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ORIGINAL ARTICLE

Wound Healing Potential of *Couroupita guianensis* Aubl. Fruit Pulp Investigated on Excision Wound Model

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Abstract

Wound care management aims at stimulating and improving healing process without scar formation. Although various plants have been reported to possess wound healing properties in tribal and folklore medicines, there is a lack of scientific data to validate the claim. In this aspect, it becomes inevitable to prove the efficacy of naturally derived products at pharmacological levels. *Couroupita guianensis* as a whole plant has been reported to exhibit wound healing activity. The leaves and fruit of this plant have been utilized in folkloric medicine to cure skin diseases and infections for many years. However, to the best of our knowledge, no scientific studies have been conducted to verify the wound healing properties of *C. guianensis* fruit pulp. Therefore, the present study seeks to investigate the wound healing potential of *C. guianensis* fruit pulp using an excision wound model in Wistar albino male rats. This study indicated that the ointment prepared from crude ethanolic extract of *C. guianensis* fruit pulp facilitated wound contraction that were evidenced by a greater reduction in the wound area and epithelialization period and increased hydroxyproline content. The experimental groups treated with low and mid dose of *C. guianensis* ethanol extract (CGEE) ointments had shown a wound closure of 80.27% and 89.11% respectively within 15 days, which is comparable to the standard betadine ointment which showed 91.44% healing in the treated groups. Further, the extract influenced the expression of genes VEGF and TGF-β on post wounding days that clearly explained the strong correlation between these genes and wound healing in the experimental rats. The animals treated with 10% CGEE ointment showed a significant upregulation of both VEGF and TGF-β as compared with other test and standard groups. These findings provide credence to the conventional application of this plant in the healing of wounds and other dermatological conditions, and may represent a therapeutic strategy for the treatment of wounds.

Keywords *Couroupita guianensis* · Wound healing · Epithelization period · Wound contraction · VEGF · TGF-β

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Introduction

In the course of an organism's life, wounds are unavoidable, yet occasionally they can be dangerous or even fatal. A wound is identified by a break in the epithelium's continuity, which disrupts or damages the structure and defenses of the skin [1]. The wounds can be characterized in a variety of various ways. In many cases, the wound will be made up of a variety of different types. The classification of the wounds takes into account factors like thickness, complexity, etiology, physiology of wound healing, levels or stages of damage, and contamination levels. Acute wounds heal in a short period of time, typically between 5 and 10 days or within 30 days, restoring the integrity of the skin, whereas chronic wounds fail to go through reparative processes in a timely and controlled manner, resulting in a pathological state. It is characterized by a delayed healing process that takes at least 12 weeks to complete [2].

Wound healing is a biological survival process initiated at the onset of tissue injury by which the skin restores itself normally. It is a complicated and dynamic process which involves the collaborative effect of cells including epithelial cells, endothelial cells, inflammatory cells, fibroblasts, macrophages and platelets, cytokines, and extracellular matrix for the proper restoration of anatomical continuity and functionality of the skin. The healing process of acute skin wound is arbitrarily divided into different and at the same time overlapping time-dependent phases. They include coagulation and hemostasis, inflammation, proliferation or granulation, and remodeling or maturation [3].

Wound management entails establishing a conducive healing environment through various approaches and preventing skin disintegration. Certain factors such as location, size, depth, and severity of wound have to be considered for appropriate management [4]. Earlier wound care involves addressing the underlying cause and then either allowing the body to heal itself or using skin grafts to replace any tissue that is lost during the healing process. While nowadays, with advancement of regenerative medicine together with better understanding of cellular and biological mechanisms enhancing tissue repair, several new technologies have emerged that aim to promote wound healing with minimal scarring [5]. However, majority of people across the world rely on traditional therapy utilizing plant and plant-derived products because of safety, reliability, and cost-effectiveness.

The ability of human skin to promote its own self-healing property after injury is constrained by a number of diseases, including pathological illnesses, deep burns, non-healing ulcers, substantial tissue loss, and chronic wounds. For a long time, many nations have relied on conventional medicines to help with tissue repair. Utilizing living organisms, silver, and other conventional dressings, traditional treatments include best practices and knowledge from around the world [6].

Plants and plant-derived compounds are commonly used as natural remedies for wound. They are utilized as extracts, ointments, creams, emulsions, and traditional dressings. They are administered through topical, oral, or systemic route. A great number of plants from different regions across the world have been validated scientifically to possess wound healing activity. Either whole plants or their parts (leaf, root stem, flowers, and seeds) are usually used in wound therapy.

The current high demand for plant-based products has prompted the pharmaceutical industries to concentrate on using plants and their products in the preparation of drugs. *Couroupita guianensis* Aubl. (common name: cannonball) as a whole plant has been reported to exhibit wound healing activity. For many years, folkloric medicine has used the leaves and fruit of this plant to treat skin diseases and infections. Native Americans used

all parts of the tree to treat a wide range of human illnesses. The leaves, bark, and flowers are used to treat microbial infections, malaria, scabies, skin diseases, allergies, inflammation, bleeding, piles, ulcers, dysentery, and other stomach problems, toothaches, and kidney problems [7].

Indigenous people use fruit pulp to treat wounds, wherein no scientific studies have been conducted to verify the wound healing properties of *C. guianensis* fruit pulp. Therefore, the current study seeks to ascertain the wound healing potential of *C. guianensis* fruit pulp employing an excision wound model in Wistar albino male rats.

Materials and Methods

Chemicals and Reagents

All chemicals and reagents were of analytical grade. The solvents used for extraction was from MERCK and fine chemicals for the reagent preparation for the experimental study were purchased from SRL laboratories.

Sample Collection

Fresh fruits of *C. guianensis* were collected from the Ayurveda Research Centre Kerala during the months of October and November, 2019. They were authenticated by Prof. Dr. Jayaraman, Institute of Herbal Science, Plant Anatomy Research Center, Chennai, Tamil Nadu, India, with certificate number PARC /2018/3859.

Preparation of Crude Extracts of C. guianensis

To extract bioactive components, scientists often employ dried powdered plants. In order to extract phytochemicals, a variety of solvents with different polarities must be used, and the phytochemicals will dissolve properly in a solvent with polarity that are identical to them [8].

Following a review of the literature, the solvents petroleum ether, chloroform, ethanol, and hydroalcohol were selected for the extraction of *C. guianensis* fruit pulp. Fruits devoid of fungus were chosen. Fruit pulp was extracted, air-dried, crushed, and stored. The powdered sample was subsequently processed using the hot percolation procedure. To obtain *C. guianensis* ethanol extract (CGEE), the sample powder (1 kg) was extracted using a Soxhlet device with ethanol. The obtained extract was then subjected to a rotary evaporator and the final extracts obtained were stored at 4 °C for further use [9].

Preparation of Test Samples

The crude extract (CGEE) was reconstituted in a simple ointment base material (polyethylene glycol) as a carrier and made into an ointment comprising various quantities (2.5%, 5%, and 10%) of test samples for topical administration. Polyethylene glycol is a water-soluble greaseless base material that can spread evenly in the applied area and more suitable in tropical and subtropical climates [10, 11].

Experimental Grouping of Animals

For the present study, healthy male Wistar albino rats, 6–8 weeks of age and weighing 150–180 g, were procured from the Central Animal House Facility of King's Institute, Chennai, India. They were maintained under standard housing conditions and were given standard diet (Biogen Foods, India) and water, ad libitum. After receiving approval from the Institute's Animal Ethics Committee, all animal experiments were carried out in accordance with NIH protocols. The animals were grouped into six groups of six animals each, where group I served as the untreated group (negative control), group II received topical application of polyethylene glycol (vehicle control), group III received topical application of standard betadine ointment (standard control), and group IV, group V, and group VI animals were treated with topical application of ointment formulated with CGEE at dose of 2.5%, 5%, and 10%, respectively.

Wound Healing Activity

The wound healing activity of CGEE was determined as described by Morton and Malone [12]. The animals in each group were anesthetized using the open mask approach. The rats were depilated on their dorsal sides. The anticipated area of the wound was marked on the shaved skin. The wound area of 2-cm diameter of skin was removed from the marked region using a toothed forceps, surgical blades, and pointed scissors, and the wound was left uncovered. All the procedures were carried out under aseptic conditions. The ointments were applied to the wounds in each group once a day until the wound healed completely, for about 21 days. The size of the wound area was measured (in cm^2) from the initial day of wounding that is on days 0, 5, 10, 15, and 20 and the average reading was considered as wound area. The wound area measurement in turn provides details regarding the contraction of wound. Hence, following wound area measurement, % of wound contraction was calculated. Further epithelialization period was also noted. After complete wound healing, animals were sacrificed and the newly formed regenerated tissues were harvested for biochemical and histological studies.

Measurement of Wound Healing Parameters

Measurement of Physical Parameters.

(a) Wound area measurement

The wound area in all the groups was measured following a planimetrical method with a transparent graph paper on days 0, 5, 10, 15, and 20.

(b) Determination of % of wound contraction

The percentage of wound contraction is a measure of change in initial wound area and was calculated as follows [13]:

$$\%\ \text{Wound contraction} = \frac{\textit{Initial wound area}\ \left(cm^2\right) - \textit{Wound area on day}\ \left(cm^2\right) \times 100}{\textit{Initial wound area}\ \left(cm^2\right)}$$

(iii) Epithelialization period

The epithelization period was determined by monitoring the number of days required for the shedding of eschar tissue from the wound surface without leaving any trace of raw wound behind [14].

Determination of Biochemical Parameters

Newly formed tissues from all the groups were collected in ice-cold petridish separately and were washed in normal saline to remove blood. A part of the collected tissues was cut into pieces. 0.15 M KCl solution containing 5 mM EDTA was chilled and added to the chopped tissues. The mixture was homogenated in a homogenizer to obtain a 10% homogenate. The homogenate was then centrifuged and the aliquots were used for lipid peroxidation analysis [15]. The remaining homogenate was mixed with Triton X-100 and kept at 4 °C for 2 h. The supernatant was used for enzymatic analysis such as catalase [16].

Another part of collected tissues was dried at 60 °C and used for the analysis of hexosamine using Ehrlich's reagent [17], hydroxyproline using Chloramine T reagent [18], and nitric oxide by Griess assay [18] after subjecting the dried tissues to acid hydrolysis.

Histological Evaluation

The excised skin tissue specimens were preserved in 10% formalin, dehydrated using increasing grades of alcohol (30–100%), washed in xylene, and finally fixed in paraffin wax. For histological studies, 0.5-µm-thick tissue sections prepared using a microtome were stained with hematoxylin and eosin and examined under a light microscope.

Gene Expression Studies

The gene expression studies of VEGF and TGF-β genes were analyzed by real-time reverse transcription PCR (qRT-PCR). The relative expression of VEGF and TGF-β genes in test and control groups was studied using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as a housekeeping gene [19]. The tissue collection and RNA extraction were described briefly as follows:

Tissue Collection

The wound and an approximately 1 mm of surrounding skin were harvested from control, standard, and treated rats on 10^{th} and 20^{th} post wounding days. The collected tissues were kept frozen at a temperature of − 80 °C until they were used for RNA extraction.

RNA Extraction

RNA extraction was done using FavorPrep™ Tissue Total RNA Mini Kit (Favorgen, Taiwan). All the extracted RNA was examined for the quality and quantity using a spectrophotometer. For the determination of total RNA quantity, A260/A280 was utilized.

RT-PCR analysis of mRNA expression of vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was assessed using primers. cDNA from each sample was synthesized using MultiScribe Reverse Transcriptase (Thermo Fisher Scientific, USA) as per the manufacturer's protocol. Relative expression was then analyzed using cDNA of specific targets (VEGF and TGF) and control (GAPDH) gene using QuantiNova SYBR Green PCR Kits (Qiagen, Germany). A total of 72 reactions were carried out. Each reaction was carried out in triplicate and the specificity of the PCR products was confirmed by agarose gel electrophoresis and melting curve analysis. The electrophoresis was carried out on 1.5% ethidium bromide–stained agarose gel at 100 V using 1 × Tris–acetate acid-EDTA (TAE) buffer (pH 8.0). Finally, the gel image was seen using the UV transilluminator. The expression of each gene was standardized to GAPDH expression and the change in gene expression, indicated as fold-change, was determined using the $2^{-\Delta\Delta Ct}$ method or Livak method.

Statistical Analysis

All analyses were performed in triplicate, and the results were reported as mean ± standard deviation. One-way ANOVA using SPSS was used to assess significant differences among the groups, followed by the Duncan's multiple range test. Values equivalent to $p < 0.05$ were considered to be statistically significant.

Results

Using an excision wound model, the ethanolic extract of *C. guianensis* fruit pulp was investigated for its ability to promote wound healing. The ethanolic extract of *C. guianensis* fruit pulp was formulated into ointment in three doses (2.5%, 5%, and 10%) using PEG. Wistar albino rats were used as a test subject for its ease to undergo surgical procedure and to subsequent measurement of wound healing parameters.

Evaluation of In Vivo *Wound Healing Activity of CGEE Using an Excision Wound Model*

(a) Effect of CGEE on body weight of rat models during post wounding days

The body weight of the rats was monitored and recorded every 5 days until the period of complete wound healing. The results are given in Table 1. It was observed that the body weight of the rats in the control and treatment groups increased constantly with neither weight loss nor extensive weight gain. Group VI animals did not show any significant change in body weight from day 10 to day 20. A similar result was observed with group II, III, and IV animals from days 5 to 10, days 5 to 15, and days 10 to 15, respectively.

Table 1 Effect of CGEE on the body weight of Wistar albino rates during post wounding days

Body weight (g)						
Day	Group I (NC)	Group II (VC)	Group III (SC)	Group IV (LD)	Group V (MD)	Group VI (HD)
0	145.50 ± 3.78^{a}	151.83 ± 2.15^{a}	151.50 ± 3.53^{a}	153.83 ± 2.76^{a}	155.51 ± 3.67^{a}	157.16 ± 2.76^{a}
5	147.16 ± 2.84^{a}	157.50 ± 1.94^{ab}	154.83 ± 3.18^{ab}	158.83 ± 3.35^{a}	161.00 ± 3.61^{ab}	161.16 ± 2.60^{a}
10	150.83 ± 1.95^{ab}	159.66 ± 2.59^{ab}	158.50 ± 3.29^{ab}	170.50 ± 2.80^{b}	166.00 ± 2.90^{bc}	169.00 ± 1.86^{b}
15	157.33 ± 2.61^{b}	161.66 ± 3.87^{b}	162.00 ± 3.68^{ab}	173.83 ± 2.44^{bc}	169.67 ± 2.12^{bc}	171.00 ± 2.85^{b}
20	159.00 ± 2.80^{b}	165.83 ± 2.52^{b}	164.16 ± 3.55^{b}	179.50 ± 2.64^{c}	175.17 ± 2.60^{c}	175.50 ± 2.53^{b}

Values are expressed as mean ± SD ($n = 6$). Statistical means followed by the same letters are statistically not significant at $p < 0.05$ by Duncan's multiple range tests. The data were analyzed using one-way ANOVA and means were compared using DMRT

NC negative control, *VC* vehicle control (PEG), *SC* standard control (betadine), *LD* low dose (2.5% CGEE), *MD* mid dose (5% CGEE), *HD* high dose (10% CGEE)

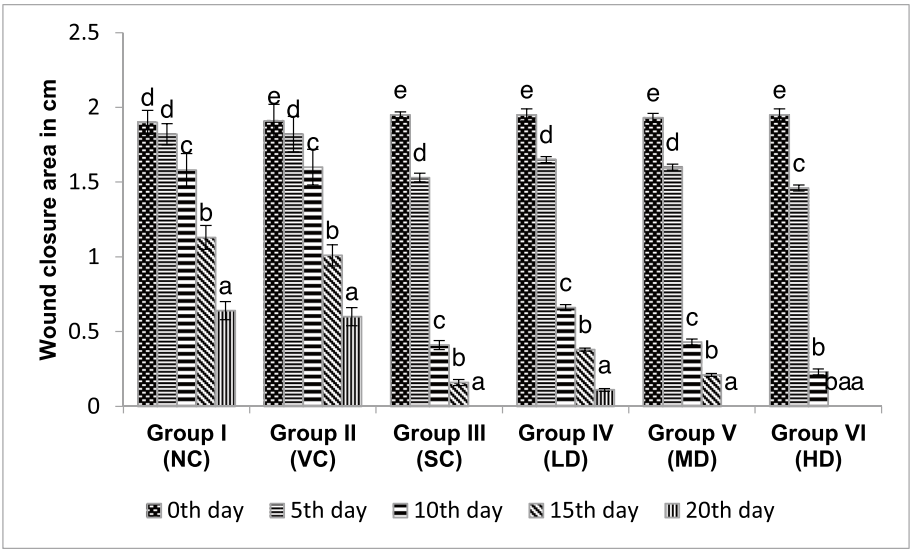


Fig. 1 Effect of CGEE on wound closure in excision wound rat model. Values are expressed as mean ± SD ($n = 6$). Statistical means followed by the same letters are statistically not significant at $p < 0.05$ by Duncan's multiple range tests. The data were analyzed using one-way ANOVA and means were compared using DMRT. NC, negative control; VC, vehicle control (PEG); SC, standard control (betadine); LD, low dose (2.5% CGEE); MD, mid dose (5% CGEE); HD, high dose (10% CGEE)

(b) Effect of CGEE on wound contraction and epithelialization

The ethanolic extracts of the fruit pulp of the plant *C. guianensis* in three doses were evaluated to analyze in vivo wound healing efficacy by the excision method ($n = 6$). The topical application of CGEE ointments at low dose (2.5% w/w), mid dose (5% w/w), and high dose (10% w/w) observed an enhanced statistically significant ($p < 0.05$) wound

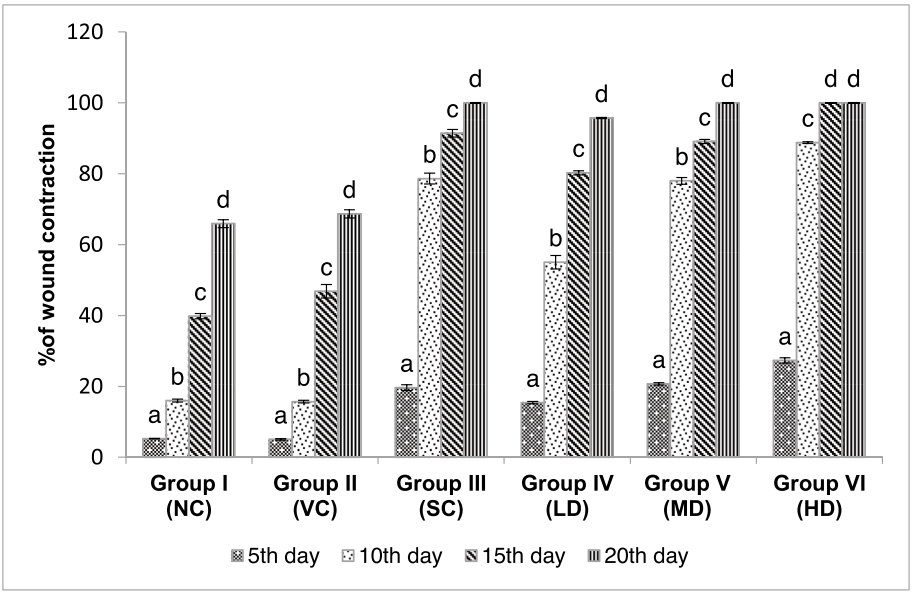


Fig. 2 Graphical representation of effects of different doses of CGEE ointment and standard ointment on percent wound contraction in an excision wound rat model. Values are expressed as mean ± SD ($n = 6$). Statistical means followed by the same letters are statistically not significant at $p < 0.05$ by Duncan's multiple range tests. The data were analyzed using one-way ANOVA and means were compared using DMRT. NC, negative control; VC, vehicle control (PEG); SC, standard control (betadine); LD, low dose (2.5% CGEE); MD, mid dose (5% CGEE); HD, high dose (10% CGEE)

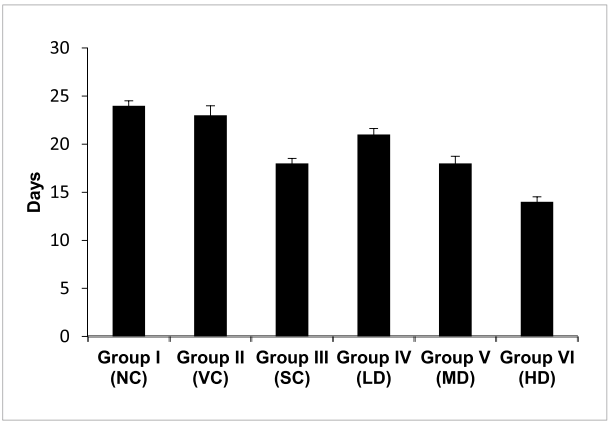


Fig. 3 Effect of CGEE on the period of epithelialization in an excision wound rat model. NC, negative control; VC, vehicle control (PEG); SC, standard control (betadine); LD, low dose (2.5% CGEE); MD, mid dose (5% CGEE); HD, high dose (10% CGEE)

healing activity in terms of wound closure area (Fig. 1) and percentage of wound contraction (Fig. 2) from post wounding days 0 to 20. However, group VI treated with high dose showed a statistically significant difference in area of wound closure as compared to other groups. The results clearly demonstrated that complete wound healing was observable in groups treated with 10% CGEE ointment within 15 days, while it required more than 20 days for the negative and vehicle control groups (Fig. 3). On the other hand, groups

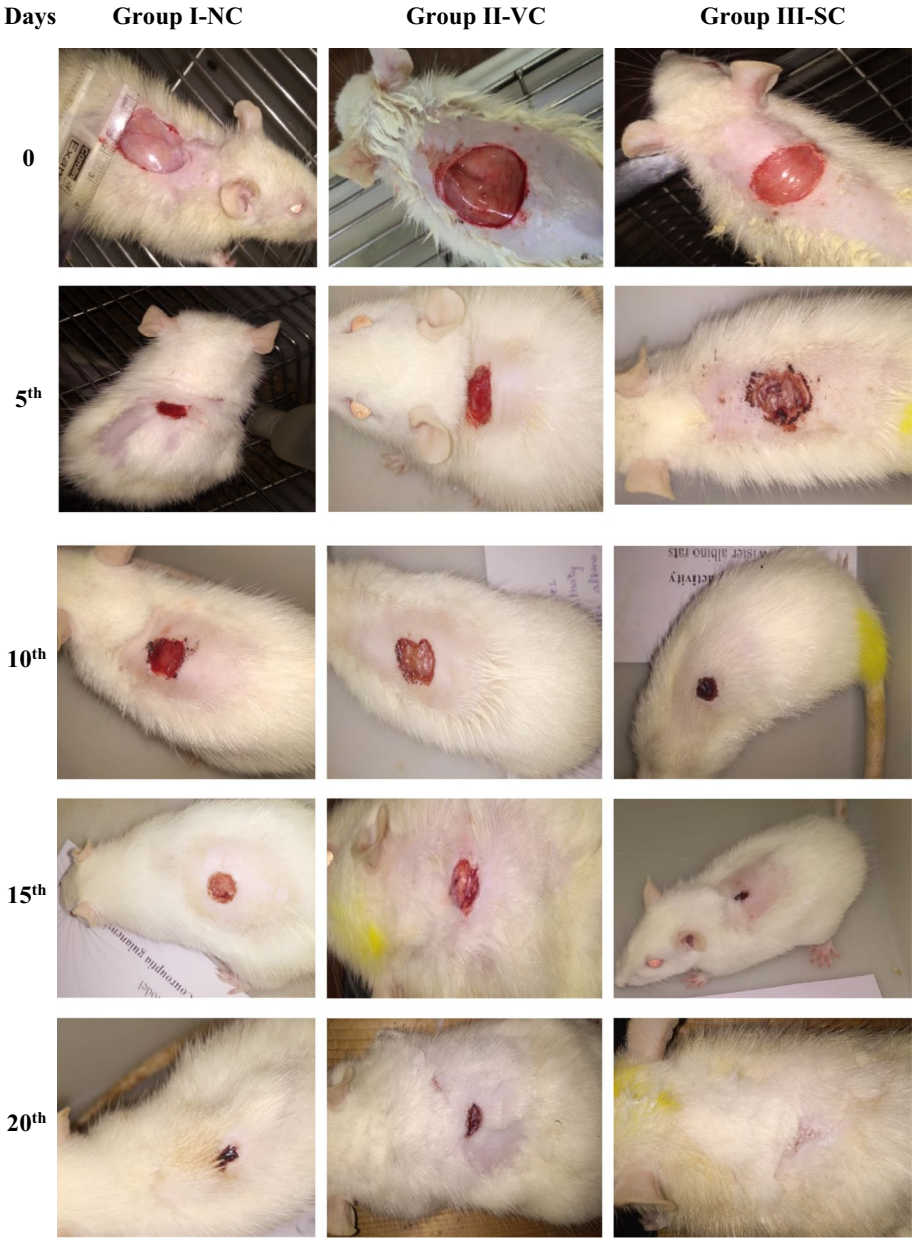


Fig. 4 Photographic images of wound healing of negative, vehicle, and standard control groups in an excision wound model

treated with low and mid dose of CGEE ointments had shown a wound closure of 80.27% and 89.11% respectively within 15 days, whereas standard betadine ointment had shown 91.44% healing in the treated groups. Complete wound healing was accomplished within

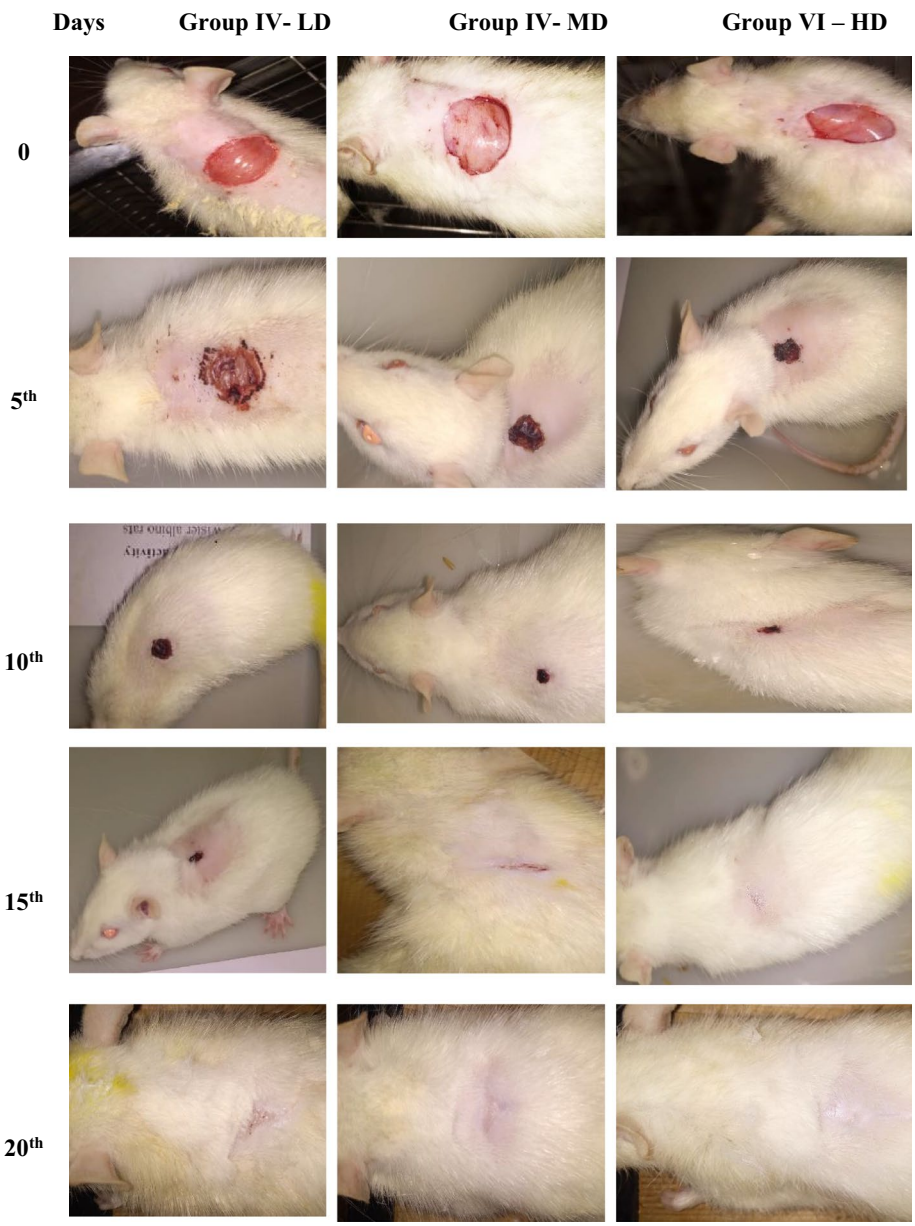


Fig. 5 Photographic images of wound healing efficacy of CGEE in an excision wound model

20 days for the groups treated with standard betadine, and low and mid dose of CGEE ointments (Figs. 4 and 5).

Furthermore, on comparing the epithelialization period for test and control groups, epithelialization period (Fig. 3) was significantly reduced in groups applied with high dose of

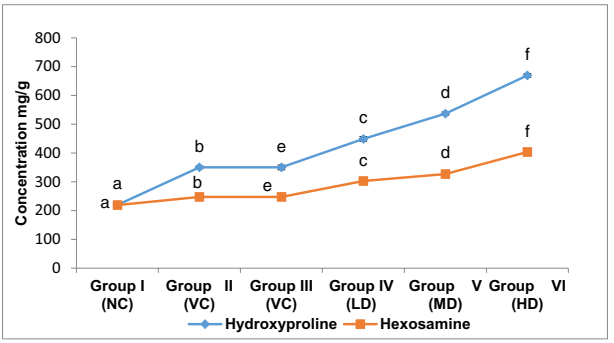


Fig. 6 Graphical representation of the effect of different doses of CGEE ointment and standard ointment on various wound tissue parameters of an excision wound model in rats. Values are expressed as mean ± SD ($n = 6$). Statistical means followed by the same letters are statistically not significant at $p < 0.05$ by Duncan's multiple range tests. The data were analyzed using one-way ANOVA and means were compared using DMRT. NC, negative control; VC, vehicle control (PEG); SC, standard control (betadine); LD, low dose (2.5% CGEE); MD, mid dose (5% CGEE); HD, high dose (10% CGEE)

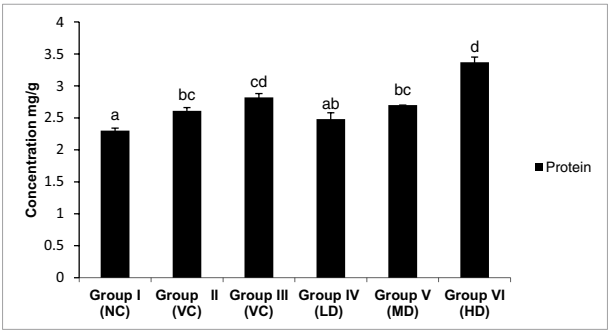


Fig. 7 Graphical representation of the effect of different doses of CGEE ointment and standard ointment on protein content in the granulation tissue of an excision wound rat model. Values are expressed as mean ± SD ($n = 6$). Statistical means followed by the same letters are statistically not significant at $p < 0.05$ by Duncan's multiple range tests. The data were analyzed using one-way ANOVA and means were compared using DMRT. NC, negative control; VC, vehicle control (PEG); SC, standard control (betadine); LD, low dose (2.5% CGEE); MD, mid dose (5% CGEE); HD, High dose (10% CGEE)

CGEE ointment. No significant difference in epithelialization period was, however, noted in groups I, II, and III.

(iii) Effect of CGEE on tissue biochemical parameters

As pointed out in the obtained results, the levels of hydroxyproline, hexosamine, and protein in granulation tissue from the test and standard groups were higher compared with control groups. Similarly, an appreciable difference in the amount of hydroxyproline, hexosamine, and protein was noted in the groups treated with standard betadine as well as low and mid dose of CGEE ointments. Further, it can be observed that the tissue levels of hydroxyproline and hexosamine (Fig. 6) and protein (Fig. 7) in the test groups increase in a dose-dependent way.

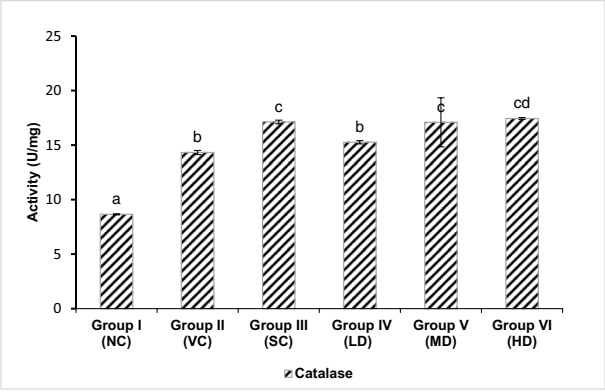


Fig. 8 Graphical representation of the effect of different doses of CGEE ointment and standard ointment on antioxidant enzyme in the granulation tissue. Values are expressed as mean ± SD ($n = 6$). Statistical means followed by the same letters are statistically not significant at $p < 0.05$ by Duncan's multiple range tests. The data were analyzed using one-way ANOVA and means were compared using DMRT. NC, negative control; VC, vehicle control (PEG); SC-standard control (betadine); LD, low dose (2.5% CGEE); MD, mid dose (5% CGEE); HD, high dose (10% CGEE)

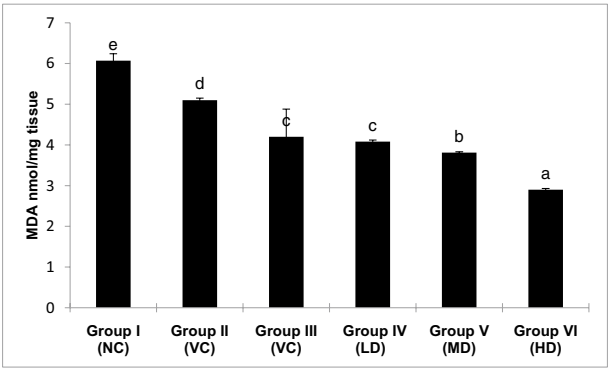


Fig. 9 Graphical representation of the effect of different doses of CGEE ointment and standard ointment on lipid peroxidation in the granulation tissue. Values are expressed as mean ± SD ($n = 6$). Statistical means followed by the same letters are statistically not significant at $p < 0.05$ by Duncan's multiple range tests. The data were analyzed using one-way ANOVA and means were compared using DMRT. NC, negative control; VC, vehicle control (PEG); SC, standard control (betadine); LD, low dose (2.5% CGEE); MD, mid dose (5% CGEE); HD, high dose (10% CGEE)

Effect of CGEE on Free Radicals and Antioxidant

Levels of free radicals including nitric oxide, lipid peroxidation product (MDA), and antioxidants such as catalase were measured in the granulation tissue obtained from control, standard, and test groups and the results are given in Figs. 8, 9, and 10. Among

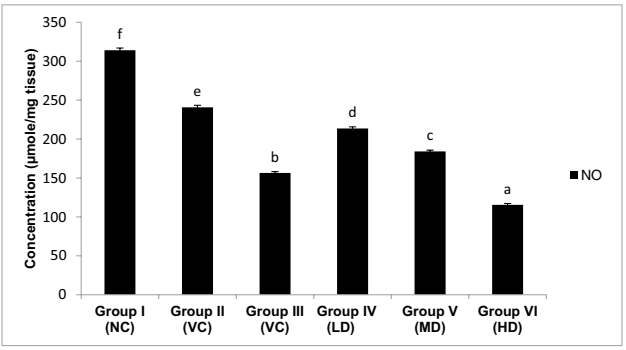


Fig. 10 Graphical representation of the effect of different doses of CGEE ointment and standard ointment on nitric oxide level in the granulation tissue. Values are expressed as mean ± SD ($n = 6$). Statistical means followed by the same letters are statistically not significant at $p < 0.05$ by Duncan's multiple range tests. The data were analyzed using one-way ANOVA and means were compared using DMRT. NC, negative control; VC, vehicle control (PEG); SC, standard control (betadine); LD, low dose (2.5% CGEE); MD, mid dose (5% CGEE); HD, high dose (10% CGEE)

all the groups, the catalase activity appeared to be higher in group VI that received 10% CGEE ointment. On comparing with negative control and vehicle control groups, there was a significantly marked decrease in the values of nitric oxide and lipid peroxidation in all the test groups and standard group. However, no significant difference was noticeable in lipid peroxidation (MDA) between the standard group (4.20 ± 0.68 nmol/mg tissue) and the test group treated with 2.5% CGEE ointment (4.08 ± 0.04 nmol/mg tissue).

Effect of CGEE on Histological Investigations of Healed Tissue

Wound healing is the process of restoring damaged tissues to a normal or near-normal state, which is determined by factors such as the injured tissue's overall health, as well as the nature and degree of injury. The primary mechanisms of wound healing are epithelialization, contraction, and connective tissue deposition. The epidermal area of the untreated (negative control) and vehicle control animal groups showed a high degree of necrosis, as shown in Fig. 11. In the case of the negative control, skin regrowth was observed to be incomplete, with loosely attached collagen fibers. In contrast, no fibrinoid necrosis or inflammation was observed in animals treated with different doses of plant extract. Furthermore, re-epithelialization was complete in all treated groups, with the wound area covered in high fibrous tissue and the tissues surrounding the wound area producing high fibrous content, with collagen fibers arranged parallel to each other to form a highly organized epidermal layer.

Gene Expression Studies

Recognizing expression of VEGF and TGF-β is critical to analyze the wound healing potential of CGEE at the molecular level. Expression studies were performed with RT-qPCR for the tissue of control, standard, and test groups using VEGF and TGF-β primers at 10^{th} and 20^{th} post wounding days. A considerable increase ($p < 0.05$) in the expression of

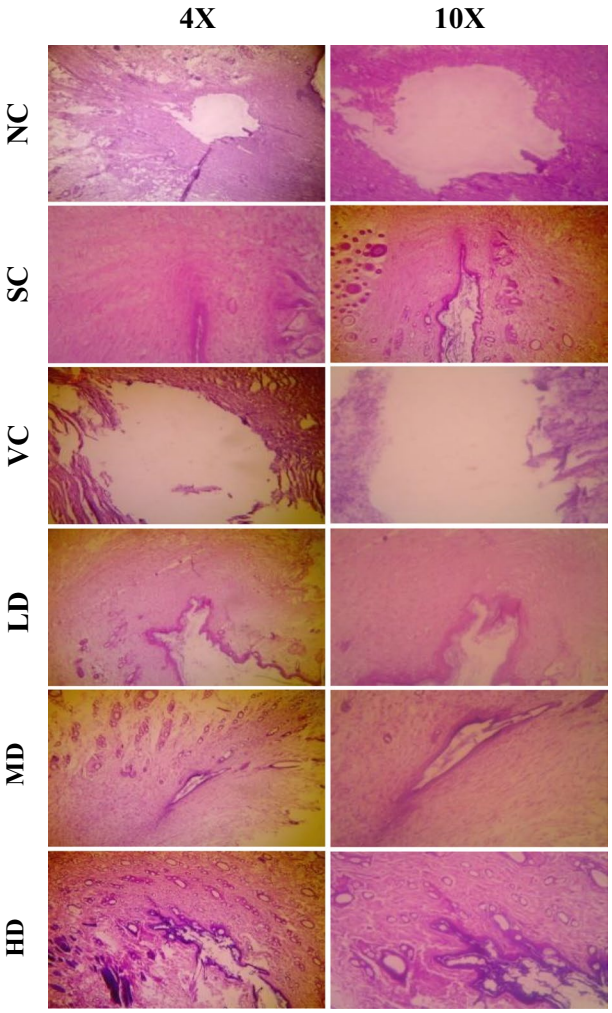


Fig. 11 Effect of different doses of CGEE ointment on histopathological examination of granulation tissue

VEGF and TGF-β in the wound tissue of test group animals at 10^{th} and 20^{th} post wounding days has been noticed as compared with control group animals during the treatment period. The results clearly stated dose-dependent and day-dependent effects of CGEE on VEGF and TGF-β expression (Fig. 12). VEGF mRNA expression of the test and standard groups reached peak at the 10^{th} post wounding day and later on it gets subsided and reached minimum at the 20^{th} post wounding day. A similar such result was observed with TGF-β mRNA expression as well. In addition, animals treated with 10% CGEE ointment showed a significant upregulation of both VEGF and TGF-β as compared with other test and standard groups. Figures 13 and 14 demonstrate the changes in the expression of VEGF and TGF-β in comparison to GAPDH (housekeeping gene). Further, these results have been shown to be correlated with the wound closure and wound tissue parameter measurements.

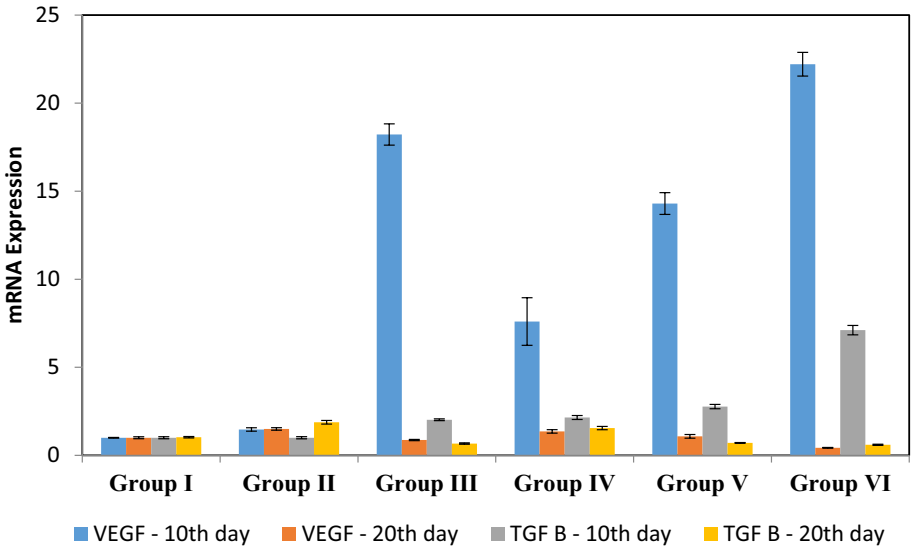
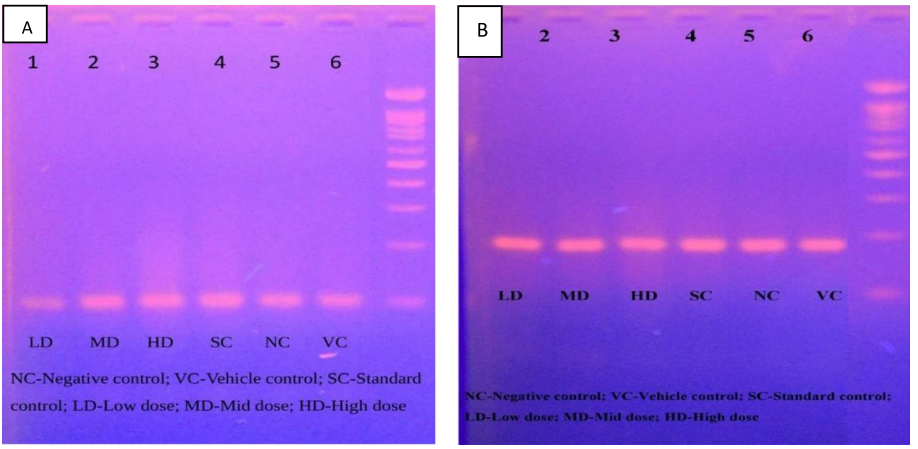


Fig. 12 Relative expression of VEGF and TGF-β at 10th and 20th post wounding days

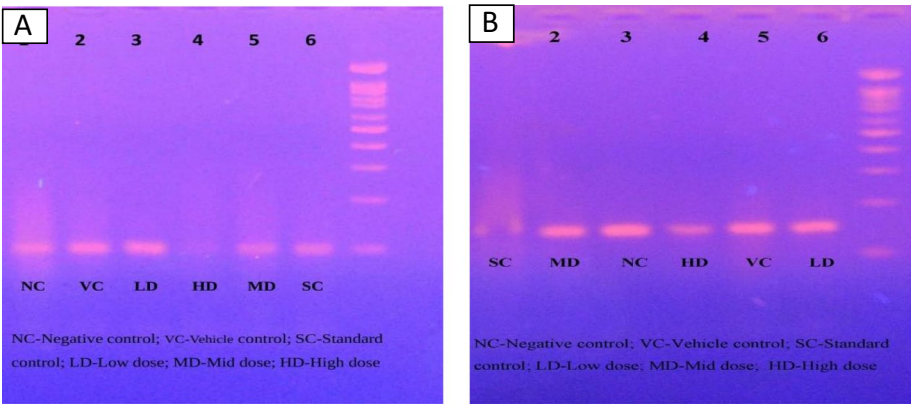


(A) VEGF Expression at the 10th day (B) TGF-β expression at the 10th day

Fig. 13 Agarose gel electrophoresis results of VEGF and TGF-β expression at the 10th day

Discussion

Wounds are considered a major health issue worldwide that implies socio-economic stress on health care organizations, primary health care providers, patients, and their family members [20]. Wound care management aims at stimulating and improving healing process without scar formation. Nowadays, researchers have indulged in manufacturing novel products which will be an amalgamation of traditional and modern techniques. There is a



(A) VEGF Expression at the 20th day

(B) TGF-β expression at the 20th day

Fig. 14 Agarose gel electrophoresis results of VEGF and TGF-β expression at the 20th day

prominent surge in search of newer wound healing agents from nature on the grounds of economy, safety, and availability.

Leaves and fruit pulp of *C. guianensis* have been employed in folklore medicine since ages to cure skin diseases and infections. But there is no direct indication of the use of the plant in wound healing in folklore applications. However, recently, a study has established the efficacy of *C. guianensis* as a whole plant (leaves, fruit, and flower) in healing wound [21]. To explore further, proper validation of individual part of the plant is necessitated. In addition, the fruit of *C. guianensis*, though widely used among tribal people, is less explored scientifically. In this context, the present study was undertaken to validate the wound healing activity of fruit pulp of *C. guianensis* at the pharmacological level.

Skin wound repair mechanisms and pathology can be better understood with different in vivo models that includes excision wound model, incision wound model, burn wound model, and dead space wound model [22]. Excisional wound is a common model for investigating wound healing mechanisms and pathophysiology [23, 24]. This model can be superficial, or partial or full in thickness and illustrates acute wound where the skin is not sutured [25].

The present investigation initiated with CGEE ointment at three doses (2.5%, 5%, and 10%) revealed significant dose-dependent and day-dependent wound healing effects in the excision wound model. This can be supported by a greater reduction in the wound area and epithelialization period on comparing with negative and vehicle control groups. The observation in turn reflects the efficiency of the extract in accelerating the wound closure [26]. In the process of wound healing, injured skin tissue attempts to regain its normal state by undergoing complex events which involve the participation of various cells, growth factors, biochemicals, and proteins. Figure 5 depicts epidermis remodelling, progressive wound contraction, and wound healing facilitated by different concentrations of CGEE ointment. The animals treated with a 2.5% of CGEE ointment developed a dark reddish, dense scab over the wound region, fully covering it. The animals in the groups treated with standard ointment and 5% and 10% CGEE ointment had substantially less inflammation than the other groups. The untreated animals (negative control) and vehicle control group showed

the most necrosis on the first day of the experiment, as evidenced by swollen, bruised, and inflamed structures at the wound site, as compared to the standard ointment and CGEE ointment–treated groups (Fig. 4). A high degree of necrosis was found around the wound edges, with no epidermis layer visible. The negative and vehicle control animals showed considerable inflammation surrounding the wound site on the 10^{th} day of the experiment.

Negative and vehicle control animals reported skin regrowth covering the main areas of the wound on the 20^{th} day of the experiment (Fig. 4). The animals treated with the standard ointment and low (2.5%) and mid (5%) doses of CGEE ointment showed full wound reduction with new skin covering over the original wound region (Fig. 5). The newly formed skin appears very similar to normal skin. The same observation was monitored on the 15^{th} day for the groups treated with high-dose (10%) CGEE ointment (Fig. 5). Furthermore, CGEE ointment–treated animals (low, medium, and high dose) had completely re-established the damaged tissues, as well as a normal dermal region and average hair growth all over the wound area. The presence of complete epithelial development on fibrous tissues has been confirmed.

Collagen is a prominent extracellular protein that plays an important role in wound healing at various stages, particularly during the proliferation and remodelling phases. Soon after an injury, there is an enhanced synthesis of collagen and glycosaminoglycans and glycoproteins such as hexosamine and hexuronic acid by the fibroblast cell in the wound area. Hexosamine and hexuronic acid are matrix molecules capable of forming a new matrix scaffold on which collagen gets deposited, and stabilized. These matrix molecules help to stabilize collagen molecules through electrostatic and ionic interactions [27]. Collagen synthesis can be monitored from their constituent amino acid hydroxyproline measurement and their stabilization can be ensured from tissue hexosamine level [28]. Throughout the course of the healing process, both hydroxyproline and hexosamine were found to have increased in all the treated test groups and standard groups than untreated vehicle and negative control groups. Increased wound healing rate observed with test groups and standard groups can be correlated with these higher concentrations of hydroxyproline and hexosamine measured in their granulation tissue which in turn reflects increased cellular proliferation and collagen formation. So, measurement of both these compounds is regarded as an index of wound healing process [29]. Similarly, decreased wound healing in the control groups can be explained by their lower hydroxyproline and hexosamine content. Among the test groups, animals treated with 10% CGEE ointment show to increase collagen synthesis significantly. These findings clearly state that extract-containing ointments similar to standard ointment influence collagen synthesis and stabilization and accelerate healing process at a faster rate. Collagen has shown to have an impact on epithelialization at the latter phase of the healing process [30]. Reduced epithelialization noticed with treated and standard groups on comparing with control may be corroborated to the increased collagen synthesis.

Free radicals produced due to lipid peroxidation exacerbate tissue damage and delay the wound healing process. They tend to inhibit granulation tissue formation and epithelialization as well [31]. Antioxidants such as catalase, glutathione peroxidase, myeloperoxidase, and superoxide dismutase have postulated to eliminate these free radicals and heal wound at a faster rate [32]. Hence, evaluating antioxidant, free radicals, and lipid peroxidation status in the granulation tissue following the application of plant extract could be a strategy to validate their role in the healing process. The studies on catalase, nitric oxide, and lipid peroxidation revealed that the extract-containing ointment possessed significant antioxidant activity preventing oxidative stress and promoting wound healing process.

Preclinical studies on the wound healing efficacy of the ethanolic extract of *C. guianensis* fruit pulp have clearly stated that the extract regenerates damaged cells and heals wound more efficiently comparing with control groups. However, finding out the underlying molecular mechanism of CGEE extract in accelerating wound healing process is now extremely important and highly valued.

Growth factors are biologically active molecules rendering a crucial role in each phase of the healing process such as chemoattractant, cellular proliferation, angiogenesis, synthesis and degradation of extracellular matrix, and synthesis of cytokines and growth factors by the nearby cells. The tissue repair process is triggered, advanced, and eventually ended as a result of the cascade of growth factor signals. It is possible to gain insight into the progression of wound healing by determining the time course of growth factor expression.

One of the important aspects in healing process is the formation of new blood vessels (angiogenesis). High levels of expression of vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF-β) have been observed in the test groups and standard group at the 10^{th} post wounding day reflecting proliferative phase of wound healing process. Especially, the expression was maximum in the animal groups treated with high-dose (10%) CGEE ointment. This showcases the dose-dependency effect of CGEE in stimulating the expression of VEGF and TGF-β. Wound contraction starts soon after cutaneous injury and peaks within the 2 weeks after the onset [33]. Both are proangiogenic growth factors increasing vascularization at the early stages of injury. As a result, there is a considerable rise in the blood vessel density in the granulation tissue to provide oxygen and nutrients [34]. VEGF tends to increase vascular permeability which is an important step at early stages of healing. VEGF expression has been increased in keratinocyte making them to proliferate and migrate to repair epidermal barrier during re-epithelialization [35]. TGF-β produced by macrophages, fibroblasts, keratinocytes, and platelets is important in inflammation, angiogenesis, re-epithelialization, and connective tissue regeneration. TGF-β attracts fibroblasts to the site of injury and stimulates their proliferation. TGF-β initiates fibroblast to produce extracellular matrix and collagen. Further, TGF-β drives the wound healing process into the maturation phase by converting fibroblasts to myofibroblast initiating wound contraction event [36].

Both the expressions were normalized and reached to basal levels at the 20th post wounding day in the test and standard groups. This hallmarks the end of angiogenesis, reduced blood vessel density, and reduced granulation tissue formation [37]. The decreased TGF-β expression results in low scar formation. Finally, the study can be ascertained that CGEE can modulate the expression of VEGF and TGF-β thereby accelerating the wound closure with minimal scarring effect.

Conclusion

In brief, the research has determined that the ethanolic extract of *C. guianensis* fruit pulp exhibits a significant wound healing activity. As a result, they can be investigated further as a source of a cost-effective therapeutic agent in the role of pro-healer, as well as faster wound healer, avoiding the formation of scar tissues. The current study, like any research, required more detailed investigations, carrying out in vivo wound healing studies using different wound models including the incision wound model and burn models. Further, it has opened up a number of potentially lucrative possibilities to determine the expression

patterns of various wound healing–associated genes and proteins in excision wound model–induced tissue exudates through genomic and proteomic analytic methods.

Author Contribution Anna Sheba L.: conception, strategy, formal analysis and research, writing—first draft preparation, composing, reviewing, and rewriting the manuscript. Anuradha V.: review and editing the manuscript. Syed Ali M.: contributed statistical tools and analyzed the data. Yogananth N.: contributed statistical tools and analyzed the data.

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code Availability Not applicable.

Declarations

Ethics Approval The animal experiment protocol was approved by the Institute Animal Ethics Committee (IAEC no: LVII/02/321/PO/Re/S/04/CPCSEA).

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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