Clinical Phytoscience

ORIGINAL CONTRIBUTION

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Evaluation of the wound healing effect of Nishamalaki, an Ayurvedic formulation comprising *Curcuma longa* and *Phyllanthus emblica* in aging rats

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Abstract

Background There are very few drugs available for healing wounds in the aged population, which is more prone to chronic cutaneous wounds that are particularly hard to heal and require a long healing process. This study which deals with age-related wound healing, investigates the healing effect of Nishamalaki, a classic antidiabetic Ayurvedic formulation comprising turmeric (*Curcuma longa*) and Indian gooseberry (*Phyllanthus emblica*), on cutaneous wounds in aging rats.

Methods Rats with excision wounds of 7 mm created on their dorsal side received Nishamalaki (500 mg/kg p.o) daily, or a combination of Nishamalaki (500 mg/kg p.o) with 1% Nishamalaki gel applied on the wound or the reference standard metformin (2 µmol) applied on the wound daily till the scabs fell off.

Results All treatments enhanced the rate of formation of granulation tissue and wound contraction. All treated rats showed lower blood glucose levels compared with their 1st-day values and significantly lower blood glucose levels when compared with the Aged Control rats. A significant restoration of the aging-depleted L-hydroxyproline, hexosamine, ascorbic acid, PDGF, AMPK, and mTOR levels, and attenuation of the aging-elevated IL-6 and TNF-α levels was elicited by all treatments. The treatments significantly restored the aging-depleted endogenous antioxidants. The Nishamalaki combination treatment of the oral extract and topical gel displayed a better wound-healing effect than the oral treatment alone. The histopathological studies on skin ulceration, hair follicles, granulation tissue, and collagen fiber formation of the wound tissues corroborated the biochemical findings.

Conclusion Curcumin and other antioxidant polyphenolic components of Nishamalaki may be responsible for its wound-healing effect. For the first time, the present study has investigated the action of PDGF, AMPK, and mTOR on cutaneous wounds. They seem to be acting together to promote wound healing and repair.

Keywords *Curcuma longa*, *Phyllanthus emblica*, Cutaneous wounds, Wound healing, Metformin

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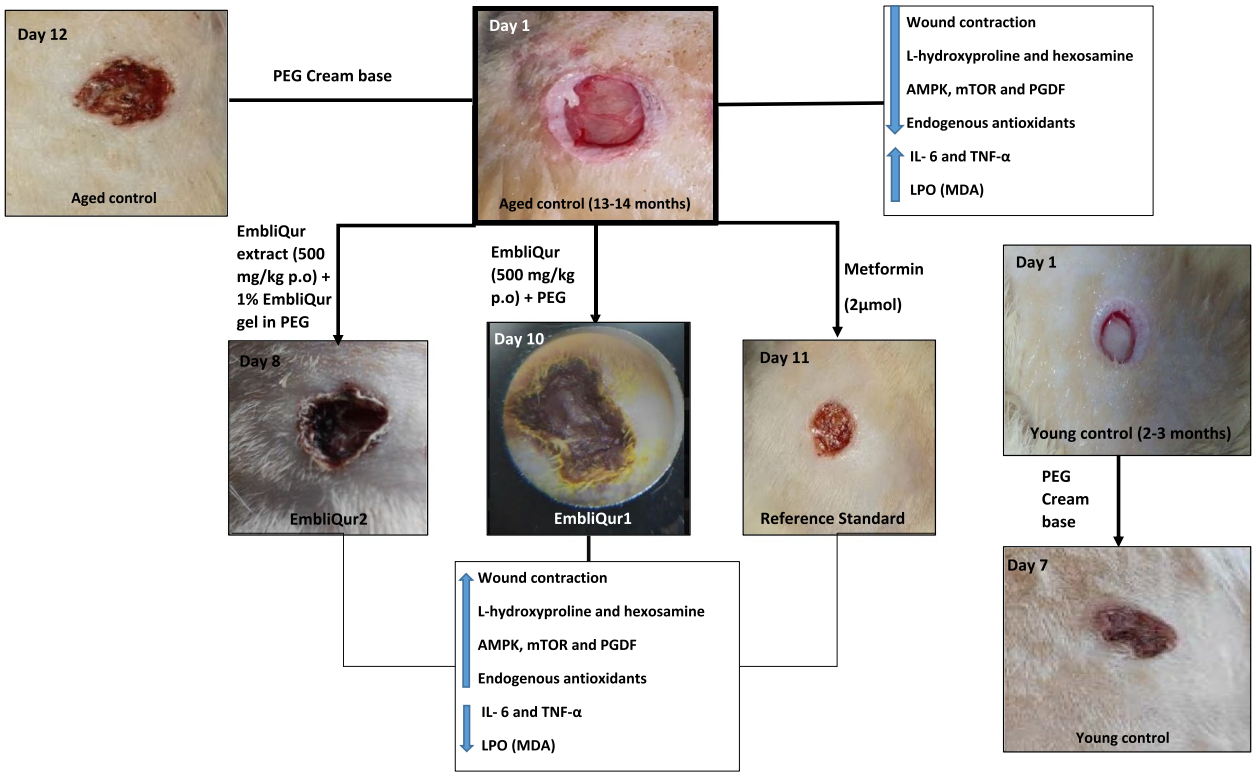
Graphical Abstract



Background

Aging is associated with an increased risk of chronic slow-healing cutaneous wounds. The reasons for slow healing are decreased growth factors, delayed infiltration of macrophages and B lymphocytes into wounds, decreased capillary permeability, decrease in macrophage phagocytic function, reduced cell proliferation and migration, diminished extracellular matrix secretion, delayed re-epithelialization, and collagen synthesis [1]. Also, remodeling in aging tissue retards wound healing [2].

The risk of uncontrolled diabetes may exist in the aged population, leading to poor blood circulation to the wound, and further delaying the healing process [3]. Malnutrition, which is common in the elderly, may impair the repair of wounds and is a significant risk factor for developing poorly healing pressure wounds [4]. Other factors affecting the age-related wound healing process are systemic factors such as stress, sex hormones, ischemia, obesity, and local factors like oxygenation, infection, foreign body, and venous efficiency [5].

Nishamalaki is an Ayurvedic formulation comprising turmeric (*Curcuma longa* Linn.) and the Indian gooseberry (*Phyllanthus emblica*) also called amla, which is used for the treatment of diabetes [6]. Curcumin, the principal curcuminoid found in turmeric, has been reported to possess anti-inflammatory, antioxidant, and wound-healing effects [7]. It has been shown to act on various stages of the wound healing process to expedite healing. Apart from inhibiting inflammation and oxidation at the wound site, curcumin promotes fibroblast migration, granular tissue formation, re-epithelization, and collagen deposition in the proliferation stage of wound healing [8]. In the remodeling stage, it promotes wound contraction and scar tissue formation. Amla is known to increase the formation of granulation tissue and re-epithelialization as well as cross-linking of collagen at the wound site, as evidenced by an increase in the activity of extracellular signal-regulated kinase 1/2 (ERK1/2), along with an increase in DNA, type III collagen, acid-soluble collagen, aldehyde content, shrinkage temperature, and tensile strength [9]. Being rich in vitamin C, it is effective in slowing the aging process.

The potent antioxidants of Nishamalaki (curcuminoids from turmeric; ascorbic acid, polyphenols, and tannins from amla) may scavenge ROS in the wound's

microenvironment and enhance the repair process. The potent anti-inflammatory effects of both herbs may aid in the wound-healing process. Thus, both herbs of Nishamalaki may exhibit an age-related healing effect by increasing wound healing.

Metformin, a biguanide used for treating diabetes (T2DM), is known to improve vasculature of the wound beds and increase the healing process in aging skin by activating adenosine monophosphate-activated protein kinase (AMPK) and its downstream component p-acetyl-CoA carboxylase [10]. In aged skin of rats, analysis on proliferative and senescent markers revealed significant rejuvenating effects of metformin, demonstrating increases in proliferative cells and epidermis and around hair follicles. The effect is triggered by AMPK stimulation [11]. AMPK signaling is known to promote angiogenesis by stimulating the vascular endothelial growth factor (VEGF), which is necessary for angiogenesis [12]. Metformin is also reported to promote collagen formation, elevate the cellularity of granulation tissue, and accelerate wound closure [13].

While many drugs are available for healing wounds in the younger population, very few are available for the elderly, who are more prone to chronic cutaneous wounds, which are particularly hard to heal and require a long healing process. Thus, this study which deals with age-related wound healing effects in the elderly, was undertaken to investigate the beneficial effects of Nishamalaki in cutaneous wounds of aged rats.

Material and methods

Drugs and chemicals

Nishamalaki extract and Nishamalaki topical gel were obtained from Pharmanza Herbals Pvt Ltd., India. SRS Pharma, Mumbai, gifted metformin. Thiobarbituric acid, trichloroacetic acid, reduced glutathione, oxidized glutathione, nicotinamide adenine dinucleotide phosphate epinephrine, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were procured from Sigma Chemical Co., St. Louis MO, USA. All other chemicals were obtained from local sources and were of analytical grade.

Plant material

The Nishamalaki extract comprising extract of *Phyllanthus emblica (Emblica officinalis)* fruit and extract of *Curcuma longa* rhizome in a 2:1 proportion was procured from Pharmanza Herbals Pvt. Ltd., India. It was a standardized extract containing: a) Total curcuminoids – 23.89% (by HPLC) b) Total gallic acid—5.27% (by HPLC) and c) Total tannins – 25.44% (by Titration) [14]. The Nishamalaki topical gel comprising Nishamalaki extract (1%) in polyethylene glycol (PEG) base was also procured from Pharmanza Herbals Pvt. Ltd.

Animals

Male Wistar rats aged 2–3 months and 13–14 months were included in this study. Animals were procured from the registered breeders Bharat Serums Ltd. Thane, India. They were housed in clean polypropylene cages under standard conditions of humidity (50 ± 5%), temperature (25 ± 2 °C), light (12 h light/12 h dark cycle), and fed with a standard diet (Amrut Laboratory Animal Feed, Pranav Agro Ltd., Pune, India) and water ad libitum. The protocol for animal studies was approved by the Institutional Animal Ethics Committee which conforms to the national guidelines for the use and care of experimental animals in research.

Preparation of test solutions

The Nishamalaki extract was dissolved in distilled water, and the fresh solution was administered orally to rats. Metformin (0.3 mg, i.e., 2 µmol) was dissolved in ethanol, and this solution was applied immediately to the wounds created in rats [15].

Experimental

After acclimatization for seven days in the animal quarters, the aged rats were randomly divided into four groups of 6 rats in each group. The 5th group comprised six young rats. All rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). The hair on the dorsal side of the rats was shaved, and the skin was cleaned with 70% ethanol. A dermal biopsy punch in all rats created an excision wound of 7 mm in diameter. Following was the treatment regimen of the rats assigned to various groups:

Group I (Young Control): Rats (2–3 months old) were applied the PEG base on the excision wound daily till the scab fell off.
Group II (Aged Control): Rats (13–14 months old) were applied the PEG base on the excision wound daily till the scab fell off.
Group III (Nisha1): Rats (13–14 months old) received Nishamalaki extract (500 mg/kg p.o.) and a gel containing only the PEG base on the excision wound daily till the scab fell off.
Group IV (Nisha2): Rats (13–14 months old) received Nishamalaki extract (500 mg/kg p.o.) and the gel containing 1% Nishamalaki extract in the PEG base on the excision wound daily till the scab fell off.
Group V (Reference standard): Rats (13–14 months old) were applied metformin (2 µmol) on the excision wound daily till the scab fell off.

The wound closure rate was studied by tracing the raw wound on a transparent plastic paper every day till the scab was formed. Wound areas were measured by retracing the wound on a millimeter scale graph paper for wound healing assessment. The number of days required for the dead tissue remnants to fall off without any residual raw wound gave the period of epithelization. The wound closure was calculated using the formula [16]:

$$\%\ \text{Wound closure} = \frac{\text{Wound area on the day '0'} - \text{Wound area on the day'n'}}{\text{Wound area on the day '0'}} \times 100$$

Blood glucose levels were measured at the start and end of the experiment using an automated digital glucometer (Accu-Chek, Roche Diabetes Care, Inc.). The scab (granulation tissue) formed on the wound tissue was collected from all animals, washed in cold saline (0.9% w/v NaCl), lyophilized, and used for L-hydroxyproline and hexosamine estimations. At the end of the treatment period, all animals were fasted for 6 h and sacrificed by cervical dislocation. Blood was collected from the retro-orbital plexus, and serum was separated by centrifugation at 2500 rpm at 30^0 C for 15 min and used for the estimation of ascorbic acid, mammalian Target of Rapamycin (mTOR), Interleukin-6 (IL-6), Tumor Necrosis Factor (TNF-α) and 5' adenosine monophosphate-activated protein kinase (AMPK). Plasma was also separated from blood and used to estimate platelet-derived growth factor (PDGF).

Wound tissues and livers of all animals were dissected immediately, washed with ice-cold saline, and weighed. The livers were used to prepare a 10% (w/v) homogenate in phosphate buffer (50 mM, pH 7.4). An aliquot was used for the estimation of lipid peroxidation (LPO). The homogenates were centrifuged at 7000×g for 10 min at 4 °C, and the supernatants were used for the assays of reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT). Wound tissues were excised and fixed in formalin for histopathological studies.

Estimation of hexosamine and L-hydroxyproline in granulation tissue

A part of the collected scab was used for L- Hydroxyproline estimation using the method of Bergman & Loxley [17]. Hexosamine was estimated in the other part of the scab by a modified method of Elson and Morgan [18].

Determination of TNF-α, IL-6, mTOR, and AMPK and PDGF levels

TNF-α, IL-6, mTOR, AMPK, and PDGF levels were determined using ELISA kits based on standard sandwich enzyme-linked immunosorbent assay technology supplied by Allianz Bioinnovation, India.

Estimation of Ascorbic acid

Ascorbic acid was estimated in serum using the method of Day et al. [19].

Estimation of LPO

The quantification of LPO was carried out by determining the concentration of malondialdehyde (MDA) in the liver using the method of Ohkawa et al. [20].

GSH, SOD, and CAT estimation

GSH was estimated in the liver homogenate using DTNB by Ellman [21]. SOD was assayed by the method of Sun and Zigman [22]. CAT was estimated by the method of Clairborne [23].

Histopathological studies

The wound tissue was excised from all rats in full depth with a margin of at least 5 mm of healthy, uninjured skin around each wound. The dissected tissue was washed immediately with ice-cold saline and stored in 10% buffered formalin. After dehydration, the pieces of skin of the wound were embedded in paraffin wax, cut into 4-6 µm thick sections, and stained with hematoxylin and eosin. These sections were then examined under a light microscope for histoarchitectural changes.

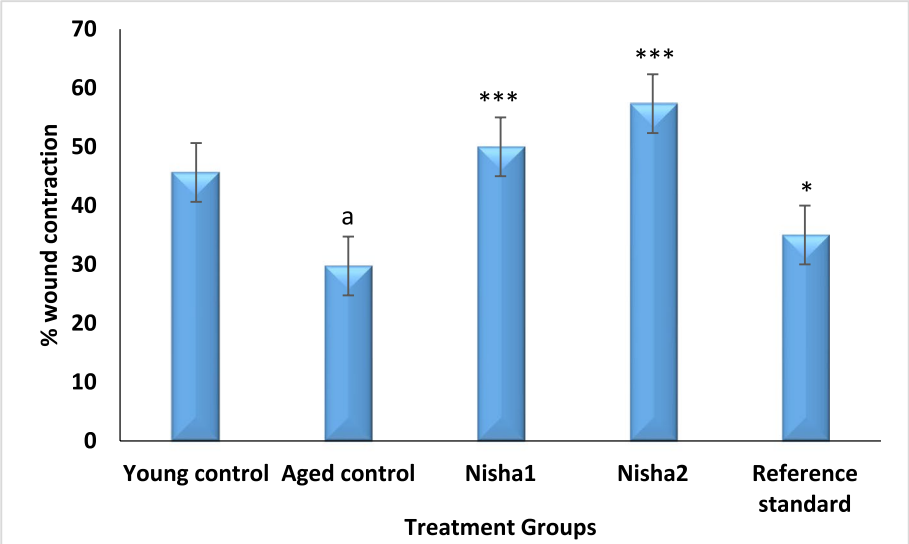
Statistical analysis

The results are expressed as mean ± SEM from 6 animals in each group. Results were statistically analyzed using one-way ANOVA followed by Tukey's Multiple Comparison Test; $P < 0.05$, 0.01, 0.001 was considered significant. GraphPad InStat version 8.00 of Graph Pad Software Inc., San Diego, USA, was used for statistical analysis.

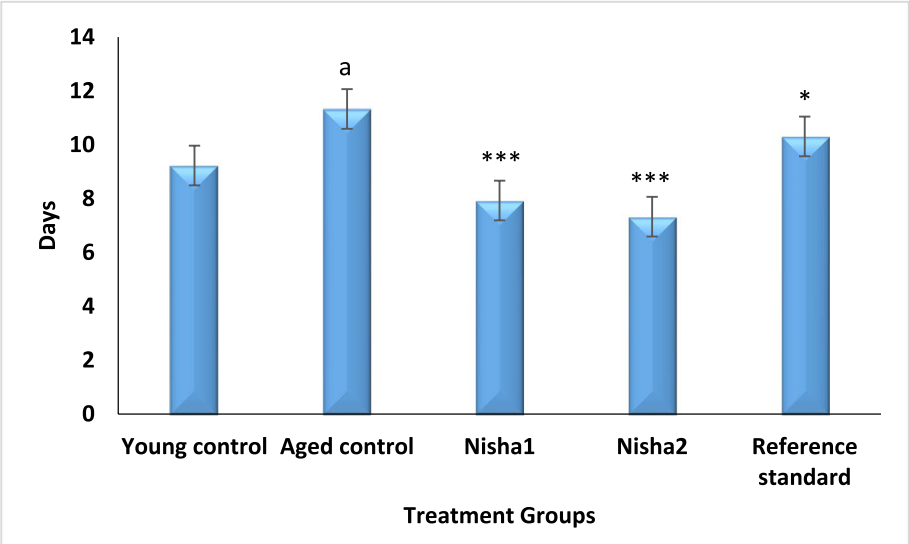
Results

Effect on wound contraction

The wound area was measured by tracing the wound using a transparent paper at a 4-day interval, and the healed area was calculated by subtracting it from the original wound area. The wound contraction (%) of the aged control rats was found to be significantly (***$P < 0.001$) lower than the Young Control rats (Fig. 1a). The wound contractions in the Nisha and metformin-treated rats were significantly higher than in the Aged Control rats (Fig. 1a). The Nisha1 and Nisha2



a. Effect of treatments on percent wound contraction



b. Days required for the formation of granulation tissue

Fig. 1 **a** Effect of treatments on percent wound contraction. **b** Days required for the formation of granulation tissue. All values are mean ± SEM; $N = 6$ in each group; One-way ANOVA followed by Tukey–Kramer post-test is applied for statistical analysis. P values: [a] < 0.001 when Aged Control is compared with Young Control *** < 0.001 & * < 0.05 when Experimental groups are compared with Aged Control

treatments showed better wound contraction than even Young Control rats. Figure 1b shows the period of granulation tissue formation (epithelization period) for all the animal groups. It was observed that the mean epithelization period was significantly ($P < 0.001$) higher in the Aged Control group of rats when compared with the Young Control rats. Nisha1 and Nisha2 treatments elicited the lowest epithelization periods, even lesser than the Young Control rats.

Effect on blood glucose levels

All rats exhibited similar blood glucose levels at the beginning of the experiment. However, on the 10th day or when the scab was formed, rats of all the treatment groups showed lower blood glucose levels compared with their 1st-day values and significantly lower blood glucose levels when compared with the Aged Control rats (Fig. 2).

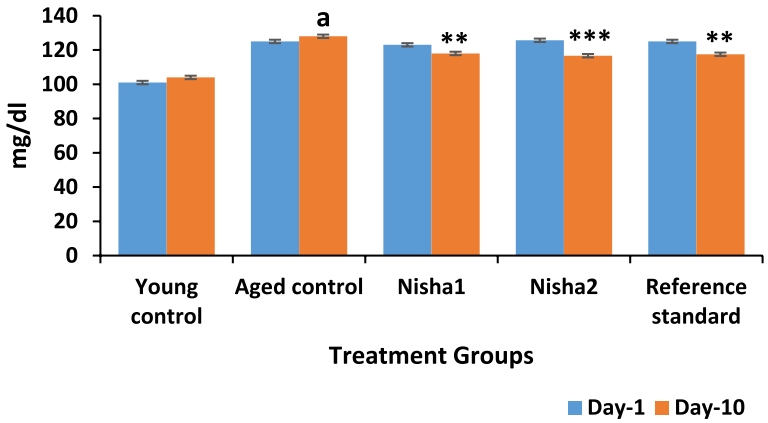


Fig. 2 Effect of treatments on Blood glucose levels. All values are mean ± SEM; *N* = 6 in each group; One-way ANOVA followed by Tukey–Kramer post-test is applied for statistical analysis. *P* values: [a] < 0.001 when Aged Control is compared with Young Control; *** < 0.001 & ** < 0.01 when Experimental groups are compared with Aged Control

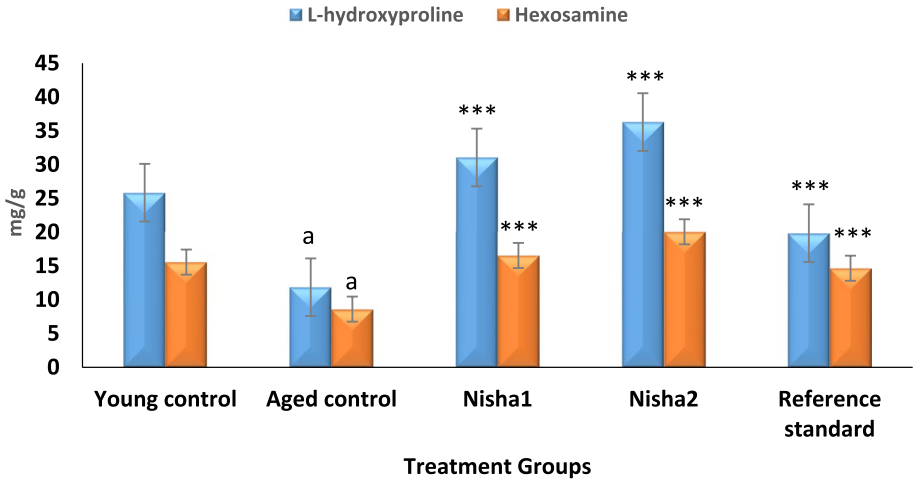


Fig. 3 Effect of treatments on L-hydroxyproline and hexosamine content. All Values are mean ± SEM; *N* = 6 in each group; One-way ANOVA followed by Tukey–Kramer post-test is applied for statistical analysis. *P* values: [a] < 0.001 when Aged Control is compared with Young Control; *** < 0.001 when Experimental groups are compared with Aged Control

Effect on L-Hydroxyproline and hexosamine content

The Aged Control group of rats exhibited significantly reduced (***$P < 0.001$) hydroxyproline and hexosamine contents compared with the young control rats. At the end of the study, all treatments, including the reference standard metformin, significantly restored ($P < 0.001$) the aging-depleted hydroxyproline and hexosamine levels (Fig. 3).

Effect on AMPK, mTOR, and PDGF levels

Rats from the aged control group exhibited a significant reduction (***$P < 0.001$) in AMPK, mTOR, and PDGF levels compared to the young control rats. At the end of the study, Nisha1 (**$P < 0.01$ for AMPK and

Table 1 Effect of treatments on AMPK, mTOR and PDGF

Groups	AMPK (ng/ml)	mTOR (ng/ml)	PDGF (ng/ml)
Young control	16.00 ± 1.38	226.89 ± 4.60***	3.55 ± 0.08
Aged control	8.26 ± 0.62[a]	134.57 ± 8.71[a]	2.90 ± 0.05[a]
Nisha1	13.36 ± 0.40**	186.11 ± 5.44***	3.53 ± 0.06***
Nisha2	15.70 ± 0.89***	197.79 ± 1.86***	3.70 ± 0.02***
Reference standard	17.00 ± 1.03***	184.76 ± 4.18***	3.20 ± 0.07

All Values are mean ± SEM; *N* = 6 in each group; One-way ANOVA followed by Tukey–Kramer post-test is applied for statistical analysis *P* values: [a] < 0.001 when Aged Control is compared with Young Control; *** < 0.001 & ** < 0.01 when Experimental groups are compared with Aged Control

$P < 0.001$ for mTOR & PDGF) and Nisha2 treatments ($P < 0.001$) significantly restored the aging-depleted AMPK, mTOR and PDGF levels (Table 1). The reference standard also significantly (***$P < 0.001$) restored the aging-depleted AMPK and mTOR levels.

Effect on Tumor necrosis factor-α (TNF-α) and Interleukin-6 (IL-6)

The Aged Control group of rats exhibited a significant (***$P < 0.001$) increase in serum levels of the inflammatory mediators IL- 6 and TNF-α when compared with the young control rats (Fig. 4). All treatments significantly (**$P < 0.01$ for Nisha1 and reference standard; ***$P < 0.001$ for Nisha2) attenuated the aging-elevated IL-6 and TNF-α levels.

Effect on LPO (MDA) and endogenous antioxidants

The effect of the treatments on LPO, ascorbic acid, GSH, SOD & CAT is shown in Table 2. The aged control rats elicited significantly (***$P < 0.001$) higher levels of MDA, the gold standard for LPO, than the young control rats. A significant (***$P < 0.001$) depletion in the ascorbic acid, GSH, SOD, and CAT levels was also noted in the aged control rats compared with the young control rats. All treatments successfully attenuated the aging-elevated MDA levels and restored significantly (***$P < 0.001$) the aging-depleted vitamin C, GSH, SOD, and CAT levels.

Histopathology of wound tissue

Histopathology of the wounds' tissue was carried out to estimate skin ulceration, hair follicles, granulation tissue, and collagen fiber formation on the wounded tissue. The wound tissue of the young control rats showed skin ulceration with a plasmaceous necrotic exudate covering it. A thickening of the skin at the edges of the ulcers was observed (Fig. 5a.i). The dermis displays epithelioid cell granulomas with multi-nucleated giant cells (Fig. 5a. ii) and lymphocytic infiltrate in the surrounding tissues. There was minimal granulation tissue and more collagen fibers (Fig. 5a.iii).

The wounds of the Aged Control rats showed skin ulcerations covered by neutrophils and a plasmaceous

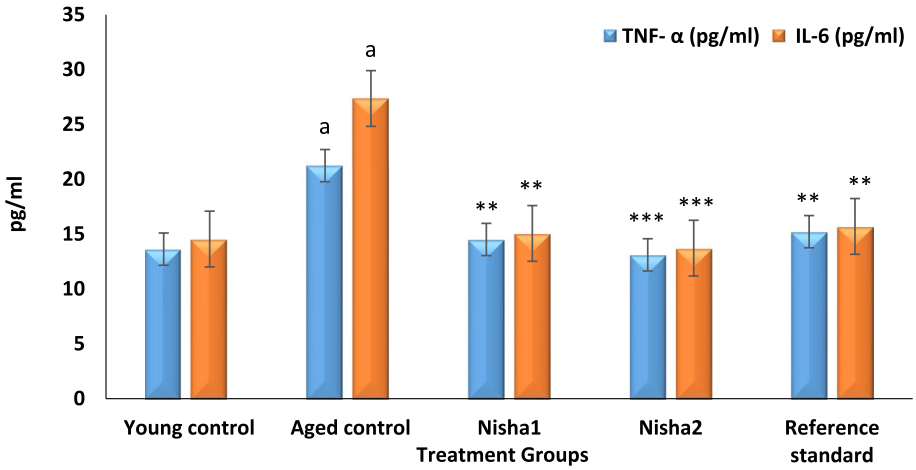


Fig. 4 Effect of treatments on TNF-α and IL-6. All Values are mean ± SEM; $N = 6$ in each group; One-way ANOVA followed by Tukey–Kramer post-test is applied for statistical analysis. *P* values: [a] < 0.001 when Aged Control is compared with Young Control; *** < 0.001 & ** < 0.01 when Experimental groups are compared with Aged Control

Table 2 Effect of treatments on LPO and endogenous antioxidants

Groups	LPO (nmol/MDA/min/mg protein)	GSH (U/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	Ascorbic acid (gm/dl)
Young control	22.81 ± 0.28	16.62 ± 0.45	37.27 ± 0.75	9.02 ± 0.06	4.59 ± 0.04
Aged control	35.63 ± 0.49[a]	7.41 ± 0.27[a]	26.56 ± 0.27[a]	5.20 ± 0.21[a]	3.48 ± 0.01[a]
Nisha1	26.54 ± 0.37***	12.5 ± 0.33***	31.25 ± 0.45***	8.11 ± 0.15***	5.03 ± 0.06***
Nisha2	24.41 ± 0.46***	14.58 ± 0.32***	32.62 ± 0.24***	8.75 ± 0.07***	5.36 ± 0.08***
Reference standard	27.64 ± 0.30***	11.41 ± 0.15***	29.65 ± 0.37**	7.50 ± 0.17***	4.64 ± 0.08***

All Values are mean ± SEM; $N = 6$ in each group; One-way ANOVA followed by Tukey–Kramer post-test is applied for statistical analysis *P* values: [a] < 0.001 when Aged Control is compared with Young Control; *** < 0.001 & ** < 0.01 when Experimental groups are compared with Aged Control

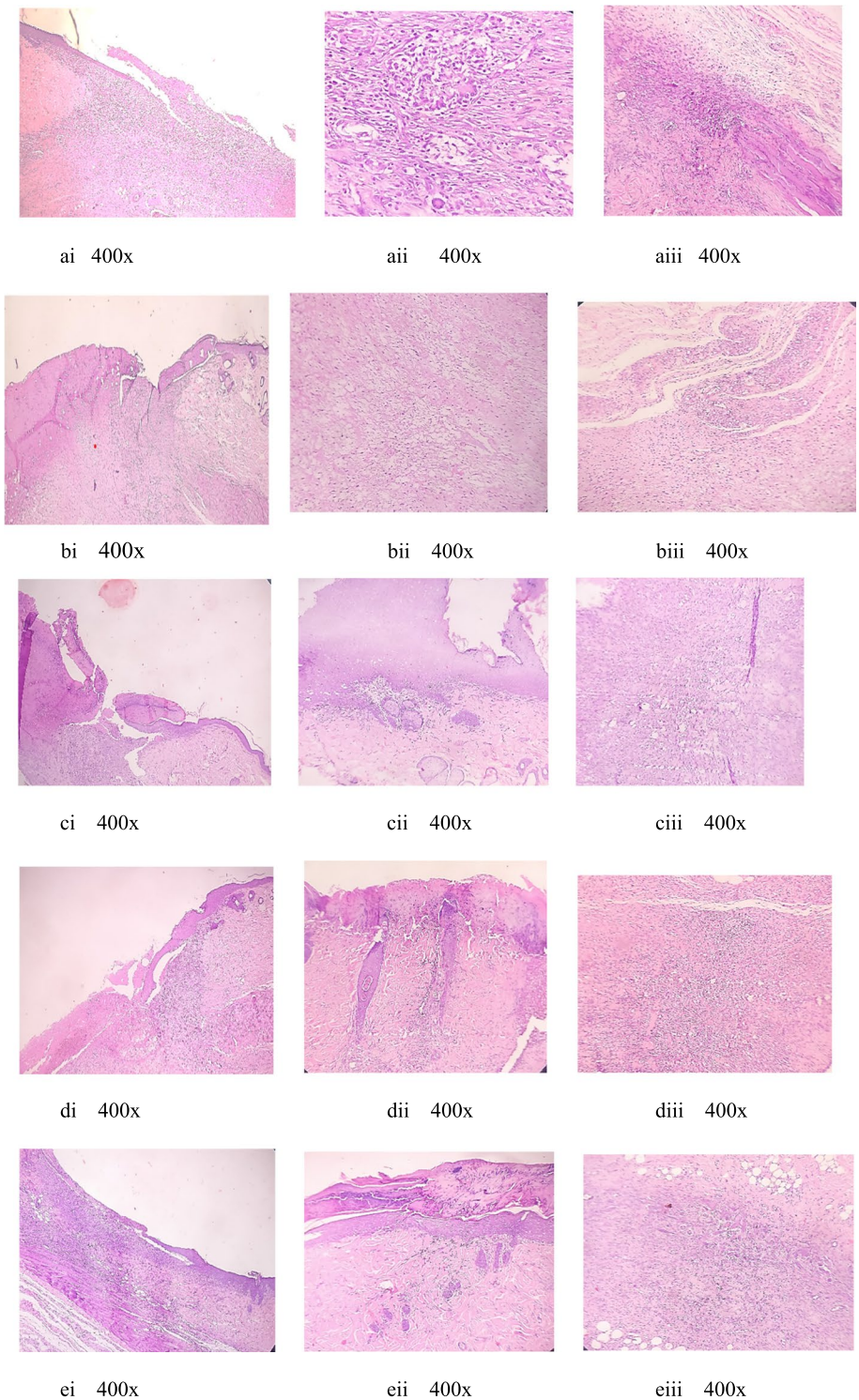


Fig. 5 **a**i H & E staining of skin ulceration of Young Control rat 40 X 10x. **a**ii H & E staining of hair follicles of Young Control rat 40 X 10x. **a**iii H & E staining of granulation tissue and collagen fibers of Young Control rat 40 X 10x. **b**i H & E staining of skin ulceration of Aged Control rat 40 X 10x. **b**ii H & E staining of hair follicles of Aged Control rat 40 X 10x. **b**iii H & E staining of granulation tissue and collagen fibers of Aged Control rat 40 X 10x. **c**i H & E staining of skin ulceration of Nisha1 group rat 40 X 10x. **c**ii H & E staining of hair follicles of Nisha1 group rat 40 X 10x. **c**iii H & E staining of granulation tissue and collagen fibers of Nisha1 group rat 40 X 10x. **d**i H & E staining of skin ulceration of Nisha2 group rat 40 X 10x. **d**ii H & E staining of hair follicles of Nisha2 group rat 40 X 10x. **d**iii H & E staining of granulation tissue and collagen fibers of Nisha2 group rat 40 X 10x. **e**i H & E staining of skin ulceration of reference standard (Metformin) group rat 40 X 10x. **e**ii H & E staining of hair follicles of reference standard (Metformin) group rat 40 X 10x. **e**iii H & E staining of granulation tissue and collagen fibers of reference standard (Metformin) group rat 40 X 10x

necrotic exudate on the ulcerated skin surface (Fig. 5b.i). The dermis lost hair follicles (Fig. 5b.ii) with plenty of granulation tissue and few collagen fibers (Fig. 5b.iii).

The wound tissue of the Nisha1 treated rats showed the skin with large excavated ulcers covered by hemorrhage and a plasmaceous necrotic exudate with a thickening of the skin at the edges of the ulcer (Fig. 5c.i). A hypertrophied epidermis with many hair follicles at the edges was observed (Fig. 5c.ii). The skin showed a dermis with minimal granulation tissue and few collagen fibers (Fig. 5c.iii). The wound tissue of the Nisha2-treated rats showed minimal skin ulceration covered by neutrophilic inflammatory exudate and hemorrhage (Fig. 5d.i). The hair follicles were preserved in the dermis below the superficial ulcer (Fig. 5d.ii). The dermis also showed abundant granulation tissue and collagen fibers (Fig. 5d.iii).

The wound tissue of the metformin (reference standard) treated rats showed minimal ulceration, an absence of exudate or hemorrhage, and sound healing (Fig. 5e.i). Many hair follicles were observed in the dermis below the partially healed ulcer (Fig. 5e.ii). The dermis showed minimal granulation tissue and dense collagen fibers (Fig. 5e.iii).

Discussion

The aged skin reveals fewer fibroblasts, diminished vascularity, reduced sebum secretion, decreased elasticity, and a loss in extracellular matrix components such as collagen and glycosaminoglycan compared with young skin [24]. These changes in aging skin retard wound healing and make the skin prone to easy injury. Age-related changes negatively affect all phases of wound repair, and disruption of any phases may delay healing by up to 60% [25].

Micro-inflammation has now been understood to accelerate skin aging by augmenting the loss of dermal elasticity, resilience, and firmness through the destabilization of collagen, hyaluronan, and glycosaminoglycans. Several pro-inflammatory cytokines, notably TNF-α and IL-6, cause collagenolysis and deterioration of the skin's connective tissue [26]. The anti-inflammatory phytoconstituents of Nishamalaki like curcumin, tannins, and polyphenols inhibit the production of IL-6 and TNF-α, thereby decreasing inflammation. *Phyllanthus emblica* is known to elevate the mitochondrial activity of human skin fibroblasts and promote procollagen production [27]. *Curcuma longa* has been shown to improve the elasticity, and hydration properties of the aging skin may be due to the synergistic antioxidant, anti-inflammatory, and photo-protective properties of its constituents.

Every wound undergoes scar remodeling over several weeks to months. The scar that forms gradually contracts centrally over the wound through the myofibroblast's action, increasing wound contraction by inducing the α-smooth muscle actin expression in the granulation tissue [28]. Contraction pulls normal surrounding skin over the wounded one. The curcuminoids of Nishamalaki are known to increase wound contraction by increasing the proliferation of myofibroblasts, thus, accelerating wound healing [29]. Gallic acid (GA), a potent polyphenolic acid from amla, may improve wound healing by increasing fibroblast cells, reducing inflammatory cell infiltration, and inducing TGF-β expressions as per published reports [30]. GA is also reported to activate enzymes essential for wound healing, such as focal adhesion kinases (FAK), c-Jun N-terminal kinases (JNK), and extracellular signal-regulated kinases (Erk) [31]. Tannins of Nishamalaki may enable fibroblast proliferation and their migration into wounds, promote wound shrinkage, and improve the healing of chronic wounds due to their antibacterial activities and cell pro-proliferative effect at the wound site [32]. The Nishamalaki treatment, thus, exhibited an increase in wound contraction, indicating its ability to increase the degree of epithelialization and tissue strength.

As the worldwide population continues to age, the number of older persons with diabetes mellitus increases. When blood glucose remains permanently high, it impairs white blood cells from functioning correctly, and the body is less able to fight bacteria and close wounds [3]. Also, uncontrolled diabetes may lead to poor circulation and difficulty delivering nutrients to damages [31]. As a result, the injuries heal slowly or may not heal at all. In the cutaneous wound healing process, fibroblasts migrate, proliferate, and take the form of newer cells that break down fibrin clots and create new extracellular matrix and collagen structures to support other cells associated with effective wound healing. However, these effects are impaired in patients with diabetes. GA has a migratory impact on keratinocytes and fibroblasts in normal and diabetic conditions to aid wound healing [31].

It has been well-established that reasonable blood glucose control prevents the development of microangiopathy, including neuropathy, which is implicated in the diabetes mellitus-related delay in healing wounds [33]. Curcuminoids and flavonoids of Nishamalaki may be responsible for its anti-hyperglycemic effect through multiple actions like increasing insulin sensitivity, increasing glucose uptake, decreasing α-glucosidase activity, decreasing amylase activity, increasing insulin release, and reducing oxidative stress and inflammation [34]. Evidence exists about GA upregulating PPARγ expression and activating Akt to increase β-cell mass and survival, and improve glucose tolerance in mice, showing proof of anti-hyperglycemic activity [35]. The Nisha

treatments, thus, controlled the aging-elevated blood glucose levels to promote wound healing.

Collagen is a major component of extracellular tissue mainly composed of hydroxyproline, the amino acid that strengthens and supports the skin. Hydroxyproline is also involved in scar formation during the healing of connective tissue [36]. Measurement of the hydroxyproline content has been used as an index for collagen turnover. An increase in the concentration of hydroxyproline indicates an increase in collagen synthesis, which leads to improved wound healing with a concurrent increase in the tensile strength of the wounds [36]. The curcuminoids, tannins, and ascorbic acid in Nishamalaki are known to increase collagen synthesis at the wound site, thus stimulating new tissue growth and promoting debridement and angiogenesis re-epithelialization in the wound bed, all conducive to healing [37].

Hexosamines are amino sugars of hexoses that act as building blocks for glycosaminoglycans. These chain-like disaccharides connect two collagen fibers to form the collagen-glycosaminoglycan matrix on which collagen can be laid down during the early stages of wound healing. Accumulation of glycosaminoglycans is necessary for granulation tissue formation and wound closure [38]. In our study, the hexosamine content was significantly increased by the Nishamalaki treatments compared to the Aged Control group, indicating the stabilization of collagen fibers and thus an enhancement of the wound healing process. Curcumin and ellagitannins of Nishamalaki may be responsible for this effect.

Ascorbic acid is a co-factor in the hydroxylation of proline and lysine, which is necessary for collagen formation. It helps in cross-linking collagen molecules to enhance their strength, which increases the healing of wounds [39]. Ascorbic acid also increases collagen synthesis, enhances neutrophil migration at the wound site, and increases angiogenesis, leading to fast healing effects [40]. Ascorbic acid is also an antioxidant that possesses potent free radical scavenging activity, inhibits lipid peroxidation, and reduces damage from inflammation and oxidation. Compared to the Aged Control group of rats, the Nisha-treated rats showed a significant increase in the ascorbic acid content. Amla being a rich source of ascorbic acid (Vitamin C), is responsible for the treatment group increase in ascorbic acid content.

Platelet-derived growth factor (PDGF) is a cytokine present in platelets and released at the instance of platelet activation at sites of injury. PDGF was the first growth factor shown to be chemotactic for cells migrating into the healing wound, such as neutrophils, monocytes, macrophages, and fibroblasts. PDGF appears to carry out its signaling through wound macrophages and is known to increase the rate of formation of granulation tissue and blood vessel maturation, which increases angiogenesis, cell proliferation, and collagen synthesis within the healing wounds [41]. PDGF administration has been reported to have contributed to increased wound tissue antioxidant capacity [42]. It is also known to play a role in maintaining the oxidative balance in normal metabolic processes [42]. Aged Control rats exhibited lower PDGF levels than the Young Control rats due to an accelerated dwindling of growth factors with aging. The Nishamalaki constituents, which are potent antioxidants, supported PDGF and its wound-healing process in this study by a mechanism that needs to be investigated.

A master kinase known as the mammalian target of rapamycin, or mTOR, is required for the physiological processes of the cell, including protein synthesis and cell growth in response to growth hormones, nutrition, energy, and stress. The mTOR pathway is the critical step in regulating epithelial cell growth and proliferation, re-epithelialization, collagen synthesis, extracellular matrix deposition, and neovascularization [43]. Therefore, activation of this pathway in wounds contributes to skin tissue repair by accelerating wound contraction rate. Thus, mTOR expression is essential for the progression of the wound-healing process. Though mTOR expression was promoted by Nisha extracts, whether curcumin and other antioxidants had a role in it needs to be investigated because the interplay between oxidative stress and mTOR signaling is still not understood.

Adenosine monophosphate-activated protein kinase (AMPK), the fundamental regulator of cellular energy homeostasis, is a key mediator in aging and wound healing. AMPK activation regulates inflammatory responses either by increasing the release of anti-inflammatory cytokines and decreasing the release of pro-inflammatory cytokines or by increasing macrophage phagocytosis [44]. Wounds are characterized by tissue injury and bacterial infection, leading to long-term inflammation in the aged population. The increased inflammation hinders angiogenesis, inhibiting the wound from entering the proliferation phase. AMPK activators are reported to alleviate tissue inflammation and promote re-epithelialization in wounds to accelerate healing [45]. In the present study, Aged Control rats showed a significant decrease in AMPK levels, probably due to inhibited AMPK signals and increased inflammation with aging. Curcumin and polyphenols are known to upregulate AMPK and its signaling and thus exert a beneficial role in alleviating tissue inflammation and improving the wound healing process by enhancing re-epithelialization in the aged skin [27].

The effects of PGDF, mTOR, and AMPK in chronic wounds on aged skin have been investigated for the first time. The outcomes have been novel and promising and require further investigation to understand the interplay between them and endogenous antioxidants. Interleukin 6 (IL-6) is a pro-inflammatory cytokine secreted to infection and tissue injury leading to inflammation. It is one of the main signaling pathways implicated in aging and inflammation. Tumor necrosis factor-α (TNF-α) is a cytokine, a cell-signaling protein that acts as a central regulator of inflammation. Different cell types produce it, the most important sources being macrophages and monocytes at inflammatory sites. In the early wound, the polymorphonuclear leukocytes released immediately after injury appear to be the source of these cytokines [46]. Their expression is upregulated during inflammation, particularly in the inflammatory phase of the wound healing process—increased TNF-alpha levels are responsible for delays in wound healing [47]. The anti-inflammatory phytoconstituents of Nishamalaki like curcumin, tannins, and GA inhibit the production of IL-6 and TNF-α, thereby decreasing inflammation and increasing re-epithelialization to enhance wound healing.

ROS generated continuously in the metabolic processes of the body are implicated as culprits in bringing about extracellular matrix degradation and cumulative skin damage, employing oxidative stress induction to cells of the skin [48]. This process of skin damage is accelerated in the aging skin by excessive ROS produced during aging. Wounds provide favorable conditions for ROS formation from leukocytes activated during injury and inflammation. As more leukocytes are attracted to the wound, the process of production of ROS is intensified, and untoward structural changes occur that contribute to reversible or irreversible cell injury due to the overpowering of the natural defense systems by the ROS. ROS mainly react with polyunsaturated fatty acids of the membrane to oxidize them and cause lipid peroxidation (LPO), leading to the membrane's structural and functional compromise. The total amount of MDA formed by LPO is used as an index to determine lipid peroxidation.

GSH, the frontline non-enzymatic tripeptide, and the enzymes SOD and CAT present a formidable antioxidant defense against oxidative injury inflicted by ROS in the body [49]. This trio, along with other GSH-dependent enzymes, efficiently scavenges ROS such as superoxide anions, alkoxy radicals, and H_2O_2, and decomposes O_2 and H_2O_2 before they form the very harmful hydroxyl radical. In this study, the endogenous antioxidants depleted due to natural diminishing on aging and excessive ROS generation due to the injury were restored significantly by Nishamalaki treatments by their powerful antioxidant phytoconstituents, viz. curcumin, and curcuminoids, ascorbic acid, gallotannins and polyphenols such as GA. Their anti-lipid peroxidative effect and presumably the high reactivity of the OH group of the phenolic components are responsible for this free radical scavenging activity. GA is reported to increase the expression of the antioxidant enzymes CAT, SOD, and glutathione peroxidase, thus, having beneficial effects on wound healing and skin aging [31]. Since AMPK activation plays a role in redox homeostasis and increases gene expression of antioxidant enzymes, AMPK activation by GA might positively influence, at least in part, the antioxidant effect of GA [31]. In the histopathological study of the wounded skin (bed), the aged control animals showed more skin ulcerations covered by neutrophilic necrotic exudate. Their skin dermis showed hair follicles and minimal granulation tissue loss with fewer collagen fibers than the young control animals. The Nishamalaki and reference standard treatments significantly reversed these changes, the histological studies thus corroborating the biochemical findings.

Conclusion

The Nishamalaki oral and combination treatment of oral and topical gel exhibited significant wound healing effects in the excision wound produced in aging rats. The combination treatment displayed a better wound-healing effect than the oral treatment alone, indicating that optimal wound healing occurs when local factors and systemic factors influencing wound healing are addressed adequately. The curcuminoids, gallic acid, polyphenols, and ellagitannins of Nishamalaki are responsible for this effect. The age-old formulation of Nishamalaki has been successfully used to treat diabetes in Ayurveda. With the present work, Nishamalaki's use may be extended to chronic and slow-healing cutaneous wounds of aging people with proper clinical studies. A future extension is to evaluate Nishamalaki's healing effect on diabetic wounds, especially in the elderly.

Abbreviations

T2DM	Type 2 Diabetes Mellitus
VEGF	Vascular endothelial growth factor
PDGF	Platelet-derived growth factor
AMPK	AMP-activated protein kinase
mTOR	Mammalian target of rapamycin
5,5′	Dithiobis (2-nitrobenzoic acid)- DTNB
1,1	Iphenyl-2-picrylhydrazyl- DPPH
LPO	Lipid peroxidation
MDA	Malondialdehyde
SOD	Superoxide dismutase
CAT	Catalase
GSH	Reduced glutathione
GA	Gallic acid
TNF-α	Tumor Necrosis Factor -α
IL-6	Interleukin-6
ROS	Reactive Oxygen Species

Supplementary Information

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Additional file 1.

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Authors' contributions
LH and AD conceived the project and revised the MS. VP designed and supervised the study and wrote the MS. LV performed the study SS performed the histopathological studies.

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Availability of data and materials
The dataset analyzed during the current study is available from the corresponding author on request.

Declarations

Ethic approval and consent to participate
The protocol for animal studies was approved by the Institutional Animal Ethics Committee (Animal House Registration No.25/1999/CPCSEA) which conforms to the national guidelines for the use and care of experimental animals in research.

Consent for publication
Not applicable.

Competing interest
The authors declare that they have no competing interests.

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