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# DEVELOPMENT OF HERBAL NANO EXTERNAL FORMULATION AS OINTMENT WITH *URENA LOBATA* EXTRACT AND VALIDATION OF ITS WOUND HEALING ACTIVITY IN EXPERIMENTALLY INDUCED OPEN WOUNDS IN SWISS ALBINO RATS

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**Keywords:**

*Urena lobata*; Antioxidant; Histopathology; Ointment formulation; Wound healing

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**ABSTRACT:** *Urena lobata* is used by traditional practitioners for treating external wounds. A preclinical study was conducted to evaluate the efficacy of the ointment formulations containing ethanolic extract of *Urena lobata* (EO) (5%) and nanosized extract (NO) of *U. lobata* (5%) for their wound healing activity by assessing wound contraction, tensile strength, superoxide dismutase, catalase, reduced glutathione, myeloperoxidase, hydroxyproline, lipid peroxidation and his to pathological studies in experimentally-induced open wounds in Swiss albino rats. Quercetin, an active principle of the formulation, was estimated qualitatively and quantitatively through HPLC studies. Topical application of NO and EO formulations promoted faster reepithelialization, wound contraction and maximized tensile strength, increased SOD, catalase activities and GSH, hydroxyproline levels with lower myeloperoxidase and lipid peroxidation in comparison with control. Histopathological studies revealed lesser inflammatory cell proliferation, degeneration, with marked fibroblast proliferation and collagen maturation than the control group. Results indicate that both NO and EO formulations showed benefits in the management of wounds when compared to the standard povidone ointment.

**INTRODUCTION:** Wound healing is a complex process involving four overlapping phases, beginning with homeostasis, followed by inflammation, proliferation and finally maturation or remodeling [1].

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At the site of an injury, homeostasis begins with platelet aggregation to form a fibrin clot to seal the bleeding. During the inflammatory phase, immune cells are in flux to the site of the wound and secrete growth factors, pro-inflammatory cytokines and extracellular matrices [2].

Neutrophils protect the wound from infection by producing reactive oxygen species (ROS) and thereby provide the first line of defense [3]. Monocytes exit the blood vessels and differentiate into macrophages, which phagocytose the damaged

cells, debris and bacteria by providing the second line of defense and clean the wound area [4, 5]. These macrophages also produce pro-inflammatory cytokines and angiogenic factors, which cause the infiltration of keratinocytes and fibroblasts to the site of a wound. Keratinocytes initiate the tissue reepithelialization [6] while fibroblasts build the extracellular matrix (ECM) by producing matrix proteins (collagen and glycosaminoglycans) and forms the granulation tissue [7].

Neovascularization occurs to deliver oxygen and nutrients to the emerging tissue. The remodeling phase starts with the reorganization and aligning of collagen fibers, mend the disrupted wound surface, cause wound contraction, reduce wound size, increase the tensile strength, thus completing the healing process [7]. Plants are a promising source for a variety of natural products for wound healing property, but are relatively less explored. *Urena lobata*, one such herb used traditionally against different skin ailments, especially wounds and burns.

Decoction of the leaf is recommended by traditional practitioners for reducing blood pressure, relieving body ache and rheumatic pain. The extracts of leaves and roots are also advocated to treat gonorrhea, hematemesis, malaria, fever, toothache, diarrhea, gastritis, nephritis, bronchitis, pneumonia, menorrhagia and inflammation [8]. Pharmacological studies revealed the antibacterial, amoebicidal, analgesic properties of *U. Lobata* [9, 12].

Despite the extensive use of this plant in folklore medicine, there is hardly any scientifically validated report to assess the wound healing effect of *U. lobata*. In recent years, plants provide a better platform for nanoparticles synthesis as they are inexpensive, easily scaled up, environmentally benign and free from toxic chemicals. Hence, in the present study, silver nanoparticles were prepared and characterized using the ethanolic extract of *U. lobata* and incorporated into a simple ointment base. The emulsifying ointment formulations prepared with the ethanolic extracts of *U. lobata* and silver nanoparticles of the ethanolic extract were formulated and evaluated for their wound healing activity in experimentally-induced open wounds in Swiss albino rats.

**Methods:**

**Plant Material and Preparation of the Extract:** The whole plant of *U. lobata* was collected from Tambaram region, Chennai, Tamilnadu, in March 2013. The plant was taxonomically identified by Prof. P. Jayaraman, Director, Plant Anatomy Research Center (PARC), Chennai. The aerial parts of *U. lobata* were shade-dried, coarsely powdered, and macerated sequentially with petroleum ether, chloroform, ethyl acetate, ethanol, and hydro alcohol (water: ethanol; 1:1) at room temperature for 72 h followed by 48 h and 24 h with extractive values of 0.623, 0.787, 0.632, 2.936 and 1.923 respectively. All the fractions were collected separately, filtered through filter paper (Whatman filter paper number l), and the extracts were dried under a vacuum desiccator to remove the solvents. All 5 extracts were subjected to antioxidant activity. Among the five extracts, ethanolic extract showed pronounced antioxidant activity [13]. Hence the ethanolic extract was selected to evaluate wound healing properties.

**Preparation of Nanoparticles of Ethanolic Extract of *U. Lobata*:** Silver nitrate ($AgNO_3$) (0.017 g) (1 mM) solution was prepared with 100 ml of distilled water and stored at 4 °C for further use for the synthesis of silver nanoparticles using ethanolic extract of *U. lobata*. 1 g of ethanolic extract was added to 250 ml of silver nitrate solution containing 1 mM of silver nitrate in an Erlenmeyer flask and sonicated at intervals of 5, 20, 40 min, and 1 h interval in the sonicator (Sonics) having 750 W. The samples were again sonicated for 10 min for equal distribution of particles using a bath sonicator. The reaction mixtures were subjected to centrifugation, and the obtained pellet was dissolved in deionized water. The formation of silver nanoparticles was confirmed visually with naked eyes by the change from greenish-yellow to dark brown and qualitatively characterized by UV-Visible spectrophotometer.

**Formulation of Ointment With Ethanolic Extract and Its Nano Extract:** Ointments of the extract and nano extract were prepared by emulsifying oil phase in water under 70 °C to 75 °C, then allowing the formulation to congeal at room temperature. The aqueous phase containing the extract (either 5% ethanol or 5% nanosized

ethanol extract) formed a stable emulsion in the presence of an emulsifying agent, and the formulation was congealed. Among the extracts prepared from *U. lobata*, since ethanolic extract showed pronounced antioxidant activity, 13 this extract was tested further for wound healing activity in this study. The herbal extract and nano-herbal extract were incorporated into the base separately and named extract ointment (EO) and Nano-ointment (NO). The ethanolic extract and the nano extract separately were incorporated into the molten simple ointment base **Table 1.** and allowed to congeal by stirring. After the ointment was formulated, they were packed in collapsible tubes separately. Betadine ointment was used as a standard reference.

**Experimental Design**

**Animals:** Swiss albino rats weighing 200 to 250 g were procured from Biogen Laboratory Animal Facility (CPCSEA-Reg no.971/bc/06), Bangalore. The rats were kept in polyacrylic cages and maintained under hygienic conditions at ambient temperature 24 ± 2 °C with 12 h light: dark cycle. Animals were allowed free access to a standard rat pellet diet and water throughout the experimentation period. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC/XXXIV/SRU/284/2013). Screening for wound healing activity was carried out using incision and excision wound models. Animals from each of these wound models were grouped into four, containing 6 animals each and received the following regimen of treatment:

- **Group I:** Wound was created and served as placebo-control (negative control)
- **Group II:** Rats received 5% of ethanolic extract ointment (EO) topically once daily.
- **Group III:** Rats received 5% nanosized extract ointment (NO) topically once daily.
- **Group IV:** Betadine cream (0.5 g) was applied once daily (positive control)

**Incision Wound Model:** After giving anesthesia, the backside of each rat was shaven, and a longitudinal 6 cm long and 1 cm deep paravertebral incision lateral to the midline of the vertebral column was made through the skin. After complete hemostasis, the wounds were closed with 0.5 cm spaced interrupted sutures to secure the edges. Wounded animals were kept in separate cages. Animals were divided into four groups and received the application of betadine ointment (0.5 g), which served as a reference standard control, ethanolic extract ointment (EO) (0.5 g), and NO (0.5 g) every day, while the placebo control rats were left untreated. All the above-mentioned ointment formulations were applied over the wound daily, starting from 0 days of wound infliction, continued till $10^{th}$ day of healing, and the tensile strength was measured by continuous constant water supply technique [14].

**Excision Wound Model:** Under ether anesthesia, the dorsal fur region of each rat was shaven, and a circular area of 500 mm 2 areas was marked using a circular stamp. The entire thickness of the demarcated area of the skin was then cut carefully using sterile scissors and forceps without causing any injury to the muscles. The wounded animals were kept separately. Animals were divided into four groups and received topical application of drugs daily, as mentioned above under the incision wound model. Wounds were traced on graph paper (1 mm 2) from day 0 of wound infliction and subsequently on 1, 4, 7, 10, 13, 16, and 19 post-wounding days. Animals were sacrificed by cervical dislocation on day [20]. A bit of healed skin tissue was removed for biochemical and histological studies. The following physical and biochemical parameters were studied at the end of the treatment period.

**Biophysical Parameters:** The percentage reduction of the wound size was determined on 1, 4, 7, 10, 13, 16, and 19 post-wounding days. The tensile strength of the incision wound tissues was determined on $10^{th}$ day [14].

**Measurement of Wound Area:** The progressive changes in wound area were measured planimetrically by placing transparent paper and defining the borders of the wound on days 1, 4, 7, 10, 13, 16, and 19 post-wound days. The tracing paper was placed on a sheet of graph paper (1 mm 2), and the number of squares within the wound area was counted. The wound area was then determined by calculating the percentage of wound

contraction, on days 1, 4, 7, 10, 13, 16 and 19 post-wound days using the following formula:

Wound area on day 0 - Wound area on day n% wound contraction × 100 Wound area on day 0

**Biochemical Studies:** Hydroxyproline [15], myeloperoxidase (MPO) [16], total proteins [17] lipid Peroxidation [18] and antioxidants such as superoxide dismutase (SOD) [19], catalase [20] and reduced glutathione (GSH) were estimated in skin tissue homogenates [21].

**Hydroxyproline:** Wound tissues were analyzed for hydroxyproline, a basic constituent of collagen. The method of Newman and Logan15 was adopted to estimate hydroxyproline. Tissues were hydrolyzed in HCl (6 N) at 110 °C for 4 h, after drying the tissue to constant weight at 60-70 °C in a hot air oven. The hydrolysate (0.3 ml) was oxidized by vigorously shaking with NaOH (2.5 N) and $H_2O_2$ (6%) in the presence of $CuSO_4$ (0.01 M) and subsequently complexed with freshly prepared paradimethyl amino benzaldehyde (5%) in n-propanol (0.6 ml) and 1.2 ml of $H_2SO_4$ (3 N). The absorbance was read at 540 nm against the reagent blank. The concentrations of hydroxyproline were determined using the standard curve, prepared from a stock solution of standard 4-Hydroxy-L-proline, and diluted to concentrations of 1.0-100 µg/ml.

**Myeloperoxidase (MPO):** The method of Bradley *et al.*, 1982 was used to estimate the myelo peroxidase activity. One ml of the sample was homogenized with 1 ml of hexadecyl trimethyl ammonium bromide (0.5%) and potassium phosphate buffer (50 mM, pH 6). After centrifugation, 0.5 ml of supernatant was mixed with 3.5 ml of phosphate buffer (pH 6.0) followed by 1.5 ml of dinizidine. The absorbance was measured at 450 nm at 0 seconds and 60 sec against a reagent blank 16 using Thermo Scientific Multiskan spectrophotometer, USA. The amount of enzyme activity that liberates 1 $\mu$mol of $H_2O_2$ to water in 1 min per mg protein at 25 °C is considered as one unit.

**Lipid Peroxidation:** Lipid peroxidation was quantified in wound tissue homogenates of all groups by estimating the malondialdehyde (MDA) content, a direct product that reflects the per oxidation of lipids. MDA reacts with thiobarbituric acid to form thiobarbituric acid reacting substance (TBARS) which developed a pink color chromophore with an absorption maximum at 532 nm and expressed as nanomoles of MDA formed per mg protein.

**Preparation of Tissue Homogenates for Antioxidant Assays:** Wound skin tissues from all animals were homogenized in ice-cold 10% potassium phosphate buffer (50 mM, pH 7.8), centrifuged, and the supernatant was used for the estimation of protein and antioxidants.

**Total Protein by Biuret Method:** Sample (50 µl) was incubated with saline (0.6 ml) and a working biuret reagent (1.25 ml), at room temperature for 15 min. The color intensity developed was read at the wavelength of 540 nm in Thermo Scientific Multiskan spectrophotometer, USA.

**Super Oxide Dismutase Activity (SOD):** The assay of SOD is based on the inhibition of the formation of NADH-phenazine methosulphate nitro blue tetrazolium formazan. The assay mixture contained 50 µl of the sample, 0.025 M sodium pyrophosphate buffer (pH 8.3), 186 M phenazonium methosulphate, and 300 µM nitro blue tetrazolium chloride. The reaction was started by incubating 780 µM of reduced nicotinamide adenine dinucleotide at 30 ºC for 90 sec. The reaction mixture was shaken with 2.0 ml of n-butanol, allowed to stand for 10 min, and centrifuged. n-Butanol (1.5 ml) alone was served as blank. The color intensity of the chromogen was measured at 560 nm using Thermo Scientific Multiskan spectrometer, USA. One unit of SOD activity is the amount of enzyme required to produce 50% dismutation of O2- radicals.

**Total Reduced Glutathione (GSH):** GSH level in the homogenate was quantified as per the protocol of Moran *et al.*[21] . The sulfhydryl groups of reduced glutathione react with dithiodinitrobenzoic acid (DTNB) and give a yellow-colored compound. Reduced glutathione was quantified by mixing equal volumes of the sample (0.25 ml) with ice-cold 5% TCA, and the precipitate was centrifuged. To the supernatant obtained, 0.25 ml of phosphate buffer (0.2 M, pH 8.0) and 0.5 ml of DTNB (0.6 mM in 0.2 M phosphate buffer, pH 8.0) were added.

The absorbance was read at 412 nm using Thermo Scientific Multiskan Spectrophotometer, USA.

**Catalase:** Catalase activity was determined by estimating the ability of the enzyme to oxidize hydrogen peroxide. The assay mixture contained 100 µl of a sample, 0.4 ml of $H_2O_2$ (2 mM), and 0.5 ml of phosphate buffer (10 mM, pH 7.4). The above mixture was stirred well and incubated with dichromate acetic acid reagent (5% potassium dichromate and glacial acetic acid in the ratio of 1:3). Absorbance was measured at 570 nm against a blank containing only dichromate acetic acid reagent using Thermo Scientific Multiskan spectrophotometer, USA. One unit (U) is the amount of catalase needed to decompose; one mM of $H_2O_2$/min at 25 °C is equivalent to one unit.

**Histopathology Studies of Skin Tissues:** Pieces of healed skin tissues from all experimental groups were fixed in 10% neutral buffered formalin for 48 h. The skin tissues were subjected to dehydration in alcohol series, embedded in paraffin wax, sectioned (5 µm), and stained with hematoxylin and eosin. His to pathological changes were observed under light microscopic examination. Healed skin samples after processing were stained with Masson's Trichrome (Sigma, USA) to visualize collagen and fibroblast proliferation.

**Statistical Analysis:** Data obtained from the experiments are represented as mean ± standard error of the mean (SEM) and examined by one-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (Version 19.0). p values of $< 0.05$ were considered as significant.

## RESULTS AND DISCUSSION

**Wound Contraction:** The percentage of wound closure at days 1, 4, 7, 10, 13, 16, and 19 post-wound infliction is presented in **Fig. 1.** The rate of wound contraction was time-dependent and markedly greater in all the groups from day 4 onwards. On day 4, wound contraction was two-fold higher in rats treated with betadine, while it was four and five-fold greater in NO and EO treated rats, respectively. The topical application of NO or EO or betadine caused faster wound healing as compared to the negative control group. These groups showed minimal scar formation when compared to the placebo control group on 19th day. Although rats that received betadine (positive control) effectively promoted the closure of the wound faster than that observed in placebo control rats, the degree of wound healing was comparatively much slower than that observed in NO and EO treated rats. The NO and EO formulations accelerated 93-94% wound contraction, which was greater than that caused by the standard drug, betadine, which showed 87.04% contraction of the wound at the end of 16 days.

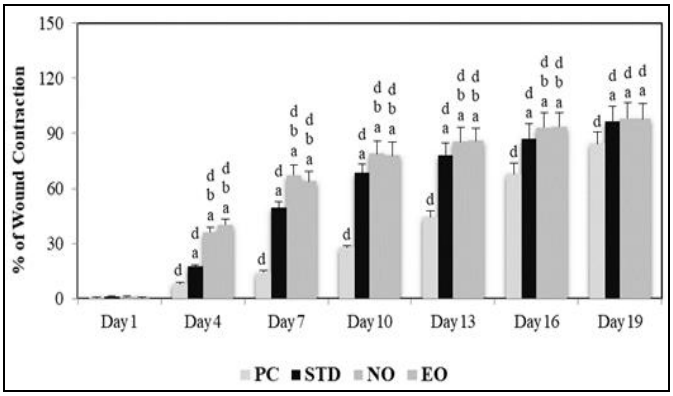


**FIG. 1: % OF WOUND CONTRACTION**

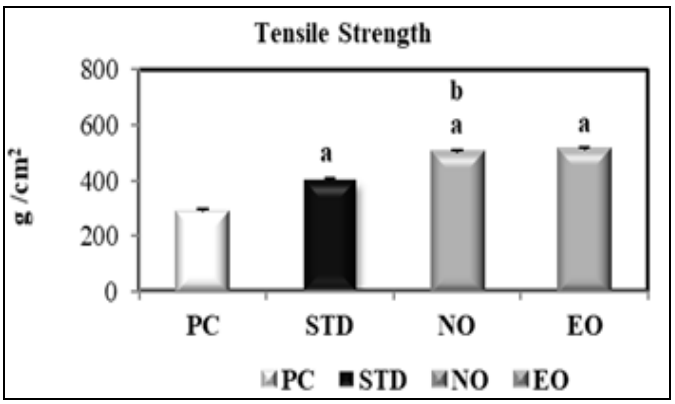


**FIG. 2: DETERMINATION OF TENSILE STRENGTH**

In rats, the topical application of betadine cream, although the wound closure was gradual, complete recovery was witnessed only by the 19th day of post-surgery equivalent to that of NO and EO treated groups. NO, and EO formulations increased the percentage of wound closure area more than that was seen in placebo and positive controls. Nevertheless, in rats with wounds dressed with NO, the degree of wound healing was on par with that dressed with EO formulation.

**Measurement of Tensile Strength:** Healing of incision wounds was assessed by measuring the tensile strength **Fig. 2.** Topical application of NO or EO, or betadine brought about a remarkable

improvement in tensile strength in comparison with the placebo control group. NO (74%) and EO (78%) formulations maximized the tensile strength of wound tissue as compared to the betadine ointment treated group (39%). The tensile strength of the wound in rats undergone NO (74%) was approximately the same to EO (78%) formulation. These observations confirm the excellent wound healing properties of EO and NO formulations as compared to the reference drug, betadine.

**Hydroxyproline:** The hydroxyproline concentration of wound tissues of various treatment groups is shown in **Fig. 3A.** NO, and EO formulations, as well as betadine augmented hydroxyproline.

Levels as compared to placebo control group, indicating efficient wound healing. Nevertheless, hydroxyproline levels did not show any appreciable difference among the three treatment groups.

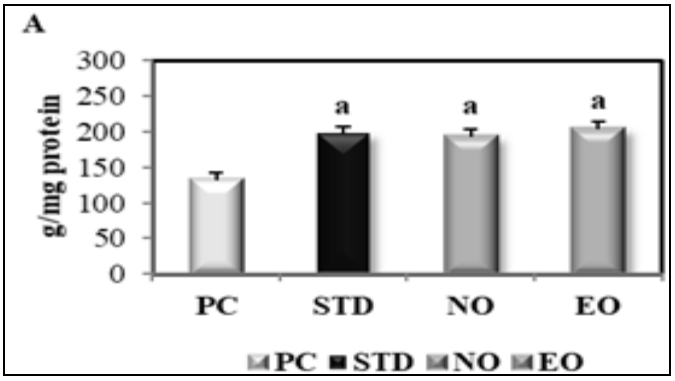


**FIG. 3A: ESTIMATION OF HYDROXYPROLINE**

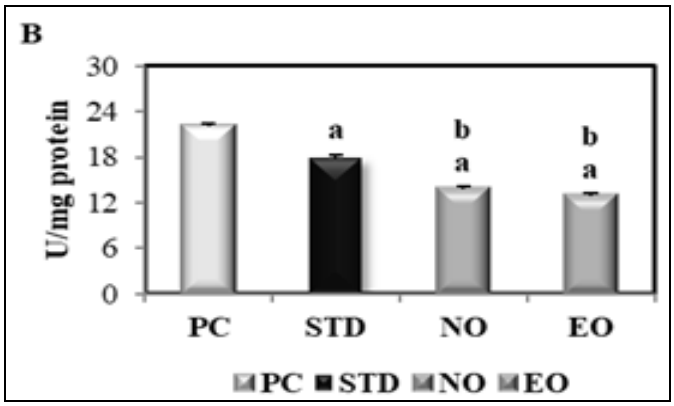


**FIG. 3B: ESTIMATION OF MYELOPEROXIDASE**

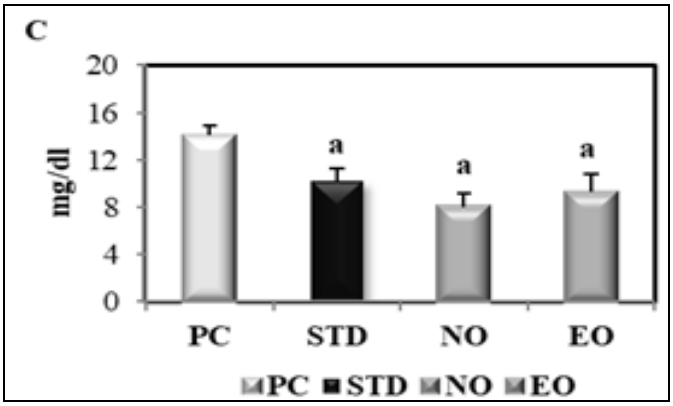


**FIG. 3C: ESTIMATION OF LIPID PEROXIDATION**

**Myeloperoxidase Activity (MPO):** The MPO activity of wound tissues of various treatment groups is shown in Figure 3b. MPO activity was significantly raised in rats applied with NO and EO formulations or betadine treatment as compared to placebo control. Never the less. the activity observed in betadine treated group was comparatively higher than that of the activity observed in NO or EO treated groups. However, no marked difference in the activities was observed between NO and EO treated groups **Fig. 3B.**

**Lipid Peroxidation:** MDA level was reduced significantly in the tissues of rats treated with NO, EO formulations or betadine drug as compared to placebo control group. Lipid peroxidation was similar in rats received NO and EO formulations **Fig. 3C.**

**Antioxidant Enzyme Activities:** As shown in **Fig. 4A-4C**, topical administration of EO and NO formulations enhanced the activities of SOD and catalase and the levels of GSH significantly in wound tissues in comparison with that of placebo control group.

A two-fold increase in SOD and catalase activities was noticed in rats in which betadine was applied topically as compared to control. There was no statistical difference in SOD or catalase activities between NO and EO treated groups.

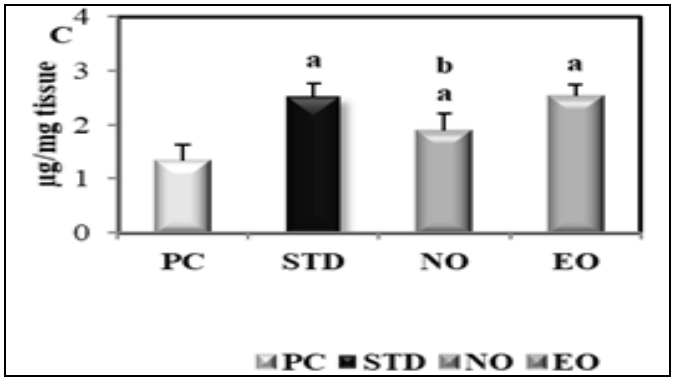


**FIG. 4A: DETERMINATION OF SUPEROXIDE DISMUTASE**

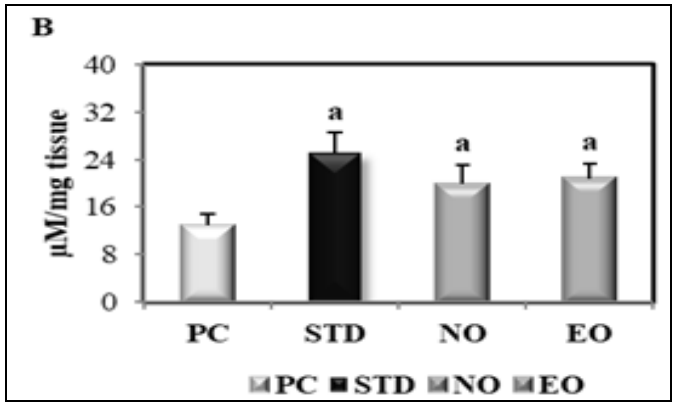


**FIG. 4B: DETERMINATION OF CATALASE ACTIVITY**

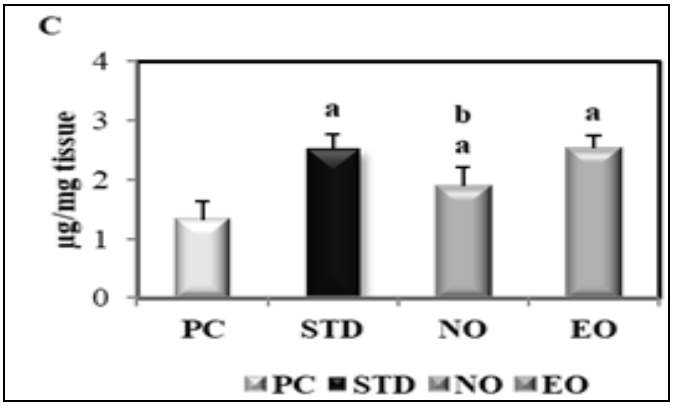


**FIG. 4C: DETERMINATION OF GSH**

**Histopathological Studies of Skin Tissue:** The skin from the control group **Fig. 5A** revealed normal histology showing epidermis, dermis, and hypodermis. Histology of normal rats showed mononuclear inflammatory cells, few proliferating vasculatures with minimal fibrosis. The microscopic appearance of skin from the placebo control rats **Fig. 5B** revealed epithelial erosions, ballooning, degeneration of epithelium, incoherent and flaky layers of keratin, many invasive inflammatory cells, a moderate degree of polymorphonuclear leucocytes, and mononuclear cells infiltration minimal signs of collagen formation in the dermis. There were empty spaces in the dermal region, indicating evidence of edema. The skin of rats treated with NO and EO ointment formulations **Fig. 5C-5F** revealed absence of epithelial erosions, re-epithelization, thin epidermis formation, a mild degree of inflammatory infiltrates, neovascularization, moderate collagen deposition, and mild to moderate degree of fibroblast proliferation in the dermis wound healed region, indicative of healing by fibrosis. The proportion of inflammatory cells was reduced than those observed in the placebo control rats. Masson trichrome staining was performed to assess the collagenous nature of scar formation. Rats received topical application of NO and EO formulations **Fig. 6B-6C** displayed a completely reconstructed collagen deposition as compared with the placebo control group **Fig. 6A.** These histopathology findings suggest that topical application of nano herbal and extract ointment revealed better-wound healing as evident by fibrosis, well-formed collagen fibers, and reduced inflammatory infiltrates when compared to placebo control group. The fibrosis was further confirmed by Masson's trichrome staining in skin tissues of nano herbal and ethanolic extract ointment dressed groups.

The present study provided evidence for the wound healing properties of *U. lobata* as evident in the incision and excision wound model rats by the topical application of two formulations, namely NO and EO to wound-inflicted skin. Wound contraction is the shrinkage of the unhealed area due to the regeneration of damaged tissue back to a normal state. Thus, the faster the rate of wound contraction, the better the efficacy of a medication. A drug to be used for effective wound healing should be able to clear the wound by 20th day after infliction.

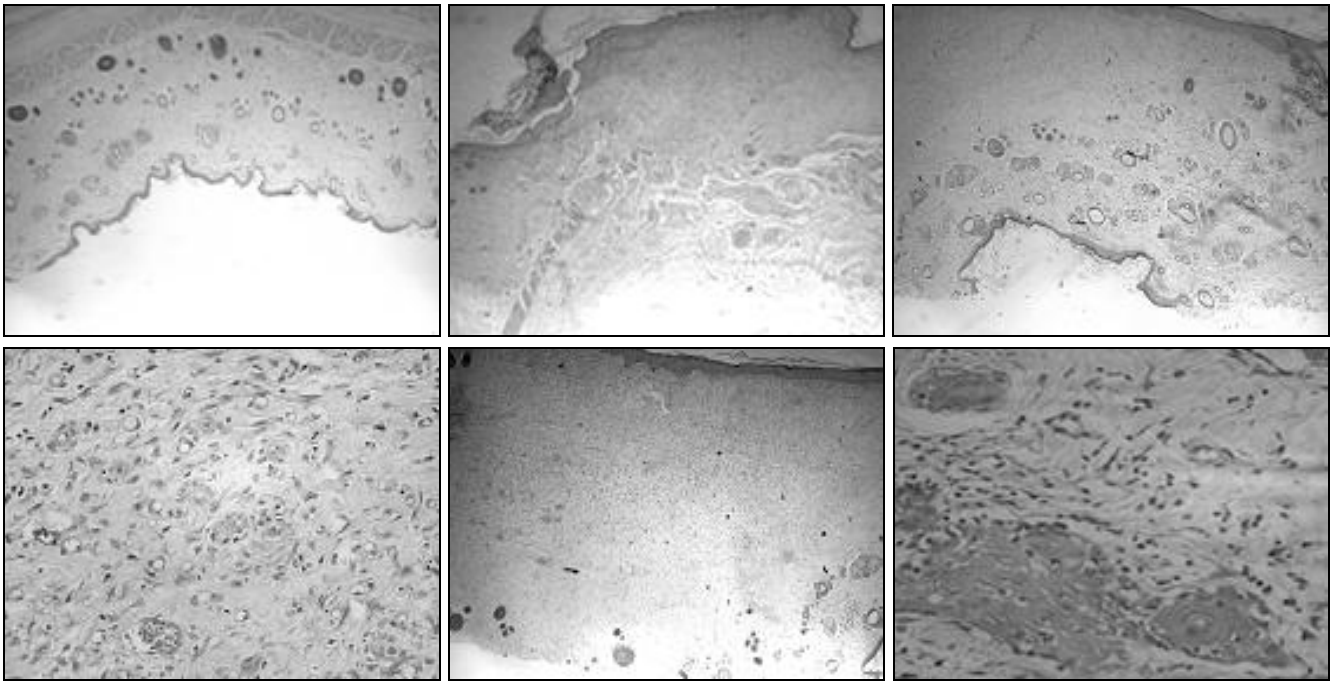

**FIG. 5: HISTOLOGICAL ASSESSMENT**

The curative effect of NO and EO formulations after 20 days is distinctly obvious in the present study, as these formulations promoted the percentage of wound closure significantly much higher than that of placebo control rats. NO and EO formulations exhibited potential wound healing activity than the reference betadine cream (positive control) in terms of wound closure and epithelialization as evident from the percentage of wound contraction, which is faster in rats that received NO and EO formulations (93-94%), in comparison with betadine treatment which caused only 87.04% wound contraction by day [16]. Further, in the present study, topical application of NO and EO improved the wound contraction and the strength of the regenerated tissue, which is evident by the maximal tensile strength observed in these groups of rats.

**FIG. 6: HISTOLOGY OF COLLAGEN FORMATION**

Breaking strength indicates the resistance of healed tissue to breaking under tension and the quality of regenerated tissue. In the present study, enhanced wound healing by the applied formulations may also be attributed to the improved tensile strength, which was substantially greater in EO treated animals *vis* a *vis* the placebo control group but approximately the same as that found in the reference group. These findings further confirm the excellent wound healing properties of EO and NO formulations. Collagen, a core component needed to confer strength and integrity to the tissue matrix and is a critical contributing factor in different phases of wound healing [22]. In the present study, wound strength acquired may be due to the remodeling and the reorganization of stable intra- and intermolecular cross-links of collagen [23]. Collagen breakdown releases free hydroxyproline. The hydroxyproline concentration is an index of collagen content. Higher the hydroxyproline

concentration, faster the rate of wound healing. Therefore, the enhanced hydroxyproline levels observed in the present study is another significant factor contributing to rapid healing with a concurrent increase in the breaking strength of the treated wounds observed in NO and EO treated groups. In accordance with these findings, the increased collagen formation and stabilization of the fibers observed in the histological studies corroborates with the increased collagen formation as reflected by the significant enhancement in hydroxyproline concentration. Lipid peroxidation was determined in terms of MDA released from the breakdown of polyunsaturated fatty acids [24]. The generation of free radicals in wound tissues causes tissue damage by inducing lipid peroxidation of cellular membranes [25].

Lipid peroxidation impairs the progression of the wound-healing process [26]. The enhanced lipid peroxidation as evidenced by the enhanced MDA level in placebo control rats clearly indicates the poor scavenging of free radicals and increased oxidative damage to wound tissue. Conversely, the level of lipid peroxidation was decreased significantly in rats with topical application of NO and betadine as showed by the decreased levels of MDA in the wounded skin of rats. However, the lack of significant difference in the MDA levels between NO/ EO and betadine treated rats indicates that both NO and EO attenuated lipid peroxidation with comparable results with the positive control. These findings collectively imply that NO and EO accelerated the wound healing process remarkably by protecting the wound tissue from lipid peroxidation, MPO catalyzes the formation of potent ROS and reactive nitrogen intermediates [27].

Though MPO has a beneficial role in killing ingested bacteria [28] it promotes oxidative tissue damage when released into tissues. In the present study, the observed higher MPO activity may be due to higher inflammatory response and oxidative damage leading to impaired wound healing. On the other hand, Lesser MPO activity observed in NO, EO and betadine treated rats is indicative of lesser infiltration of inflammatory cells and oxidative stress. Antioxidants play a vital role as the primary defense against tissue damage of the skin [29, 30]. Since superoxide is the major ROS generator and a vital mediator of oxidative injury in tissues leading to delayed wound healing [31] detoxification of free radicals could be an important strategy in wound healing. Therefore, the measurement of antioxidants such as SOD, catalase, and GSH in the wound site is more appropriate as the antioxidants hasten wound healing by quenching the ROS. SOD causes the dismutation of superoxide anions to $H_2O_2$, thereby preventing the generation of deleterious ROS and oxidative stress. The enhanced activity of SOD results in excess production of $H_2O_2$, which might be neutralized by catalase [32]. GSH, a tripeptide, scavenges the free radicals directly and acts as an electron donor for the glutathione peroxidase activity in reducing $H_2O_2$ and lipid peroxides [33, 34].

Antioxidants, notably SOD, catalase and GSH, that are abundantly present in skin, neutralize free radicals and protect cells from oxidative damage induced by wound injury, thereby promoting healing [30, 35, 36]. The improved wound healing observed in the rats treated with the topical application of NO or EO or betadine may be due to the enhanced activities of SOD, CAT and GSH levels, which probably would have protected the cells from ROS insults. On the contrary, the observed delay in the wound healing activity in placebo control rats may be attributed to the reduced activities of SOD, CAT and GSH levels, or alternatively overproduction of ROS, which delays the healing process. In the present study, histological sections (H & E stained) were examined for epithelialization, degeneration and leukocytic infiltration. The Masson's trichrome stained sections were similarly evaluated for fibroblast and collagen distribution and angiogenesis.

As shown in **Fig. 5A-5F** and **Fig. 6B-6C**, histological findings revealed regeneration of epithelialization, forming new blood vessels with few inflammatory cells within the dermis, implying significant improvement in wound repair NO or EO treated groups. In addition, collagen fiber synthesis and enhanced fibrosis were seen in the wound site of rats exposed with topical application of NO and EO. The collagen formation observed in histological findings is consistent with the increased levels of hydroxyproline concentration in these groups of rats. Thus, his to pathological studies add further support for wound contraction

and tensile strength. On the contrary, the placebo control group lagged behind the treated groups with respect to granular tissue formation in the dermis as evidenced by the high number of inflammatory cells with minimal fibrosis and few proliferating vasculature, indicating a poor healing process. The slow wound healing process observed in placebo control rats may also be due to the reduced blood flow, uncontrolled inflammatory and immune responses, microbial infection, and excessive ROS production at the wound site. Excessive amounts of ROS may modify and/or degrade ECM proteins and damage dermal fibroblasts and keratinocytes functions.

The decreased collagen formation as reflected by the reduced hydroxyproline levels may be due to the reduced synthesis and/or enhanced collagen degradation. These changes might have contributed to the delay in wound healing observed in placebo control rats. Antioxidants have been known to control oxidative stress at the wound site and thereby accelerate wound healing [30]. In this regard, it is worthwhile to recall the earlier studies on the antioxidant potential of *U. lobata*, [13], which may be attributed to the presence of phytoconstituents such as tannins, flavonoids, and triterpenoids with proven antioxidant activity [37, 38]. Therefore, the well-known antioxidant property of ethanolic extract of *U. lobata* helped the wounds to heal significantly faster in No and EO treated rats than the untreated control. Further investigations are necessary to determine the exact mechanism and bioactive constituents present in these formulations. Overall, the present study provided evidence for the positive effects of NO and EO on wound healing, in terms of morphological (enhanced wound contraction and tensile strength), biochemical (decreased lipid peroxidation and increased hydroxyproline concentration, SOD, catalase activities and GSH level) and histological findings in a rat model. These findings could justify the folklore use of *U. lobata* for wound healing property.

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