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Wound Healing Activities of Latex of Grassleaf Spurge (*Euphobia graminea* Jacq.)

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Abstract The present study evaluated the wound healing potentials of *E. graminea* (EG) latex. The latex of EG was screened for the presence of phytochemical constituents; *in vitro* antioxidant property and erythrocyte membrane stabilizing activity was used to investigate anti-inflammatory property of the latex. The wound healing potential of *E. graminea* latex (EGL) was investigated via incision and dead space wound models in wistar rats; eighteen wistar rats (separately for each model) were randomly divided into 3 groups (6 in each group): Group A: wound + EGL ; Group B: wound + Gentamycin (G); Group C: wound without treatment (control). Wound breaking strength (Incision model), hexuronic acid and hexosamine concentrations, activities of antioxidants biomolecules (superoxide dismutase, catalase, and reduced glutathione) as well as the levels of free radicals (nitric oxide-NO, malondialdehyde -MDA) were evaluated in the granulation tissue (dead space model) of experimental rats. The result of the study revealed that EGL tested positive for the presence of saponins, alkaloids, triterpenes, flavonoids and cardiac glycosides. Also, EGL contained an appreciable concentration of total flavonoids and phenolics. The latex exhibited mild ferric reducing power, inhibited DPPH in dose dependent manner as well as protected red blood cells against hypotonic and heat induced lyses. In wound incision model, EGL exhibited 13.6 % increase in wound breaking strength when compared to the control animals. Also, in dead space wound model, there was significant increase ($p < 0.05$) in hexoxamine, hexuronic acid and GSH concentrations as well as SOD activity in EGL and gentamycin treated wound compared to the control. Furthermore, there was a significant reduction ($p < 0.05$) in MDA concentration in EGL and gentamycin treated wound compared to the control while NO level and catalase activity showed no significant difference. This study revealed that EGL contained potentially bioactive molecules that could be employed in the treatment of wound.

Keywords: *euphorbia graminea, latex, wound, healing*

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1. Introduction

Wounds arise as a result of injuries to the skin that disrupt the soft tissue. It may be produced by the physical, chemical or microbial damage to the tissue. Wounds represent a significant burden on the affected and the healthcare professionals worldwide [1,2,3]. Wounds are the major causes of physical disabilities and affect the physical and mental health problems. Studies have revealed that nearly 6 million people suffer from chronic wounds worldwide [4]. This has drawn the attention of many scientists to identify potential means and sources of wound care.

There are a number of plants which are used traditionally for wound healing. Several plants' extracts are known as a promising alternative for wound healing agents due to the presence of various bioactive substances, accessibilty and little/no side effects [5]. Currently available therapies do not give an effective impact on tissue repairing process; hence instigate a need to develop new approach of natural origin to tissue repairing. Among such plants is *E. graminea.* It is very morphologically variable, as discussed by [6]. The possible anthropogenic uses of EGL include: treatment of skin infections such as ulcers, cancers, tumors, warts, and other diseases as practiced by Colombian traditional healers [7].

The plant belongs to the kingdom Plantae (plants), class of Magnoliopsida (dicotyledons), order of Malpighiales,

family of Euphorbiaceae (spurge family), genus of *Euphorbia* L. (spurge). It is an annual plant, generally from 15-30 cm tall, sometimes taller, often branching from the base, and dichotomously branched distally. The leaves are alternate; those toward the base of the plant are ovate or oblong with a few large distantly-spaced teeth, while those toward the top of the plants are elliptical to linear and entire [6]. Cyathia are in short- to long-pedunculate cymes and have white petaloid appendages. The capsules are exerted from the cyathia, glabrous, and about 2 x 3 mm. The seeds are angular, ovoid, ca. 1.5 x 1.3 mm, gray or whitened, and rugose [7]. There is no scientific information documented on the latex of *E. graminea*, hence this study evaluated the wound healing activity of grassleaf spurge latex in albino rats.

Plate 1. *E. Graminea* plant (Source: Botanical garden, Obafemi Awolowo University, Ile- Ife)

2. Materials and Methods

2.1. Collection of Plant Latex

E. graminea plant was collected from the Botanical Garden, Obafemi Awolowo University, Ile- Ife, Osun State, Nigeria. It was identified at IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife.The latex was obtained from the plant into several petri-dishes, covered and kept in the freezer for biochemical analyses.

2.2. Reagents and Chemicals

All reagents and chemicals used were of analytical grades, and were purchased from Sigma Chemical Company, Germany.

2.3. Phytochemical Screening of *E. graminea* Latex

The phytochemical analysis of the latex was carried out by a procedure that was based on the earlier reports of [8,9].

2.4. Evaluation of *In Vitro* Antioxidant Properties of *E. graminea* Latex

2.4.1. Estimation of Total Phenolics

Estimation of total phenol was carried out using the method described by [10] as reported by [11]. The assay involved pipetting 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml garlic acid solution (1 mg/ml) in triplicate. The volumes were adjusted to 1.0 ml with distilled water. To each of the test tubes was added 1.5 ml Follin Ciocalteu's reagent (1:10). The reaction mixture was incubated at room temperature for 15 minutes. To the mixture was added 1.5 ml of 7% (w/v) Na_2CO_3 solution. The reaction mixture was further incubated for 1 hr 30 min. The absorbance was read at 725 nm against the blank containing all reagents except the standard, garlic acid. The absorbance was plotted against the concentration to produce the standard curve. The latex (0.1 ml of 5 mg/ml) was pipetted into clean dry test tubes in triplicate and treated as standard. The concentration of phenolics from the latex was interpolated from the standard graph.

2.4.2. Estimation of Total Flavonoids

The concentration of flavonoids in *E. graminea* latex was estimated according to the procedure of [12]. The standard curve for the estimation of total flavonoids was prepared by pipetting 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of 1 mg/ml rutin (standard) into clean dry test tube. The volume was made up to 5.0 ml with distilled water. To each of the test tubes was added 0.3 ml of 5% (w/v) $NaNO_2$, 0.3 ml of 10% (w/v) $AlCl_3$ and 4 ml of 4% (w/v) NaOH. The reaction mixture was incubated at room temperature for 15 minutes. Absorbance taken at 500 nm was plotted against the concentration to give the standard curve.

The latex (0.1 g) was dissolved in ethanol {10 ml, 70% (v/v)}. The working solution (0.5 ml) was pipetted into clean dry test tubes in triplicate and treated as standard. The concentration of flavonoids from the latex was interpolated from the standard graph.

2.4.3. Ferric Reducing Antioxidant Power Assay

The reducing power of the latex and the standard drug was determined according to the method of [13]. Different concentrations of *E. graminea* latex (0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml in distilled water) were mixed with 1.25 ml of phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (1.25 ml, 1%). The mixture was incubated at 50°C for 30 minutes and allowed to cool. To the mixture was added 10% (w/v) trichloroacetic acid, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (0.625 ml) was collected and mixed with 0.625 ml distilled water and $FeCl_3$ (0.125 ml, 0.1%). The absorbance of the mixture was measured at 700 nm against water blank. Ascorbic acid (Vitamin C) was used as a standard to compare the reducing power of the latex.

2.4.4. DPPH Radical Assay

The method described by [14] was used to determine the antioxidant activity of the latex. The latex scavenging activity of the stable DPPH free radical was determined at varying concentrations of 50, 100, 150, 200, 250, 300 µg/mL. Distilled water (500 µL) was pipetted (in triplicate) into clean dried test tubes followed by the addition of 0.0, 75, 150, 225, 300, 375, and 450 µL of the latex based on the varied concentrations, then 1500, 1425, 1350, 1275, 1200, 1125 and 1050 µL of DPPH solution

was added to the test tubes respectively and mixed thoroughly. The reaction mixture was incubated in the dark for 30 minutes. The absorbance was taken at 517 nm and the percentage inhibition ability was calculated using the expression below.

$$Percentage\ Inhibition = \frac{Absorbance\ of\ blank - Absorbance\ of\ sample}{Absorbance\ of\ blank} X 100.$$

2.5. Evaluation of the Anti-Inflammatory Activity of *E. Graminea* Latex

2.5.1. Membrane Stabilization Assay

2.5.1.1. Preparation of red blood cell

The bovine red blood cell was prepared according to the method of [15]. The fresh blood sample was transferred into clean centrifuge tubes and followed by centrifugation at 3000 rpm on a Bench Centrifuge Model 90-2 (Searchtech Instrument England, UK.) for 10 min at room temperature. The supernatant was carefully removed and the packed red blood cell was washed again in fresh normal saline {0.85% (w/v) NaCl}. The process of washing and centrifugation was repeated until the supernatant was cleared (colourless). The bovine erythrocyte {2% (v/v)} was prepared by diluting 2 ml of packed cell with normal saline to 100 ml. It was kept undisturbed at 4°C in the refrigerator.

2.5.1.2. Red Blood Cell Membrane Stability Assay

The membrane stabilizing potentials of the latex was carried out according to the procedure earlier reported by [16]. Typically, varying concentrations (0, 100, 200, 300, 400 and 500 µg/ml) of the latex was pipetted into clean dry test tubes and adjusted to 1.5 ml with normal saline. To the reaction mixtures were added 0.5 ml hyposaline and 0.5 ml phosphate buffer (pH 7.4, 0.15 M) and 0.5 ml of 2% (v/v) erythrocyte. The volume was adjusted to 3.0 ml with normal saline. The blood control was prepared as above without the latex while the drug control contained all other reagents except the erythrocyte suspension. The mixtures were then incubated at 56°C for 30 min., cooled and centrifuged at 3000 rpm for 10 min. The supernatants were carefully decanted and absorbance was read at 560 nm against the reagent blank. The percentage membrane stability was calculated as:

$$Percentage\ Membrane\ Stability = 100 - \frac{(Abs_{test} - Abs_{drug})}{Abs_{blood\ control}} X\ 100.$$

2.6. Evaluation of Wound Healing Activity of *E. graminea* Latex

2.6.1. Experimental Animals

Thirty-six wistar rats weighing between 120-150g of either sex were purchased at the Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. They were acclimatised for two weeks in the Animal House, Department of Biochemistry, Adeleke University, Ede, Osun State, Nigeria. The rats were divided into three groups (separately for each model) of six rats each. Animals were provided with standard rodent pellet diet and water *ad libitum*. Principles of laboratory animal care guidelines were followed [17].

2.6.2. Incision Wound Model

Incision wound model was carried out according to the procedure described by [18]. In this model, cuts were made in the skin of the animal after giving them anaesthesia with ketamine (0.5 mL). Two para-vertebral long incision of 6 cm length was made through the skin and cutaneous muscles at distance about 1.5 cm from the midline on each side of the depilated back of the rats. After the skin incision, the parted skin kept together was stitched at 1cm intervals continuously and tightly using suture thread (No. 000) and a curved needle (No. 11). When the wounds were cured thoroughly, the sutures were removed on day 7 and tensile strength of the healed wound was measured on day 10 by continuous and constant water flow technique.

2.6.3. Measurement of Tensile Strength

The sutures were removed on 7th post wounding day and the wound breaking strength of the wounds was measured on the 10th day according to the continuous constant water flow technique as described by [19]. Two Allis forceps were firmly applied onto the line facing each other. One of the forceps was fixed, while the other was connected to a freely suspended light weight polypropylene graduated container through a string run over to a pulley. Distilled water was continuously poured slowly and steadily into the container. A gradual increase in weight was transmitted to the wound site pulling apart the wound edges as at when the wound just opened up, the water was stopped and the weight was noted [18]. The percentage increase in wound breaking strenght was calculated as follows:

$$Percentage\ increase\ in\ wound\ Breaking\ strength\ (PIWBS) = (WT/WC - 1) \times 100,$$

where W*T* and W*C* were WBS values of test and control groups respectively.

2.6.4. Dead Space Wound Model

The dead space wound model was carried out based on the procedure described by [20]. Rats were anesthetized with ketamine and 1 cm incision was made on dorsolumbar part of the back. Two polypropylene tubes (0.5×2.5 cm^2 each) were placed in the dead space of lumbar region of rat on each side, and wounds were closed with a suture material.

2.6.5. Grouping and Treatment of Animals

After the surgical wounds, all rats were randomly divided into three groups: A, B and C respectively. Group A: animals were treated with *E. graminea* latex dissolved in distilled water animals; Group B: animals were treated with an ointment, gentamycin cream (antibiotic drug) and served as the reference standard group; Group C: animals

were without treatment and served as the control group. The latex and the ointment were topically applied to the wound of the animals respectively. All rats were monitored daily for 15 days for any wound fluid, evidence of infection or other abnormalities.

2.6.7. Preparation of Granulation Tissue Homogenate

Granulation tissue homogenate was prepared as described by [21]. On the 15th day, the rats were euthanasia via cervical dislocation. Thereafter, the implanted tubes were removed cautiously through incision on the animals' skin, and weighed. Granulation tissue homogenates (10% w/v) were prepared by cutting granulation tissue (1 g) into bits and homogenized in freshly prepared phosphate buffer (pH 6.8, 100 mM). The homogenate was centrifuged at 3000 rpm with Bench Centrifuge Model 90-2 (Searchtech Instrument England, UK.) for 10 min. The supernatant was carefully transferred into clean vial bottles and kept frozen at -4°C for further biochemical assays.

2.6.8. Estimation of Wet Granulation Tissue Free Radicals

2.6.8.1. Determination of Nitric Oxide (NO) Concentration

Nitric Oxide assay was carried out using the Griess reagent [22]. The homogenized granulation tissue (500 µl) was pipetted into clean and dry test tubes and TCA (50 µl) was added to it. The resulting mixture was centrifuged for 10 min at 4000 rpm and the clear supernatant was transferred to a clean tube. The supernatant (100 µl) was pipetted into clean, labelled Eppendorf tubes and sulphanilamide (0.1 ml, 1%) in 5% phosphoric acid was added to it. The reaction mixture was incubated at room temperatute for 10 min, followed by addition of 0.5 ml 0.1% NED (N-1-napthylethylenediamine dihydrochloride). The reaction mixture was further incubated for 10 min at 60°C and the absorbance of the chromophore formed was measured at 546 nm. The standard curve was prepared using sodium nitrite (100 µM). The amount of NO in the sample was interpolated from the standard curve.

2.6.8.2. Determination of Malondialdehyde (MDA) Concentration

Total amount of lipid peroxidation product (malondiadehyde) in the homogenate was estimated according to the method described by [23]. The homogenate (0.5 ml) was pipette into clean and dry test tubes, phosphate buffer (0.5 ml, 0.1 M, pH 8.0) and TCA (0.5 ml, 24%) were added to it respectively. The resulting mixture was incubated at room temperature for 10 min followed by centrifugation at 2000 rpm for 20 min. and the supernatant was collected. TBA (0.25 ml, 0.33%) in 20% acetic acid was added to 1 ml of supernatant and the resulting mixture was boiled at 95°C for 1 hr. The resulting pink colour product was allowed to cool and absorbance was read at 532 nm. The results were expressed as nM TBARS/min/mg tissue at 37°C. The concentration of malondialdehyde was calculated using this expression:

$$Malondialdehyde\ (nM\ TBARS/min/mg) = \left(\frac{Abs\ sample}{l\ X\ \varepsilon}\right) X\ DF$$

Where l = light path
ε = Extinction coefficient of MDA at 532nm = 1.52 x 10^5 $M^{-1}cm^{-1}$
DF = Dilution factor

2.6.9. Estimation of Antioxidant Parameters in Wet Granulation Tissue

2.6.9.1. Estimation of Reduced Glutathione (GSH) Activity

Reduced glutathione (GSH) was measured by the method of [24]. Standard calibration curve was prepared using different concentrations of the standard GSH (0, 2, 4, 6, 8, 10 µg/ml). Ellman's reagent (0.5 ml, 10 mM) and phosphate buffer (2 ml, 0.2 M, pH 8.0) were added to the different concentrations of the standard and the yellow colour developed was read at 412 nm. The homogenate (1 ml) was pipetted and treated as standard. The blank contained phosphate buffer only. The amount of GSH was expressed as mg/100g tissue.

2.6.9.2. Estimation of Superoxide Dismutase (SOD) Activity

The method described by [25] was used to assay for the activity of superoxide dismutase. To 50 µl of the homogenate, 75 mM of Tris-HClbuffer (pH 8.2), 30 mM EDTA and 2 mM of pyrogallol were added. An increase in absorbance was recorded at 420 nm for 3 min by spectrophotometer. One unit of enzyme activity is 50% inhibition of the rate of autooxidation of pyrogallol as determined by change in absorbance/min at 420 nm. The activity of SOD is expressed was expressed as units/mg protein.

$$Increase\ in\ absorbance = \frac{A3 - A0}{2.5}$$

Where A0 = absorbance after 30 seconds
A3 = absorbance after 150 seconds

$$\%\ inhibition = 100 - 100X\ \frac{(inccrease\ in\ absorbance\ of\ substrate)}{increase\ in\ absorbance\ of\ blank}$$

One unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline.

2.6.9.3. Estimation of Catalase (CAT) Activity

The assay of CAT activity was carried out according to the method [26]. Typically, the homogenate (50 µL) in duplicate was added to a cuvette containing 450 µL of phosphate buffer (0.1 M, pH 7.4) and H_2O_2 (500 µL, 20 mM). CAT activity was measured at 240 nm for 60 sec (15, 30, 45, 60 sec.) against the reagent blank. The blank contained distilled water instead of the homogenate. The catalase activity was calculated using the expression:

$$\text{Catalase activity} = \frac{\frac{\Delta A}{\min} \times d \times TV}{SV \times 0.0436}$$

ΔA/min = slope of the graph of absorbance against min
d = dilution factor
SV = Sample volume (mL)
0.0436 = Extinction coefficient for hydrogen peroxide
TV = Total reaction volume

$$Catalase\ activity\ \left(U / mg\ protein\right) = \frac{\text{Unit / mL}}{\text{mg protein / mL}}.$$

One unit of activity of catalase is equal to 1 mmol of H_2O_2 degraded per minute and expressed as units per milligram of protein.

2.6.10. Estimation of Connective Tissue Parameters

Approximately 250 mg of wet tissue was dried at 50°C for 24 hr. It was weighed and kept in glass stoppered test tubes. To each test tube containing 40 mg of dried granulation tissue was HCL (1 ml, 6N) added. The tubes were kept on boiling water bath for 24 hr (12 hr each day for two days) for hydrolysis. The hydrolysate was then cooled and excess of acid was neutralized by 10 N NaOH using phenolphthalein as indicator. The volume of neutral hydrolysate was diluted to a concentration of 20 mg/ml with distilled water. The final hydrolysate was used for the estimation of hexosamine and hexuronic acid following the standard curve prepared using the proper substrate.

2.6.10.1. Determination of Concentration of Hexosamine (HXA)

The method of [27] was used to determine the concentration of hexoxamine.The hydrolysed fraction (0.05 ml) was diluted to 0.5 ml with distilled water. Acetyl acetone reagent (0.5 ml) was added to the diluted sample and heated in boiling water bath for 20 min and then cooled under tap water. Ethanol (95%, 1.5 ml) was added, followed by an addition of 0.5 ml of Ehrlich's reagent and the reaction mixture was allowed for 30 min. Color intensity was measured at 530 nm against the blank. Hexosamine content of the samples was determined from the standard curve prepared with D (+) glucosamine hydrochloride, 5 µg/0.5 ml to 50 µg/0.5 ml using 100 µg/ml working solution.

2.6.10.2. Determination of Concentration of Hexuronic acid (HUA)

The method of Bitter and Muir [28] was used to determine the concentration of hexuronic acid. Borax (2.5 mL of 0.025 M) in concentrated sulphuric acid was placed in stoppered tubes fixed in a rack and cooled to 4°C. The hydrolysate (0.125 mL) was diluted to 0.5 mL with distilled water. The hydrolysate (0.5 mL) was layered carefully on Borax-sulphuric acid mixture kept in rack at 4°C. The tubes were closed with glass stoppers and then shaken, first slowly then vigorously, with constant cooling by placing tubes in ice container. The tubes were heated for 10 min. in a vigorously boiling water bath and cooled to room temperature. Thereafter, carbazole reagent (0.1 mL of 0.125%) in absolute alcohol was added to each tube, shaken, heated in the boiling water bath for further 15 min., and then cooled to room temperature. Color intensity was measured at 530 nm against the blank. Hexuronic acid content of the samples was determined from the standard curve prepared with D (+) Glucurono-6, 3-lactone, from 5 µg/0.5 mL to 40 µg/0.5 mL using 100 µg/mL working solution.

2.7. Statistical Analysis

Experimental data were expressed as mean ± standard error of mean (SEM). Statistical significance of differences among groups were evaluated using one-way analysis of variance (ANOVA) followed by Dunnett's method of multiple comparisons using Graph Pad prism 5.0 Software. Data were considered significant at $p < 0.05$.

3. Results

3.1. *E. graminea* Latex Phytoconstituents

Phytochemical screening of EGL revealed the presence of alkaloids, flavonoids, cardiac glycosides, saponins and triterpenes (Table 1).

The total flavonoids and total phenolic contents were 6.400 ± 0. 001 (µg/g RE) and 8.504 ± 0.047 (µg/g GAE) respectively (Table 2).

Table 1. Phytoconstituents of *E. graminea* Latex

Phytochemicals	result	Phytochemicals	result
Flavonoids	+	C. glycosides	+
Alkaloids	+	Saponin	+
Phlobatannin	-	Steroids	-
Tannin	-	Triterpenes	+
Xanthoproteins	-	Anthraquinone	-

+ positive, - negative, C-Cardiac.

Table 2. *E. graminea* Latex Total Flavonoids and Phenolics Concentrations

Sample	Total flavonoid (µg/g RE)	Total phenolic (µg/g GAE)
***E.graminea* Latex (µg/mg)**	6.400 ± 0. 001	8.504±0.047

Each value represented the mean ± SEM, in triplicates (n=3) R - Rutin; G- Garlic acid E- Equivalent.

3.2. Antioxidant Capacity of *E. graminea*

The latex of *E. graminea* inhibited DPPH in dose dependent manner (Figure 1).

It also exhibited mild ferric reducing (IC_{50} - 3.304 ± 0.831 µg/ml) compared to ascorbic acid (IC_{50} -1.011 ± 0.245 µg/ml) (Table 3)

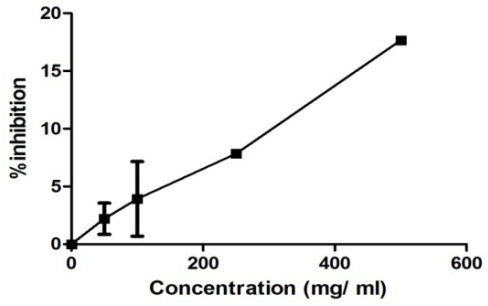


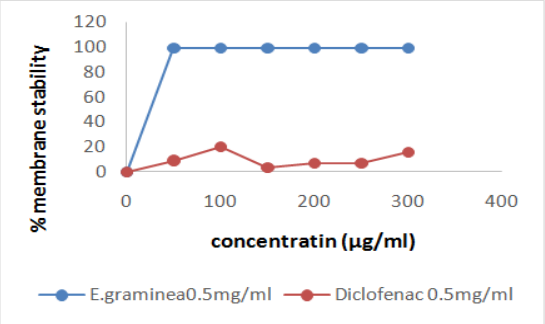
Figure 1. % DPPH Inhibition of *E. graminea* Latex

Table 3. Ferric reducing antioxidant potential of *E. graminea* latex

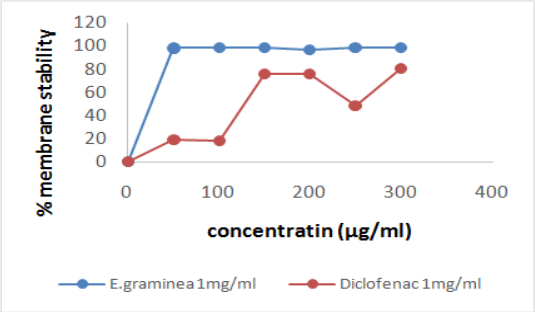
Sample	IC_{50} (µg/mL)
EGL	3.304 ± 0.831
Ascorbic acid	1.011 ± 0.245

3.3. Membrane Stability Activity of *E. graminea* and Diclofenac on Bovine Red Blood Cell

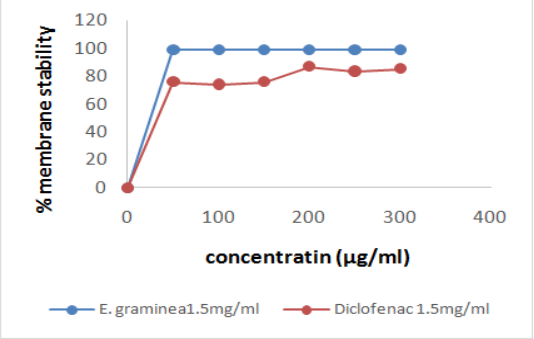
A. Percentage membrane stability of *E. graminea* and diclofenac (0.5 mg/ml)



B. Percentage membrane stability of *E. graminea* and diclofenac (1 mg/ml)



C. Percentage membrane stability of *E. graminea* and diclofenac (1.5 mg/ml)



D. Percentage membrane stability of *E. graminea* and diclofenac (2 mg/ml)

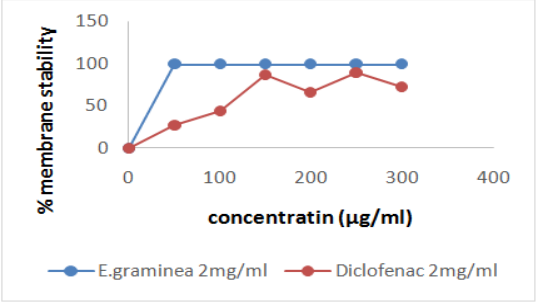


Figure 2 (A - D). Membrane Stability Profile of *E. graminea* Latex and Diclofenac at 0.5, 1.0, 1.5 and 2.0 mg/ml (Figures: A, B, C and D respectively). Each sample concentration was prepared in triplicates (n=3)

E. graminea latex exhibited minimum percentage stability of 98.98%, 96.95%, 98.95%, 98.90% at 0.5, 1.0, 1.5 and 2.0mg/mL respectively while the minimum percentage stability of diclofenac at 0.5, 1.0, 1.5 and 2.0 mg/mL were 18.94%, 18.35%, 74.00%, 27.06% respectively. The maximum percentage stability at at 0.5, 1.0, 1.5 and 2.0 mg/mL for *E. graminea* latex were 99.05%, 98.44%, 99.10%, 98,99% respectively while the maximum percentage stability for the standard (diclofenac) at the same concentrations were 76.35%, 80.94%, 86.86%, 89.06% respectively. At 0.5 and 1.5 mg/ml, *E graminea* latex stabilized the stressed erythrocyte membrane maximally at 99.05% and 99.10% better than 2 mg/ml diclofenac with maximum stability at 89.06%.

3.4. Effect of *E. graminea* and Gentamycin in Incision Wound Model

Control group rats showed wound breaking strength (WBS) value of 680.00 ± 0.00 g on 10th post wound day. EGL treated rats showed significant increase in WBS by 13.60% and the effect on WBS was comparable with Gentamycin ointment treated rats which showed 20.96% significant increase.

Table 4. Effects of *E. graminea* latex and Gentamycin on Wound Breaking Strength (WBS)

Sample	WBS (g)	(PIWBS)
Wound + EGL	$772.50 \pm 20.96^{a,b}$	13.60
Wound+G	$822.50 \pm 13.60^{a,c}$	20.96
Wound only (Control)	680.0± 0.00	0.00

Values were represented as Mean ± SEM from 5 animals in each group (n = 5).

Superscript a, b and c indicate significant ($p < 0.05$) difference from control, wound + Gentamycin (G) and wound + *E. graminea* Latex (EGL) respectively at 95% C.I.

$$\textit{Percentage increase in wound Breaking strength } (PIWBS) = (WT/WC - 1) \times 100,$$

where W*T* and W*C* were WBS values of test and control groups respectively.

3.5. Effects of *E. graminea* Latex and Gentamycin on Hexoxamine and Hexuronic Acid Concentrations

There was significant ($p < 0.05$) elevation in hexoxamine and hexuronic acid concentrations in both EGL and gentamycin treated rats when compared to the control rats.

Table 5. Effects of *E. graminea* latex and Gentamycin on Hexoxamine and Hexuronic acid Concentrations

Group	Hexosamine (µg/mL)	Hexuronic acid (µg/mL)
Wound+EGL	85.21 ± 18.18^{b}	$7.16 \pm 2.49^{a,b}$
Wound + G	$98.61 \pm 3.10^{a,c}$	$14.59 \pm 0.80^{a,c}$
Wound only (Control)	70.07± 12.89	1.45± 0.48

Values were represented as Mean ± SEM from 5 animals in each group (n = 5).

Superscript a, b and c indicate significant ($p < 0.05$) difference from control, wound + Gentamycin (G) and wound + *E. graminea* Latex (EGL) respectively at 95% C.I.

3.6. Effects of *E. graminea* on Wet Granulation Tissue Antioxidants and Free Radicals

There was significant ($p < 0.05$) increase in the level of superoxide dismutase (SOD) and reduced glutathione (GSH) in the EGL and Gentamycin treated rats when compared with the control. Gentamycin significantly ($p < 0.05$) increased the GSH concentration, when compared with the EGL treatment. There was no significant ($p < 0.05$) difference in the level of NO and catalase activity rats treated with *E.graminea* latex and Gentamycin were compared with the control rats. However, EGL treatment significantly ($p < 0.05$) decreased the lipid peroxidation end product (MDA) when compared with the control group.

Table 6. Effects of *E. graminea* Latex and Gentamycin on Antioxidants and Free Radicals

Group	SOD (mU/mg)	CAT (mg/100 tissues)	GSH mU/mg	NO (nM/mg protein)	MDA nM TBARS/min/mg
Wound+EGL	0.63± 0.15[a]	0.0058 ±0.0041	6.52± 0.55[a]	0.25± 0.06	2.32± 0.88[a]
Wound+ G	0.59± 0.18[a]	0.0026± 0.0049[a]	10.45± 0.80[a,b]	0.29± 0.15	2.96± 1.99
Wound (Control)	0.19± 0.01	0.0069 ±0.0038	8.82± 0.97	0.26± 0.07	3.16± 1.22

Values were represented as Mean ± SEM from 5 animals in each group (n = 5).

Superscript a, b and c indicate significant ($p < 0.05$) difference from control, wound + Gentamycin (G) and wound + *E. graminea* Latex (EGL) respectively at 95% C.I.

4. Discussion

Bioactive compounds of plant origin have beneficial effect on wound care and management by accelerating the rate of epithelisation with least scarring of skin tissue [4]. Wound healing process consists of re-epithelization and reconstruction of tissue matrix collagen. It is an innate immunological activity happening on its own, but factors like tissue ROS generation and inflammation not only hindered the healing process but also increased the chances of secondary infection [29,30]. Medicinal plants that have antioxidant and anti-inflammatory properties have been reported to reduce the wound healing process [31,32].

The result of this study revealed that *E. graminea* latex possesses appreciable concentrations of phenolics and flavonoids content. Phenolic compounds, especially flavonoids and phenols have been shown to possess significant antioxidant activity which can help in the treatment of wound [33,34,35,36]. Flavonoids and phenolics are powerful antioxidants against free radicals and are described as free radical scavengers [37,38].

In this study, DPPH radical scavenging assay and the ferric reducing antioxidant potential assay were used to investigate the antioxidant activity of EGL. The DPPH radical scavenging assay is a rapid, simple and convenient method of investigating the ability of phenolic compounds in extract to act as electron donor [39]. Numerous plant constituents have been demonstrated to exhibit free radical scavenging or antioxidant activity [11]. The *in vitro* DPPH radical scavenging assay revealed that EGL has a potent antioxidant activity. Evident from this study, a linear relationship was observed between the decrease in absorbance of DPPH and different concentrations. The increased concentration of the latex showed increased DPPH radical scavenging activity.

Ferric reducing antioxidant power (FRAP) is used to assess the metal (iron exclusively) ions binding ability [40]. *E. graminea* latex exhibited mild ferric reducing antioxidant activity of (IC_{50} value of 3.304 ± 0.831 mg/ml) comparable to the standard, ascorbic acid (IC_{50} value of 1.011 ± 0.245 mg/ml). IC_{50} value is defined as the concentration of substrate that causes 50% loss of free radicals activity and was calculated by linear regression of plots of the percentage of antiradical activity against the concentration of the tested plant latex. The lower the IC_{50} value, the higher the antioxidant potential. The antioxidant properties of the latex may be attributed to the presence of bioactive compounds like flavonoids and alkanoids with antioxidant potential [37,38].

Reactive oxygen species (ROS) is produced in high quantities at the site of wound as a defense mechanism against invading bacteria [41]. However, the presence of increased numbers of neutrophils and ROS overwhelm the antiprotease substances that normally protect the tissue cells and the extracellular matrix [42]. At high concentrations, ROS can induce severe tissue damage and even lead to neoplastic transformation decreasing the healing process by damages in cellular membranes, DNA, proteins and lipids [43].

The overall role of antioxidants appears to be significant in the successful treatment and management of wounds. Antioxidants reduce these adverse effects of wounds by removing products of inflammation. They counter the excess proteases and ROS often formed by neutrophil accumulation in the injured site and protect protease inhibitors from oxidative damage [44]. The most likely mechanism of antioxidant protection is direct interaction of the extracts (or compounds) and the hydrogen peroxide rather than altering the cell membranes and limiting damage [45]. Compounds with high radical-scavenging capacity have been shown to facilitate wound-healing [46].

In the present study, two different models were used to assess the effect of *E. graminea* latex and Gentamycin ointment on various phases of wound healing. In the first model, incision wound model, wound breaking strength was evaluated which depicts the strength of a healing wound and is measured experimentally by the amount of force required to disrupt it. At the initial stage wound will possess little breaking strength because the clot alone held the edges together. Thereafter, wound breaking strength increased rapidly as collagen deposition increases and cross-linkages are formed between the collagen fibres [47]. The animals treated with topical application of EGL and gentamycin exhibited 13.6 % and 20 % increase in wound breaking strength respectively. The ability of EGL to increase wound breaking strength might probably be attributed due to increased collagen synthesis. Wound

strength had been reported to be acquired from both re-modeling of collagen and formation of stable intra and inter molecular crosslinks [48,49]. The wound healing accelerating potential of the latex may be attributed to the presence of bioactive compounds such as flavonoids, alkaloids, saponins, c. glycosides and triterpenes present in the plant [50].

The enhanced capacity of wound healing accelerating potential with the plant was on the basis of anti-inflammatory effect of the plant [51]. This was demonstrated *in vitro* by the membrane stabilizing potential of the *E. graminea* latex. The latex showed highest stability of the stressed red blood cell membrane at higher percentage than the standard, anti-inflammatory drug (diclofenac).

The second model, dead space wound model, is mainly for evaluating the efficacy of drugs on the connective tissue markers which include hexosamine and hexuronic acid. Implantation of polypropylene tubes causes inflammatory response and modulates the release of inflammatory mediators which finally results in tissue proliferation and granulation tissue formation [52]. Hexosamine and hexuronic acids (glycosaminoglycans) are matrix molecules which act as ground substratum for the synthesis of new extracellular matrix [53]. Secondary metabolites like tannins, steroids and saponins are known to have antimicrobial and anti-inflammatory effect that can be responsible for the treatment of gastrointestinal infection, swellings and wounds [54]. In this study, the concentrations of hexuronic and hexosamine were significantly increased ($P<0.05$) in wounds treated with EGL when compared with the control indicated the stabilization of granulation tissue.

Furthermore, it was observed that treatment of wound with EGL caused a significant increase in wet granulation tissue GSH and SOD when compared to the control animals. Overproduction of reactive oxygen species ROS results in oxidative stress thereby causing cytotoxicity and delayed in wound healing [55]. Intercellular and extracellular antioxidants are thus important to protect tissues from oxidative injury [56]. From the study, decrease in wet granulation tissue NO (though not significant) and MDA concentrations in EGL treated wound was observed compared to control. Free radicals and ROS are constantly formed by the human body; ROS mediate cell damage. Intercellular and extracellular antioxidants are thus important to protect tissues from oxidative injury [57]. Superoxide dismutase (SOD) converts superoxide to hydrogen peroxide (H_2O_2), which is then converted into water by catalase in lysosomes or by glutathione peroxidase (GPx) in mitochondria [56]. GSH reduces H_2O_2 and lipid peroxides via reactions catalyzed by GSH peroxidase [58]. Topical application of EGL significantly ($P< 0.05$) enhanced the activity of SOD and GSH concentration but no significant effect on catalase. These prevented the harmful effects of free radicals generated in rats. Superoxide and hydroxyl radicals are vital mediators of oxidative stress that contribute to clinical disorders. Therefore, any natural or synthetic element with antioxidant properties can totally or partially reduce the harm caused by free radicals [59].

This suggests that EGL at the concentration applied probably possess very little or no influence to restrain free radicals generated stress .

5. Conclusion

This study revealed that *E. graminea* latex possessed significant antioxidant and anti-inflammatory properties as well as wound healing accelerating potential confirmed by stabilized collagen fibers.

It could therefore be surmised that the therapeutic principles contained in *E. graminea* latex could possibly be employed in boosting human defense system as well as treatment of wound.

References

[1] Nagor, B. P., Solanki, R., "Role of medicinal plants in wound healing," *Journal of Medicinal Plant Research* 5: 392-405. 2011.

[2] Das, J., Injury and Wound Healing, In: J. Das (ed.) Trauma in otolaryngology Springer Singapore, Singapore, 2018, 31-52.

[3] Mitzzy, F., Medellín-Luna, J., YordaniMartínez-Balderas, V., Cervantes-Villagrana, A. R., "Medicinal plant extracts and their use as wound closure inducing agents," *Journal of Medicinal Food*, 22: 435-443. 2019.

[4] Kumar, B., Vijayakumar, M., Govindarajan, R., Pushpangadan, P., "Ethnopharmacological approaches to wound healing-exploring medicinal plants of India," *Journal of Ethnopharmacology*, 114: 103-113. 2007.

[5] Oguntibeju, O., "Medicinal plants and their effects on diabetic wound healing," *Veterinary World*, 12 (5): 653-663. 2019.

[6] McVaugh, R. Euphorbiae Novo-Galicianaerevisae. Contributions to University of Michigan Herbarium, 1993, 207-239.

[7] Betancur-Galvis, L. A., Morales, G. E., Forero, J. E., Roldan, J., "Cytotoxic and antiviral activities of Colombian medicinal plant extracts of the *Euphorbia* genus," *Mem Inst Oswaldo Cruz*, 97 (4): 541-546. 2002.

[8] Trease, G. E., Evans, W. C., *Pharmacogosy*.15th Edn. Saunders 2002, 214-393..

[9] Sofowora, E. A. Medicinal plants and traditional medicine in Africa, Second Edition, Spectrum Books Limited, Ibadan, Nigeria, 2006, 150-153.

[10] Singleton, V., Orthofer, R., Lamuela, R., "Analysis of total phenol and other oxidation substrates and antioxidant by means of folin- ciocalteu reagent," *Methods Enzyme*, 299: 152-178. 1999.

[11] Aina, O. I., Oyedapo, O. O., "*In vitro* investigations into the antioxidant and antiinflammatory potentials of the fractions and ethanolic extract of *Cyclosorus afer* (Christ.) Ching, stalks," *Ife Journal of Science*, 15 (2): 235-249. 2013.

[12] Sun, P. X., Yie, L. K., Zhang, Z. L., Hu, M., Lu, L, "Colometric determination of the total content of the flavonoids in epicedium capsules," *Journal of Shenyang Pharmaceutical University*, 16: 68-70. 1999.

[13] Oyaizu, M., "Studies on products of browning reaction. Antioxidative activities of browning reaction products prepared from glucosamine," *Japanese Journal Nutrition*, 44: 307-315. 1986

[14] Konate, K., " *In vitro* antioxidant , lipoxiginase and xanthine-oxidase inhibitory activities of fractions from *Cienfuegosia digitata* Cav. Sida alba L. and Sida acuta Bum. F. (Mavaceae)," *Pakistan Journal of Biological Sci*ence, 13 (22): 1092-1098. 2010.

[15] Oyedapo, O. O., Akinpelu, B. A., Orfuwa, S. O., "Anti-inflammatory effects of *Theobroma cacao*, root extract," *Journal of Tropical Medicinal Plants*, 5: 161-165. 2004.

[16] Bode, S. O., Oyedapo, O. O., "Biological activities and phytoconstituents of the lower plant *Platycerium anglolense*, Welwex Hook," *Journal of Medicinal* Plants *Research*, 5: 1321-1329. 2011.

[17] NIS National Institutes of Health NIH Laboratory Animal Welfare *Guide for Grants and Contracts*, 14 (8): 25. 1985.

[18] Agrawal, N., Patil, M., Chimkode, R., Tripathi, A., "Antimicrobial and Wound Healing Activities of Leaves of *Alternanthera sessilis* Linn.," *International Journal of Green Pharmacy*, 1: 141-144. 2000.

[19] Lee, K., "Studies on the mechanism of action of salicylates III, effect of vitamin A on the wound healing retardation action of aspirin," *Journal of Pharmaceutical Science*, 57: 1238-1240. 1968.

[20] Agarwal, P. K., Singh, A., Gaurav, K., Goel, S., Khanna, H. D. Goel, R. K., "Evaluation of wound healing activity of extracts of plantain banana (*Musa sapientum var. paradisiaca*) in rats," *Indian Journal of Experimental Biology*, 47 (1): 32-40. 2009.

[21] Murthy, S., Gautam, M. K., Goel, S., Purohit, V., Sharma, H. Goel, R. K. "Evaluation of *in-vivo* wound healing activity of bacopa monniera on different wound model in rats," *Biomed Research International* 1-9. 2013.

[22] Grisham, M. B., Johnson, M. D., Gautreaux, R. D., Methods, *A Comp. to Meth. in Enz*., 7: 84-90. 1995.

[23] Ohkawa, H., Ohishi, N., Yagi, K., "Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction," *Analytical Biochemistry*, 95 (2): 351-358. 1979.

[24] Beutler, E., Duron, O., Kefly, B. M., "Improved method for the determination of blood glutathione," *Journal of Laboratpry and Clinical Medicine*, 61: 882-888. 1963.

[25] McCord, J. M., Fridovich, I., "Superoxide dismutase: An enzymic function for erythrocuprein (hemocuprein)," *Journal of Biological Chemistry*, 244(22): 6049-6055. 1969.

[26] Aebi, H. E., Catalase. In: Bergmeyer, H. U. Methods of Enzymatic Analysis, Vol. 3 (3rd ed.), VerlagChemie, Weinheim, Germany, 273-286. 1982.

[27] Dische, Z., Borenfreund, E., "A spectrophotometric method for the micro determination of hexosamines" *Journal of Biological Chemistry*, 184*:* 517-522. 1950.

[28] Bitter, T., Muir H., "A modified uronic acid carbazole reaction," *Anaytical Biochemistry*, 4 (4): 330-334. 1962.

[29] Nayak, B. S., Pinto Pereira, M., "*Catharanthus roseus* flower extract has wound healing activity in Sprague Dawley rats," *BMC Complement*ary *and Alternative Medicine*, 2006.

[30] Kumar, M. S., Kirubanandan, S., Sripriya, R., Sehgal, P. K., "Triphala promotes healing of infected full-thickness dermal wound", *Journal of Surgical Research*, 144(1): 94-101. 2008.

[31] Jonas, I., Anthony, S., Afolayan, J., Bradley, G., "Antioxidant, antibacterial and phytochemical properties of two medicinal plants against the wound infecting bacteria," *Asian Pacific Journal of Tropical Biomed*icine, 7(9): 817-825. 2017.

[32] Aryani, R., AgungNugroho, R., Manurung, H., R. Mardayanti, Rudianto, W. Prahastika, Auliana, A. BruKaro, P., "*Ficus deltoidea* leaves methanol extract promote wound healing activity in mice," *Eurasia Journal of Bioscience*, 14: 85-91. 2020.

[33] Kamath, J. V., Rana, A. C., Chowdhury, A. R., "Pro-healing effect of *Cinnamomum zeylanicum* bark," *Phytotheraphy Research*, 17: 970-972. 2003.

[34] Saroja, M., Santhi, R., Annapoorani, S., "Wound healing activity of flavonoid fraction of *Cynodon dactylon* in swiss albino mice," *International Research Journal of Pharmacy*, 3: 230-231. 2012.

[35] Kumar, S., Pandey, A., "Chemistry and biological activities of flavonoids: an overview," *Science World Journal* 2013.

[36] Muralidhar, A., Babu, S., Sankar, R., Reddanna, P., Latha, J. "Wound healing activity of flavonoid fraction isolated from the stem bark of *Buteamono sperma* (Lam) in albino wistar rats," *European Journal of Experimental Biology*, 3(6): 1-6. 2013.

[37] Ghasemzadeh, A., Ghasemzadeh, N. "Flavonoids and phenolic acids: Role and biochemical activity in plants and human," *Journal of medicinal plant research*., 5(31): 6697-6703. 2011.

[38] Sandhar, H., Kumar, B., Prasher, B., Tiwari, P., Salhan, M., Sharma, P., "A review of phytochemistry and pharmacology of flavonoids," *Internationale Pharmaceutica Sciencia*, 1 (1): 25-41. 2011.

[39] Stoilova, I., Krastanov, A., Stoyanova, A., Denev, P., Gargova, S., "Antioxidant activity of a ginger extract *Zingier officinale," Food Chemistry*, 102: 764-770. 2007.

[40] Nsimba, R., Kikuzaki, H., Konishi, Y., "Antioxidant activity of various extract fractions of *Chenopodium quinoa* and *Amaraathus* sp. Seeds," *Food Chemistry*, 106: 760-766. 2008.

[41] Reddy, B. S., Reddy, R. K. K., Naidu,V. G. M., Madhusudhana, K., Agwane, S. B., Ramakrishna, S., "Evaluation of antimicrobial, antioxidant and woundhealing potentials of *Holoptelea integrifolia*," *Journal of Ethnopharmacology*. 115:249 56. 2008.

[42] Mensah, A. Y., Sampson, J., Houghton, P. J., Hylands, P. J., Westbrook, J., Dunn, C. M., "Effects of *Buddleja globosa* leaf and its constituents relevant to wound healing," *Journal of Ethnopharmacology*, 77:219-26. 2001.

[43] Jorge, M. P., Madjarof, C., Ruiz, A. L., Fernandes, A. T., Rodrigues, R. A. F., Sousa, I. M. O., Foglio, M. A., Carvalho, J. E., "Evaluation of wound healing properties of *Arrabidaea chica* Verlot extract," *Journal of Ethnopharmacology*, 118: 361-6. 2008.

[44] Houghton, P.J., Hylands, P. J., Mensah, A. Y., Hensel, A., Deters, A. M., "*In vitro* tests and ethnopharmacological investigations: wound healing as an example," *Journal of Ethnopharmacology*, 100: 100-7. 2005.

[45] Annan, K., Houghton, P. J., "Antibacterial, antioxidant and fibroblast growth stimulation of aqueous extracts of *Ficus asperifolia* Miq. and *Gossypium arboreum* L., wound-healing plants of Ghana," *Journal of Ethnopharmacology*, 119: 141-4. 2008.

[46] Süntar, I., Akkol, E., Nahar, L., Sarker, S. "Wound healing and antioxidant properties: do they coexist in plants**?,"** *Free Radicals and Antioxidants*, (1): 1-8. 2012.

[47] Shirwaikar A. Shenoy R. Udupa A.L. Udupa S.L. Shetty S., "Wound healing activity of phenolic extract of leaves of Hyptis suaveolens with supportive role of antioxidant enzymes," *Indian Journal of Experimental Biology*, 41: 238-241. 2003.

[48] Kováč, I., Ďurkáč, J., Hollý, M., Jakubčová, K., Peržeľová, V., Mučaji, P., Švajdlenka, E., Sabol, F., Legáth, J., Belák, J., Smetana, K., Gál, J.P., "*Plantago lanceolata* L. water extract induces transition of fibroblasts into myofibroblasts and increases tensile strength of healing skin wounds," *Journal of Pharmacy and Pharmacology*, 67: 117-125. 2015.

[49] Ram, M., "Bilirubin modulated cytokines, growth factors and angiogenesis to improve cutaneous wound healing process in diabetic rats." *Internation Journal of Immunopharmacology*, 3: 137-149. 2016.

[50] Nayak, B.S., Sandiford, S. Maxwell, A., "Evaluation of the wound-healing activityof ethanolic of *Morinda citrifolia* L. Leaf," *Evidence Based Complementary and Alternative Medicine*, 6: 351-356. 2009.

[51] Shedoeva, A., Leavesley, D., Upton, Z. 1,2 Fan, C. "Wound healing and use of medicinal plants," *Evidence-Based Complementary and Alternative Medicine* Volume 2019. Article ID 2684108, 30 pages 2019.

[52] Heymann, F., Trotha, K., Preisinger, C., Lynen-Jansen, P., Roeth, A., Geiger, M.,Geisler L., Frank, A., Conze, J., Luedde, J., Trautwein, C., Binnebösel, M., Neumann, U., Tacke F., "Polypropylene mesh implantation for hernia repair causes myeloid cell-driven persistent inflammation," *JCI Insight*, 4(2): e123862. 2019.

[53] Muralidhar, A., Babu, S., Sankar, R., Reddanna, P., Latha, J., "Wound healing activity of flavonoid fraction isolated from the stem bark of *Buteamono sperma* (Lam) in albino wistar rats," *European Journal of Experimental Biology*, 3(6): 1-6. 2013.

[54] Okach, D. O., Nyunja, A. R. O., Opande, G., "Phytochemical screening of some wild plants from Lamiaceae and their role in traditional medicine in Uriri District - Kenya," *International Journal of Herbal Medicine*, 1(5): 135-143. 2013.

[55] Matthias, S., Sabine, W., "Oxidative stress in normal and impaired wound repair," *Pharmacological Research*, 58 (2): 165-171. 2008.

[56] Johansen, J. S., Harris, A. K., Rychly, D. J., "Oxidative stress and the use of antioxidants in diabetes: Linking basic science to clinical practice," *Cardiovascular Diabetology*, 4: 5-12. 2005.

[57] Asadi, S. Y., "Effect of green tea (*Camellia sinensis*) extract on healing process of surgical wounds in rat," *International Journal of Surgery*, 11, 332-337. 2013.

[58] Eroglu, A., Dogan, Z., Kanak, E. G., Atli, G., Canli, M., "Effects of heavy metals (Cd, Cu, Cr, Pb, Zn) on fish glutathione metabolism," *Environmental Science and Pollution Research*, 22, 3229-3237. 2015.

[59] Silveira, P. C. L. "Iontophoresis with gold nanoparticles improves mitochondrial activity and oxidative stress markers of burn wounds," *Materials Science and Engineering C*, 44, 380-385. 2014.