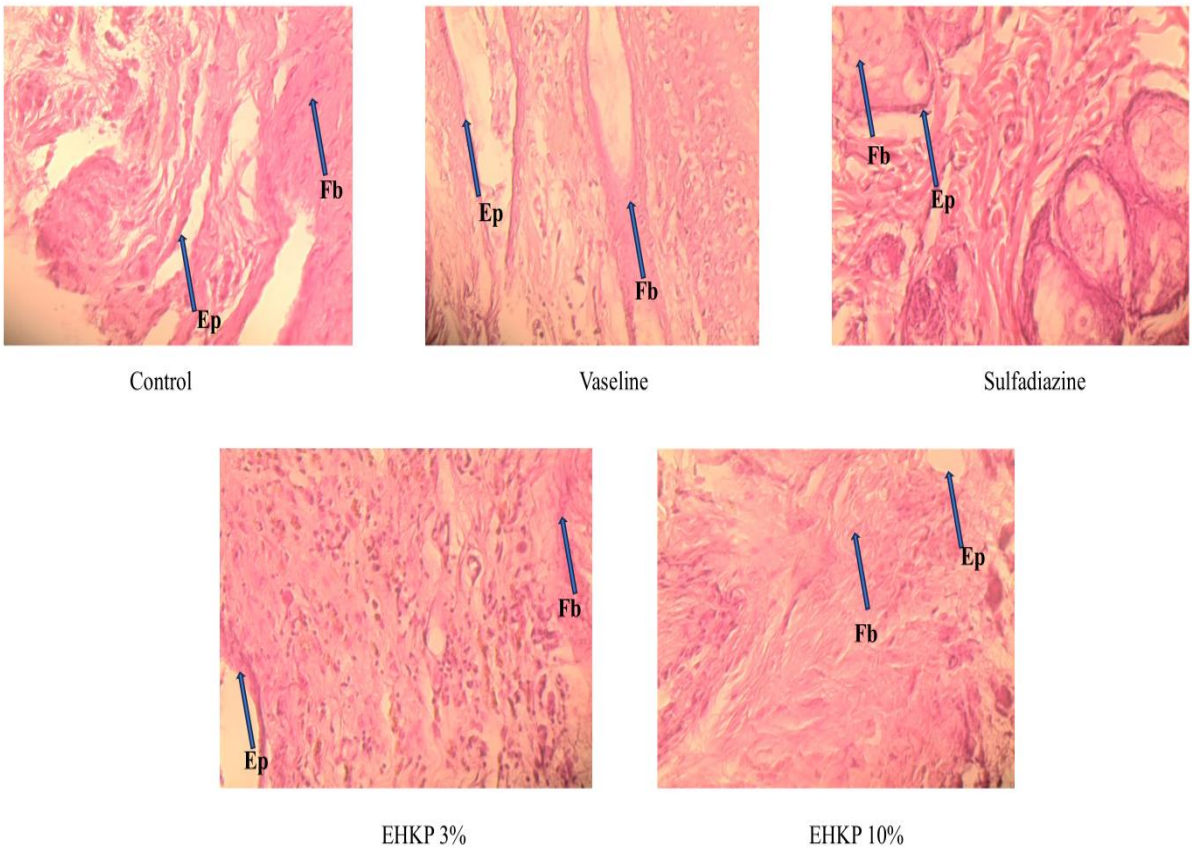


Values are expressed as means ± SEM, n = 6. Units: µg/mg dry tissue. ***P<0.0002: Compared to the Control Group on day 12. ###P<0.0002: Compared to the Vaseline Vehicle Group on day 12.

Figure 4: Hydroxyproline Content of Rat Skin Samples on the Last Day of Treatment

Histological Study

The histological sections taken on the last day showed neovascularization and fibroblastic cells were observed in the sections of rats treated with ALHE-10% and Sulfadiazine. In rats treated with petrolatum, control and ALHE-3%, there was only minor neovascularization (Figure 18 and Table 15). High re-epithelialization was observed in skin samples of rats treated with EHKP 10% and sulfadiazine. Conversely, in the control, petrolatum and EHKP 3% groups, only minor re-epithelialization was observed.



Structure of wounds under light microscope. Hematoxylin-eosin (H&E) staining; x 50 growth, scale bar is 50 µm. Abbreviations: EHKP 3%; EHKP 10% ; Fb = Fibrosis ; Ep = Re-epithelialization.

Figure 5: Histological Observation of Wounds at the Last Day

Table 3: Analysis of Histological Sections on the Last Day

Treatment	Ed	Inf	NV	Ep	Fb	U
Control	-	+	+	+	++	+
Vaseline	-	+	+	+	++	++
Sulfadiazine	-	-	++	++	++	-
EHKP 3 %	-	+	+	+	++	+
EHKP 10 %	-	-	++	++	++	-

(-) = Absent; (+) = Normal; (++) = high; Ed: Edema; Inf: Inflammatory Infiltrate; Nv: Neovascularization; Fb: Fibrosis; U: Ulceration; Ep: Squamous Epithelium

DISCUSSION

Antioxidants are useful in reducing and preventing damage caused by free radical reactions due to their ability to donate electrons that neutralize the radical without forming another one. They are compounds that can delay or inhibit the oxidation of lipids or other molecules by preventing the initiation or propagation of oxidative chain reactions[22]. The antioxidant effect is mainly due to phenolic compounds, such as flavonoids, phenolic acids and phenolic diterpenes[23]. The results obtained from our work show us that the hydroethanolic extracts of *Kalanchoe pinnata* have the ability to reduce not only free radicals but also to chelate metal ions and therefore have very good antioxidant activity. Studies carried out on different extracts of the plant corroborate our results. Tatsimo and al.[24] studied the antioxidant

activities of ethyl acetate and methanolic extract of K. pinnata and reported that the methanolic extract showed the highest activity as compared to the aqueous extract. According to their work, the methanolic extract showed the highest activity in scavenging free radicals and inhibiting microorganisms[24]. Sindhu and Manorama[25] evaluated hexane, chloroform, ethyl acetate, acetone and ethanol extracts of the leaves of the plant which showed varying degrees of antioxidant activity in different test systems in a dose-dependent manner[25]. Jaiswal and al.[26] studied the ethanolic extract of K. pinnata and concluded that it had a high potential to protect against oxidative stress compared to standard antioxidants against oxidative stress in aqueous and lipid phases[26]. Singh and al.[27] also confirmed the antioxidant activity of K. pinnata extracts prepared at different doses[27]. The healing activity of the hydroethanolic extract was evaluated using an excision wound model in Wistar rats. Treatment of excision wounds with the different formulations gave indications on the healing rate of the plant compared to the reference drug and the untreated batch. The increase in contraction and hydroxyproline levels during two weeks of treatment in rats treated with *Kalanchoe pinnata* indicates that this plant accelerates skin restoration. Topical application of these 10% ointments inhibited erythema, exudate and unpleasant odors fairly rapidly. Hydroxyproline measurement is frequently used as a reliable index to quantify collagen in tissues[28]. Collagen is the main structural protein component of tissues and is well known to effectively improve the healing process by promoting greater proliferation of fibroblasts[29]. In the present study, the hydroxyproline content of the groups treated with EHKP10% and sulfadiazine increased significantly compared to the control batches, indicating that the extract may have promoted collagen synthesis by stimulating the proliferation of fibroblasts responsible for collagen production. This observation was confirmed by the wound histology which revealed re-epithelialization and neovascularization in the groups treated with EHKP10% and sulfadiazine, while a delayed healing process was still observed in the control animals after two weeks. All of these phenomena explain the increase in the healing rate that it causes as demonstrated by [30]. We also noted that the extract induced a dose-dependent healing. The healing activity of *Kalanchoe pinnata* is demonstrated by ethanolic extracts which significantly reduce the size of cuts and wounds as well as edema in the affected area. Recent studies have shown that extracts prepared in alcohol, petroleum ether and water have wound healing potential. These experimental researches have demonstrated that aqueous extracts have more potential than alcoholic and etheric extracts[30]. The results of the present study are consistent with those of the studies of Metowogo and al.[19], which showed that another Togolese medicinal plant, *Cochlospermum planchonii*, could accelerate the healing process of burn wounds. According to these studies, the healing properties would be linked to the presence of phenolic compounds such as flavonoids in the plants. Indeed, some recent studies have shown that this healing potential may be due to the presence of phenolic antioxidants. Subramanian and al.[32], studied the healing properties of a flavonoid-rich fraction formulated from a leaf extract of *Dodonaea viscosa*. According to their work the ethyl acetate fraction of D. viscosa revealed flavonoids with high concentrations of quercetin and kaempferol. They showed that the ethyl acetate fraction of D. viscosa significantly accelerated wound healing by accelerating faster wound contraction and epithelialization, by elevating epithelialization. Treatment with ethyl acetate fraction of D. viscosa improved wound healing through re-epithelialization, collagen formation and vascularization of damaged skin samples[32]. Similarly Muralidhar and al.[33] evaluated the wound healing activity of flavonoid fraction isolated from bark of *Butea monosperma* in excision, incision models using Albino wistar rats. According to the results of their work, the flavonoid fraction showed significant wound healing activity in excision, incision and dead space wound healing models using Albino wistar rats. According to them, the increase in wound contraction rate and hydroxyproline content in animals treated with the flavonoid fraction provides a scientific basis for the ethnomedical use of *Butea monosperma*, which is largely attributable to the additive or synergistic effect of isoflavones present in the flavonoid fraction[33]. Phenolic compounds, flavonoids are known for their healing properties related to their astringent effect[30]. Flavonoids are one of the most attractive and promising families of natural products for the treatment of skin problems. The structure-activity relationship (SAR) of flavonoids is one of the key elements of this characteristic. The presence of hydroxyl groups in their chemical structure, especially at positions 5, 7, 3, and 4, is essential for their antifibrotic, antioxidant, and anti-inflammatory effects due to their high levels of hydroxylation[34]. Many flavonoids have been characterized as potent inhibitors of reactive oxygen species (ROS), making them essential antioxidant dietary components. The use of antioxidants such as most flavonoids is believed to accelerate wound healing by reducing oxidative stress in the wound[35].

Antioxidants are useful in reducing and preventing damage caused by free radical reactions due to their ability to donate electrons that neutralize the radical without forming another one. These are compounds that can delay or inhibit the oxidation of lipids or other molecules by preventing the initiation or propagation of oxidative chain reactions[22]. The antioxidant effect is mainly due to phenolic components, such as flavonoids, phenolic acids and phenolic diterpenes[23]. The results obtained from our work show us that the hydroethanolic extracts of *Kalanchoe pinnata* have the ability to reduce not only free radicals but also to chelate metal ions and therefore have very good antioxidant activity. Studies carried out on different extracts of the plant corroborate our results. Tatsimo and al.[24] studied the antioxidant activities of ethyl acetate and methanolic extract of K. pinnata and reported that the methanolic extract has the highest activity compared to the aqueous extract. According to their work, methanolic extract showed the highest activity in scavenging free radicals and inhibiting microorganisms[24]. Sindhu and Manorama[25] evaluated hexane, chloroform, ethyl acetate, acetone and ethanol extracts of the leaves of the plant, which showed varying degrees of antioxidant activity in different test systems in

a dose-dependent manner[25]. Jaiswal and al.[26] studied the ethanolic extract of *K. pinnata* and concluded that it had a high potential to protect against oxidative stress compared to standard antioxidants against oxidative stress in both aqueous and lipid phases[26]. Singh and al.[27] also confirmed the antioxidant activity of K. pinnata extracts prepared at different doses[27]. Our results therefore indicate that *Kalanchoe pinnata* leaves contain antioxidant molecules, including flavonoids. Indeed, flavonoids are recognized for their numerous biological activities that are partly attributed to their antioxidant properties. They are likely to react with most reactive oxygen species[36]. The antioxidant action of these phytonutrients is not only exerted by the inhibition and deactivation of free radicals, it is also manifested by the neutralization of oxidizing enzymes and by the chelation of traces of metal ions forming stable complexes responsible for the production of reactive oxygen species (ROS)[37]. This chelation can prevent metals from participating in oxidation processes[38]. The presence of a large quantity of flavonoids in the extract contributes to the overall antioxidant activity of the plant extract. Flavonoids are also known to modify the activities of enzymes such as protein kinase C, protein tyrosine kinase, aldose reductase, myeloperoxidase, NADPH oxidase, xanthine oxidase, phospholipase, reverse transcriptase, ornithine decarboxylase, lipoxygenase, and cyclooxygenase. Some of these enzymes are involved in immune functions, carcinogenesis, cellular transformations, tumor growth, and metastasis[36].

CONCLUSION

The present study aimed to investigate the in vitro antioxidant and wound healing properties of the hydroethanolic extract of *Kalanchoe pinnata* leaves using an excision wound model in Wistar rats. The plant leaf extract showed the iron reducing and free radical scavenging capacity of which the extract can be considered as an effective antioxidant. The contraction rate, hydroxyproline levels and the nature of histological tissues obtained after two weeks of application of the hydroethanolic extracts of *Kalanchoe pinnata*, allowed us to conclude that the hydroethanolic extract of *Kalanchoe pinnata* leaves could be a potential natural remedy for the treatment of wounds. Further studies will be conducted to elucidate the factors such as toxicity, anti-inflammatory activity and effective dose for human use.

Conflict of Interest

The authors have declared no conflict of interest.

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