RESEARCH ARTICLE

Methanolic Extract of *Neurocalynx calcinus* for Promoting Wound Healing: Characterization by Ultra-High-Performance Liquid Chromatography High-Resolution Mass Spectrometry

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Received: 21st August, 2023; Revised: 11th October, 2023; Accepted: 29th February, 2024; Available Online: 25th March, 2024

ABSTRACT

Wounds are physical injuries caused to living tissues due to discontinuation, disruption of cellular function, anatomical rupture, and functional issues in the concerned parts. Wound healing is an intricate restoration of damaged tissue by anabolic progression due to enhanced cellular function, matrix signaling, and various physiological processes. Still, a satisfactory solution is not viable for complete wound healing. Need of the hour is a wound healing product that is natural and easily available with a reduction in pain and finances, as well as restorations of tissue integration, with a minimum scar and quick healing. Based on ethno-pharmacological studies, *Neurocalynx calcinus* (Rubiaceae) was chosen for comprehensive research on *in-vitro* and *in-vivo* wound healing investigation. This plant is called "Pacha chedi" and is native to the jungles of Kerala and south India. The plant is reported to have antioxidant, analgesic, and anti-inflammatory properties, and it also showed significant wound healing, burn healing, and immune-enhancing properties. Several chemicals, such as flavonoids, were discovered in the NCME extract through ultra-performance liquid chromatography–high-resolution mass spectrometry analysis (UPHPLC-HRMS). Additionally, solutions with various NCME crude concentrations made with this extract were assessed for their anti-inflammatory, antibacterial, and acute dermal healing processes in a rat excision wound model and safety. The results of histopathology demonstrated the best rate of wound contraction. Compounds like glucoside, kaempferol, and quercetin ellagic acid were identified in NCME and are known as wound healers. The result also showed that NCME extract has good antimicrobial, anti-inflammatory, and antioxidant activity. It can probably be the next target for a novel drug from a phytochemical background, and it can play a significant role in accelerating wound healing by supporting traditional use.

Keywords: Herbal drugs, Phytoconstituents, Ethnopharmacology natural resources wound healing, UP-LC HRMS, Incision model.

International Journal of Pharmaceutical Quality Assurance (2024); DOI: 10.25258/ijpqa.15.1.39

How to cite this article: Anita A, Diwekar K, Geetha KM. Methanolic Extract of *Neurocalynx calcinus* for Promoting Wound Healing: Characterization by Ultra-High-Performance Liquid Chromatography High-Resolution Mass Spectrometry. International Journal of Pharmaceutical Quality Assurance. 2024;15(1):249-257.

Source of support: Seed Research Grant fund ref no:13150230/09-07-21, Dayananda Sagar University, Bangalore, is the source of funding for which the authors are ever grateful.

Conflict of interest: None

INTRODUCTION[1-11]

The occurrence and frequency of chronic wounds are heightened by factors such as age, nutritional status, infections, and, notably, the presence of both diabetes and coagulation disorders. These elements exert a substantial influence on the process of wound healing. Non-healing wounds continue to be a public health issue that contributes to social difficulties in patients and need increasingly potent therapy and huge financial costs as a result.[1,2] In light of this, natural resources have been studied to create chemicals to speed up the healing process increased over the past few years.[3] Many plants, according to ethnopharmacological research, and a lot more individuals now benefit from wound care due to the advancement of approaches that utilize simpler, more affordable, and safer technologies, along with the utilization of resources and raw materials available in less developed regions more people now have access to wound care, thanks to the development of strategies for utilizing less sophisticated, less expensive, and safer technologies as well as resources and raw materials present in less developed places for wound healing. In light of this, the study of natural resources has been used in the creation of chemicals to speed up the healing

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process, emphasizing plants abundant in anti-inflammatory and antioxidant chemicals[4-6] The Rubiaceae family includes the species *Neurocalynx*.

The frequency and incidence of chronic wounds are collectively referred to as "ulcers," scars that never heal. A common name for *Neurocalynx calcinus*. in Kerala is "Paccha chedi. Lately, this plant named *N. calcinus,* belonging to the Rubiaceae family, was found to be a traditional medicine used by tribal people in Western Ghat (Kerala). The leaf is the most medicinally important portion, having anti-inflammatory, antioxidant, and antibacterial properties apart from wound healing. Cuts, wounds, burns, acne, seborrhoea, dermatitis, and psoriasis heal faster with less scarring after topical application of methanolic *N. calcinus* extract, according to traditional applications and earlier reports,[7] particularly on topical application. The principal components were the iridoids, diterpenes, sterols, and flavonoids.[8,9] The major ingredient, polyphenols, is widely present in the plant (10–18% w/w on a dry basis) (8% w/w in a dry basis). It has significance in a variety of sectors, including burn wounds and raw wound healing, particularly in folk treatments.[10] Even though this plant has yet to have been systematically screened for evaluation of its numerous actions in various pharmacological models, the wound-healing potential of the traditionally used *N. calcinus* whole plant has not been explored or scientifically examined. The Cholainikan tribes of the Western Ghats have traditionally employed *N. calcinus* for medicinal purposes[11]. However, this current study aims to assess the excision wound healing capabilities of methanolic leaf extract from *N. calcinus* in rats. The focus lies in investigating its traditional use in managing different types of wounds, including burns, within the realm of traditional medicine.

MATERIALS AND METHODS[12-19]

Collection and Extraction of Plant Material

The dried plant powder used in this study was obtained from Sri Venkateshwara University Tirupathi, India. Dr. C. Madhava Chetty, Principal and Head of the Department of Botany at Venkateshwara University Tirupathi, A.P, India, conducted authentication. For future reference, a specimen copy was preserved in the department's herbarium (Herbarium No: HFP 1531, HFP 1912). The plant sample, consisting of leaves, was collected and oven-dried at 60°C for 6 hours. Subsequently, it was subjected to soxhlet extraction to obtain the methanolic extract for further analysis.[19] Preparation of NC extract (250 g): After standardization of the process, NC extract (600 g) was extracted with methanol using a reflux condenser for 6 hours. The oven temperature was kept at 45°C. To obtain a soft extract, the extract was collected and filtered using Whatman No 1 filter paper, and the filtrate was then evaporated under decreased pressure. The extract had 28% (w/w) yield.

Methods of Phytochemical Screening

Different qualitative chemical tests were used to determine the presence of various phytoconstituents in aqueous extract. Tannins, polysaccharides, saponins, diterpenes, flavonoids, and polyphenolic chemicals were found in crude methanolic extract of NC (NCME) leaf in preliminary phytochemical screenings.[20,21] Thin-layer chromatography (TLC) was used to validate the presence of the active components.

The Local Ethical Committee for Animal Experimentation (Ref no. 606/20/c/CPCSEA) authorized the study protocol for animal experimentation. The Swiss albino female rats were bought from DSU in Bangalore, India, and housed in an animal house with a 12-hour light-dark cycle and appropriate ventilation. Throughout the experiment, they were provided with food, drink, and *ad libitum*. The staircase approach was used to conduct an acute toxicity study. Toxicity tests were carried out in Swiss albino rats at dose levels up to 2000 mg/kg using an internationally recognized technique based on Organization for Economic Cooperation and Development (OECD) guidelines 420.

The animals were occupied and eating and drinking healthfully. Six animals were employed for each dose level under investigation. A fixed-dose level of 5, 50, 300, and 2000 mg/kg was selected as the starting dose for the sighting investigation to provide a dose that would cause toxicity. With a single dosage, the extracts were gavaged through a stomach tube or suitable intubation cannula. There could be a 24-hour interval between doses for each animal. Every animal was observed for fourteen days in terms of body weight, food consumption, and the relative weights of their kidneys, livers, lungs, and hearts.

Animals

All experiments utilized adult Swiss albino female rats weighing between 30 and 40 grams. The rats were housed in polypropylene cages with free access to water and food, maintained at a controlled temperature of 18 to 20°C on a 12-hour light/dark cycle. All procedures involving animals were conducted per approved protocols. The study protocol for animal experimentation was authorized by the Local Ethical Committee for Animal Experimentation (Reference number: 606/20/c/CPCSEA).

Control of Bacteria in Plant Extract

To ensure the correctness of the results, a microbiologic control was carried out prior to the investigations. Colony-forming units (CFU) assays were performed on both viral and bacterial samples. The detection of bacterial pathogens, including *Escherichia coli, Salmonella* species*, Pseudomonas aeruginosa,* and *Staphylococcus aureus*, was carried out in the presence of CFU, according to Indian Pharmacopoeia.[22] The CFU/g value of the number of live pathogens discovered in relation to the Guidelines for Assessing the Residues and Contaminants of Herbal Medicines.[23]

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relation to the Guidelines for Assessing the Residues and Contaminants of Herbal Medicines[25] With antibacterial activity tested in triplicate, the following reference strains minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) were determined: Gram-negative bacteria, such as *E. coli* (ATCC 8739), *Klebsiella pneumoniae* (ATCC 4352), and *P. aeruginosa*; and gram-positive bacteria, such as S. *aureus* (ATCC 6538), *S. epidermidis* (ATCC 12228), and *S. aureus* (ATCC 12228), and (ATCC 25853), *Enterococcus faecalis* is. For the MIC and MBC studies, the *N. calcinus* methanolic leaf extract (NCME) was dissolved in a solution of water and DMSO. Mueller-Hinton broth was analyzed using the broth microdilution technique described by the Clinical and Laboratory Standards Institute. The experiment used 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), in which metabolically active cells transform MTT into a bright, water-soluble formazan. And ATCC 25853, *E. faecalis*. For the MIC and MBC studies, the NCME was dissolved in a solution of water and dimethyl sulfoxide (DMSO). Mueller-Hinton broth was analyzed using the broth microdilution technique described by the Clinical and Laboratory Standards Institute. The experiment used 3-(4,5-dimethylthiazol-2yl)-2,5-diphenytetrazolium bromide (MTT), in which metabolically active cells transform MTT into a bright, water-soluble formazan.

The lowest extract concentration at which a color shift occurred was found to be the MIC. To find the MBC, 1 liter of broth was taken out of each well and cultured in Mueller-Hinton agar for 24 hours at 37°C. The[26] MBC was defined by Lopes *et al*. as the lowest concentration of extracts or fractions that resulted in fewer than three colonies (killing 99.9% of the colonies) or no growth at all. The positive control was trimethoprim with sulfamethoxazole, while the negative control was a bacterial inoculum in one liter of DMSO 0.4% solution.

Coagulation Assay[27-29]

The fibrinogen coagulation (FC) assay was carried out in accordance with the procedures outlined in a previous paper by Moura and colleagues.[27] In a nutshell, 250 mL of the extract solutions were added to a 10-minute incubation at 37°C with human fibrinogen (Sigma Aldrich1) solution at 2 mg/mL concentration. Then, thrombin (200 g/L) was added, and the coagulation time was tracked. Assays for prothrombin time (PT) were carried out in accordance with the manufacturer's instructions (Wiener laboratories1). About 100 mL of pre-warmed thromboplastin with calcium was added to the extract to start the coagulation process after the extract had been incubated for 10 minutes at 37°C with a commercial control plasm (Wama diagnostic 1). Different concentrations of the NCME were tested (500, 250, 200, 100, 50, 20, and 10 g/mL), and the studies' controls were carried out utilizing Assay for Folin-Ciocalteau

The Folin-Ciocalteau colorimetric method uses a variety of media, including phenolic compounds, complexes in blue form, and combinations of phosphomolybdic and phosphotungstic acid, which are based on the transfer of electrons in alkaline solutions.[28] Following the previously outlined procedure, measurements were carried out by reacting 10% (v/v) of the Folin-Ciocalteau reagent with Na2CO3. After two hours of diluting the samples in water, spectrophotometric results were visible at 760 nm. Biospectro SP-22 is used in the treatment of wounds with *N. calcinus*. Gallic acid was utilized as the calibration curve for the assay, which was developed in triplicate. The results were expressed in gallic acid milligrams. for each gram of dried extract (GAE).[29]

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Hybrid quadrupole Q Exactive GC from Thermo Scientific Liquid chromatography analysis in conjunction with high-resolution mass spectrometry or LC-MS analysis, was carried out using the Orbitrap mass spectrometer of mass spectrometer. The creation of a pharmaceutical formulation intended for topical use, chromatographic analysis, and acquisition parameters were previously described in great detail, albeit with minor modifications, such as increasing the gradient elution time from 10 to 20 minutes.[29] The crude extract formulations in this study were used with NCME concentrations of 0.5 and 1.0% (w/v) prior to the trials. The British Pharmacopoeia (1980) states that the following components were combined in a beaker placed in a water bath at 65°C to make the ointment base: Five grams each of wool fat, hard paraffin, and, 5 g of cetostearyl alcohol, and 85 g of soft white paraffin.

Preparation of Extract

The lyophilized NCME was gently integrated to create a consistent crude extract after all of the ingredients in the extract had been blended to the desired consistency. There were 0.5 g or 1.0 g of NC extract, also known as NCME 0.5% and NCME 1.0%, respectively, in every 100 g of the extract created for the current investigation. Additionally, it was ready for formulation constancy. Ointment Formulation. Simple ointment BP was prepared using hard paraffin, cetostearyl alcohol, white soft paraffin, and wool fat. The master formula used for the preparation of wool fat 50 gm, hard paraffin wax 50 gm, white soft paraffin wax 850 gm, and cetyl steryl alcohol 50 gm, a total 1000 gm. The crude control sample and the NC were put through a physical preliminary. In the Cosmetic Products Stability Guide[31] to expedite the stability research. In the ice-thaw cycles, centrifugations were used to evaluate early stability tests after performing the organoleptic, microbiologic, and pH measurements in accelerated stability. Samples were

kept at room temperature (25°C), in a refrigerator (5°C), and in a drying oven (40°C) for 24, 30, and 90 days. Following the stability test, the samples' microbiological quality was evaluated to look at the existence of E and UFC above the advised limits in the environment, bacteria such as *E. coli, Salmonella* spp., *P. aeruginosa*, and *S. aureus* as described previously for the extracts.[30,31]

Acute dermal toxicity test

The OECD recommendations (N 432, 2017)[32] were adhered to for the study. In all, there were six rats. The shaved region was uniformly covered with the limit test dose of 2000 mg/kg of the 10% formulation, or 10% of the body surface area. The shaved area was wrapped in sterile gauze and taped to the ends to prevent the animal from withdrawing or consuming the product for twenty-four hours. Up until day 14, clinical symptoms and mortality were recorded every 30 minutes, 2, 6, 24 hours, and once daily after the test drug was removed and the exposure time had passed. We measured each weight individually. In addition to the amount of food and drink consumed on days 0 (before to administration), 7, and 14 were measured every day.

Assemblages and Experimental Procedures

Six groups of ten animals each were formed by randomly dividing the animals (a total of 60 rats). The following substances were applied locally to each group of animals: I) Betadine cream as a positive control (PVPI); and III) a solution with lyophilized extract of NC at a concentration of 0.5% (CaE 0.5%); an IV solution with lyophilized extract of NC at a concentration of 1% (1%); and a V gel with lyophilized extract of NC 0.5% (0.5%); VI) gel with 1% of lyophilized NCME 1%. Two times each day, the medications (100 mg or 100 L/day) were applied topically.

Model for excisional wounds

Each rat was weighed and given an intraperitoneal injection of a mixture of chloroform in the tank and ketamine (90 mg/kg) to make them unconscious. Trichotomy of the back was performed when the animals' reflexes were lost, along with 2% chlorhexidine gluconate antisepsis. With the use of a biopsy punch, a single 6-mm-diameter cutaneous (full thickness, totally transdermal) circular wound was produced on the animal's pre-shaved dorsal surface. Animals were housed separately in cages with autoclaved paper trimmings while they recovered. They had unlimited access to food and liquids. Animals were not given any local or systemic therapies aside from the medications being studied.

Rate of wound healing

A wound was selected for daily pachymeter (ZAAS Precision-Digital 0–150 mm) measurement. The areas were determined using the formula A =. R. r, where A stands for the area (mm^2), "R" for the greater radius, and "r" for the smaller radius. The following equations were used to calculate the contraction degree and express it as a percentage.[33]

$$\% \text{ Wound contraction} = \frac{(Initial\ wound\ size) - (Specific\ day\ wound\ size)x100}{Initial\ wound\ size}$$

Histopathology

On the seventh and fourteenth days of the therapy, five mice from each group were killed in a chloroform chamber to provide tissue for the morphometric analysis of the skin lesions. Before being dehydrated in ethanol, each sample was fixed for at least 24 hours at a 10% concentration in buffered formalin. After that, the samples were routinely prepared for inclusion in paraffin by slicing them using a microtome set to 2.5 m. The sample was covered in hematoxylin and eosin and then put on slides.

Statistical Analysis

The mean ± SEM is used to present the results. One-way analysis of variance (ANOVA) was used to examine group differences, and the Tukey-Kramer test was then used. At $p < 0.05$, the data were deemed significant when compared to the control group.

Outcomes

A microbiological analysis was conducted to guarantee the security of the animals during the trials, and a microbiologic study of NCME was carried out. A minimal amount of microbial load is permitted in dry extracts, with a limit of 104 CFU/mL for bacteria and 103 CFU/mL for fungi and yeasts, and without the presence of harmful bacteria. The NCME's lack of bacterial and fungal contamination is evidence of the effectiveness of the operating practices used in the hematoxylin and eosin extraction.

Anti-microbiological action

According to antibacterial testing, experiments shows that the NCME did not show any discernible inhibitory effects as per Table 1.

Tests for coagulation

The prothrombin time test measures the amount of time it takes for blood to coagulate, while the fibrin coagulation test assessed the extract's impact on fibrin clot formation. The extract had no effect on the measured levels of fibrinogen or the prothrombin time. Hydroethanolic extract from *C. americana* leaves identified chemically using the Folin-Ciocalteau assay. The extract's gallic acid equivalent (GAE) measurements showed a total phenol content of 209 4 mg/g of NCME. The NCME extract was eluted using gradient mode, and a total ion chromatogram (TIC) was generated. obtained with the UPLC-HRMS's positive and negative modes. There were nine chemicals found, two in the positive mode and four in the negative mode. As can be observed in Figure 1 and Table 2, the identification process involved comparing the m/z ratios of the compounds with those of the metabolome platforms, accounting for an error of 10 ppm.

Formulation for topical use and stability[34,35]

As the formulation has been utilised as the dermatological standard for all skin types, it proved to be the right choice. In addition to being cheap and simple to work with, it had

Table 1: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of for extract of NCME[37]

Bacteria	*MIC (mg.ML^{-1})*	*MBC (mg.ML^{-1})*
Staphylococcus aureus	>2.5	>2.5
Staphylococcus epidermidis	2.5	2.5
Escherichia coli	0.62	>2.5
Klebsiella pneumonia	>2.5	>2.5

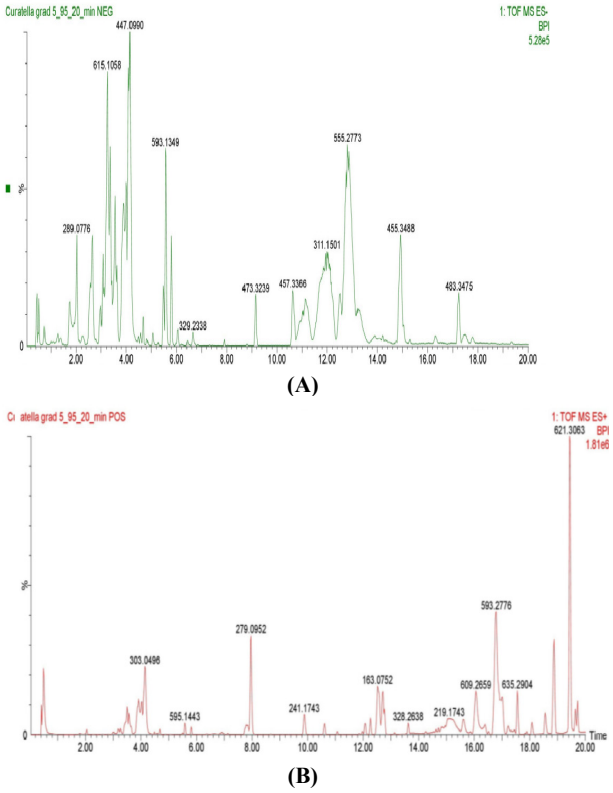


Figure 1: (A) and (B) Total ion current chromatogram of Hydroethanolic extract of NCME[43,44]

smooth spreading, and minimal occlusion, and was utilised to incorporate a variety of active chemicals, such as antibacterial and anti-inflammatory compounds.[36,37]

The tests were carried out in duplicate, and the samples were kept in a manner that sped up any potential alterations that could take place before the expiration date and provided clues as to their stability. Any changes in volume, homogeneity, creaming, or phase separation were noted in the preliminary stability in Table 3.

A limited amount of microbial load is permitted in non-sterile topical products, with a limit of 102 CFU/mL for bacteria and 101 CFU/mL for fungus and yeasts and the absence of harmful microorganisms. There were no harmful germs present or microorganisms growing beyond the recommended levels in the formulations that were examined.[38-42]

RESULTS AND DISCUSSION

Chronic Cutaneous Toxicity

Testing was done to establish the 10% formulation's safe limit dose, which is 2000 mg/kg. In this test, NC was classified as a "non-irritant" because no animal exhibited any erythema, eschar, oedema, or signs of poisoning, death, or other responses.[45-47]

Contracting a Wound

Table 4 shows that there were no variations in the wound contraction in any of the formulations, including the positive control (betadine) and the hydroalcoholic extract of NC.[48,49] On the seventh day, the wound contraction rate was highest in the group that utilized 1% NCME extract. a pathological analysis

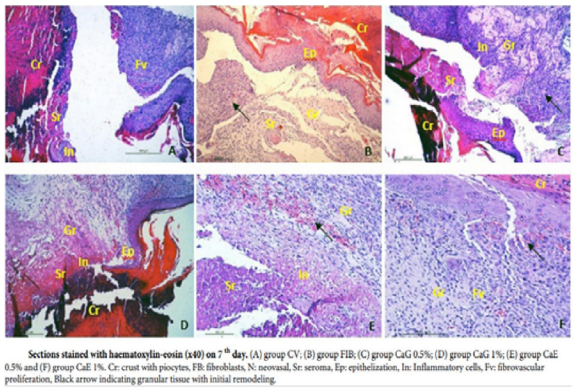
All NCME-treated groups and the betadine group exhibited active fibroplasia, vascularization, and epithelialization, according to a seven-day histological study. In the CaG 1 percent group, tissue in the early phases of remodelling and recent granulation were visible to a lesser degree. Inflammatory

Table 2: Identified compounds in the NCME with their respective retention time AND m/z ratio

S. No.	*Compounds*	*Formula*	*Class*	*rt (mn)*	*Ms mode*	*Calcd.*	*Obs*	*Error*
1	Catechin	$C_{15}H_{14}O_6$	Flavan-3-ol	1.73	$[M-H]^+$	289.0712	289.0706	2.5
2	Epicatechin	$C_{15}H_{14}O_6$	Flavan-3-ol	2.03	$[M-H]^+$	289.0712	289.0741	10
3	Kempferol-4-β-GlucoPyranoside	$C_{21}H_{20}O_{11}$	Flavanol	2.73	$[M-H]^+$	449.1084	449.1064	4.5
4	Quercetin-3-β-GlucoPyranoside	$C_{21}H_{20}O_{12}$	Flavanol	3.48	$[M-H]^+$	465.1033	465.1029	0.9
5	Apigenin-7-O- β -GlucoPyranoside	$C_{21}H_{20}O_{10}$	Flavanol	3.50	$[M-H]^+$	433.1135	433.1136	0.2
6	Quercetin -3-O-GalactoPyranoside	$C_{21}H_{20}O_{12}$	Flavanol	3.62	$[M-H]^+$	465.1033	465.1029	0.9
7	Quercetin -3-O-Arabinopyranoside	$C_{20}H_{18}O_{11}$	Flavanol	4.01	$[M-H]^+$	435-0927	435.0920	1.6
8	Quercetin -3-α-Rhamnopyranoside	$C_{21}H_{20}O_{11}$	Flavanol	4.14	$[M-H]^+$	449.1084	449.1064	4.5
9	Quercetin	$C_{15}H_{10}O_7$	Flavanol	5.51	$[M-H]^+$	303.0505	303.0531	8.6
10	Kempferol-3-0-(6"-O-E-p-coumaroyl)- β-D-GlucoPyranoside	$C_{30}H_{26}O_{13}$	Flavanol	5.54	$[M-H]^+$	595.1452	595.1492	6.7
11	Kempferol-3-0-(2"-O-E-p-coumaroyl)- β-D-GlucoPyranoside	$C_{30}H_{26}O_{13}$	Flavanol	5.77	$[M-H]^+$	595.1452	595.1492	6.7
12	Kempferol	$C_{15}H_{10}O_6$	Flavanol	6.49	$[M-H]^+$	285.0399	285.0374	8.8
13	Betulinic acid	$C_{30}H_{48}O_3$	Flavanol	15.0	$[M-H]^+$	455.3525	455.3487	8.3

cells such as neutrophils and macrophages were seen in the CaG 0.5%, CaG 1%, and CaE 0.5% groups.

All groups that had received NCME treatment showed signs of full epithelization and substantial tissue remodelling by the 14 day. In the CV group, micro abscesses were present, which resulted in cicatricial process damage despite re-epithelialization and early remodelling stages listed in Figure 2.



Sections stained with haematoxylin-eosin (x40) on 7th day. (A) group CV; (B) group FIB; (C) group CaG 0.5%; (D) group CaG 1%; (E) group CaE 0.5% and (F) group CaE 1%. Cr: crust with piocytes, FB: fibroblasts, N: neovasal, Sr: seroma, Ep: epithelization, In: Inflammatory cells, Fv: fibrovascular proliferation, Black arrow indicating granular tissue with initial remodeling.

Figure 2: Illustrates of extensive seroma with neutrophil infiltration & CV group had fibroblasts and angiogenesis

DISCUSSION

The complex physiological process of wound healing involves blood dressing, inflammation, re-epithelialization, and angiogenesis. These processes consist of three distinct steps. After 0–3 days of inflammation and 2–14 days of cellular growth, there are stages including remodelling. started in 14 days and can take up to 30. Infectious agents, chronic inflammation, and reactive oxygen species (ROS) are a few of the main causes of delayed healing. either insufficient responses or angiogenic pathways. This suggests that increased mitogenic activity to support cell division, antibacterial action, and antioxidant activity are desirable qualities in a healing agent. Thus, angiogenesis makes the contraction happen more quickly. The duration required for blood to clot has clinical significance concerning skin homeostasis damage.

All NCME concentration tests did, however, demonstrate coagulant effects; yet, this did not obstruct the spontaneous coagulation process. Neutrophils release lysosomal enzymes and reactive oxygen species (ROS) to begin the process of debridement of the devitalized tissue and the phagocytosis of infectious organisms within 24 hours of the damage. This sets off the biological reaction. The neutrophils started the process

Table 3: Accelerate stability studies

Conditions	*pH*	*Colour/smell*	*Microbial wound*	*conditions*	*pH*	*Colour/ smell*	*Microbial wound*	*conditions*	*pH*
Formulation	Gel 0.5%	Gel 1.0 %	Gel control	Gel 0.5%	Gel 1.0%	Gel control	Gel 0.5%	Gel 1.0%	Gel control
Formulation day	5.15	5.08	7.01	1/1	1/1	1/1	NR	NR	NR
Temperature (25° C ± 2)									
30 Days	5.95	5.97	6.97	1/1	1/1	1/1	-	-	-
60 Days	5.55	5.59	7.20	11/11	11/11	1/1	-	-	-
90 Days	5.50	5.6	7.22	11/11	11/11	1/1	+	+	-
Refrigerator (5° C ± 2)									
30 Days	5.98	5.97	6.98	1/1	1/1	1/1	-	-	-
60 Days	5.08	5.03	7.20	11/11	11/11	1/1	-	-	-
90 Days	5.07	5.01	7.01	11/11	11/11	1/1	-	-	-
Dry Over (40° C ± 2)									
30 Days	5.92	5.95	6.95	1/1	1/1	1/1	-	-	-
60 Days	5.06	5.21	7.30	111/11	111/11	1/1	-	-	-
90 Days	4.07	4.98	6.64	111/11	111/11	1/1	-	-	-

Table 4: Effects of NCME topical formulations

Group	*0 day*	*7th day*		*14th day*	
	Wound surface area mm²	*Wound surface area*	*% wound contracted*	*Wound area*	*% wound contracted*
CV	29.6+4.05	11.46 + 4.79	61	0.58 ± 1.2	98
FIB	27.14 ± 4.44	5.79 ± 3.6	78	0.35 ± 0.79	100
NCME 0.5%	30.02 ± 4.32	7.36 ± 3.54	73	0	100
NCME 1%	26.45 ± 6.7	6.69 ± 1.86	85	0	100
NCME 0.5%	28.02 ± 3.16	6.09 ± 1.85	74	0	99

The values are analyzed using Turkey Kramer after Annova, with an expression in ± SEM.

After 0–7 days, 0 animals were used.

*p <0.05% **p <0.01% against vehicle control cvv NCME – EX

of sanitization and disinfection, which produced ROS, and the macrophages help to complete it within 48 hours of reaching the lesion. People with chronic leg ulcers and ischemia-induced lesions have higher levels of these ROS, which often slows down the healing process. There is substantial xanthine oxidase activity in oxygen radicals.

In the present scenario, the existence of metabolites capable of capturing a sizable concentration of phenolic compounds (209 mg/g GAE) confirms the effectiveness of plant extracts with therapeutic characteristics. Lopes *et al*. and Fujishima have shown the antioxidant activity of this species by enumerating ROS. The mass spectrum supported previous findings by Gurni & Kubitzki and El-Azizi *et al*. by revealing the presence of phenolic chemicals, terpenoids, and flavonoids in NCME. However, by comparing the findings with the inclusion of real standards, it was possible to distinguish between epicatechin and catechin in this investigation. With the exception of kaempferol and apigenin-7-O-glucoside, which have never been discovered in this species, these substances have previously been discovered in the bark. Of the phenolics that have been found, flavan-3-ols such as epicatechin and catechins have demonstrated potent antioxidant activity and have the potential to inhibit xanthine oxidase.

Furthermore, pre-treating fibroblast cells with catechins boosted the proliferation of one of the key processes in wound healing. Shiroma-Lima were discovered monomeric and polymeric catechins in *N. calcinus* extract in addition to a range of procyanidins, which could perhaps account for the remarkable healing and gastro protective properties for gastric ulcers. Vascular endothelial growth factor (VEGF) expression is significantly upregulated in response to prostaglandin E2 (PGE2) in the mucosa, which causes angiogenesis. The major enzyme COX 2 was active right away after the injury, indicating that PGE2 is necessary for fibroblast proliferation and the healing process. In assays, it has been shown that COX-2 selective inhibitors promote delays in healing, and the epidermis produces more PGE2.

Prostaglandin E2 in the mucosa and the consequent production of a considerable increase in the expression of vascular endothelial growth factor (VEGF) that leads to angiogenesis are comparable to the consumption of catechins from green tea. The key enzyme that produces PGE2, cyclooxygenase 2, or COX 2, is induced in the epidermis right after injury. Assays demonstrating that the application of particular COX-2 inhibitors caused healing delays support the crucial role that PGE2 plays in fibroblast proliferation and the healing process. After consuming a catechin-rich tea for a duration of 12 weeks, human skin was infused with 12-hydroxyeicosatetraenoic acid (12-HETE), a pro-inflammatory mediator produced by UVR. Notably, PEG2 was not reduced.

Kaempferol and apigenin-7-O-glucoside are previously unidentified substances in this species. Similar to apigenin, kaempferol, and their derivatives, antioxidant and anti-inflammatory properties have been linked to quercetin and its derivatives. While kaempferol, apigenin, and nuclear transcription factor kB (NF-kB) effectively inhibit COX-2 activation, their derivatives, along with quercetin and catechins, appear to only weakly inhibit COX-2 via kB-inhibitor. To manage inflammation, this is essential. Effective wound treatment appears to benefit from the topical use of quercetin. Metalloproteinase (MMP) synthesis appears to be suppressed by a single terpenoid called botulinic acid. Since metalloproteinase, such as collagenases and elastases, overproduce and impede healing, therapeutic intervention against them is essential. The absence of a matrix to anchor newly formed cells results in a chronic illness.

The hypothesis that the stimulating effect on is made possible by the newly identified compounds. In the NCME-treated groups, fibroblasts, endothelial cells, inflammatory modulation, and 86% for the 1.0% group have wound contraction all contributed to the high degree of inflammation. The % contraction on the 7th day in CV group is deviated significantly. The two lytic enzymes that make up fibrinase are deoxyribonuclease and fibrinolysin. The treatment of dual-infection infected lesions, such as burns, ulcers, and wounds caused by accident. It is composed of a thin, 1% chloramphenicol-containing emollient ointment. It must function as a topical debriding and antibacterial agent. The NCME investigation validated this plant species' low level of antibacterial activity.

Despite the histopathological examination's indication that NCME was helpful in boosting healing, the proliferative phase of the healing process is dominated by the development of fibroblasts two to three days after the damage. the population of cells up to the first week. Fibroplasia is characterised by its first activity being limited to cellular proliferation and replication, then collagen formation. The angiogenesis phase, which comes after the fibroblast phase, is critical to healing because it creates new blood vessels that will provide oxygen and nutrients to the granulation tissue during its development. If angiogenesis is unsuccessful, the fibroblasts migrate slowly, which hinders the healing process. The process of epithelization is now complete as the epithelial cells move from the wound's edge toward its centre.

On the 7thday trial, the histological analysis showed that all groups had strong vascularization, NC new granulation tissue treatment, and epithelialization, fibroplasia, and FIB. In these groups, there is evidence of tissue that is starting to reorganise, suggesting an advanced healing stage. Usually, the remodelling phase starts on day twelve. Because there were no inflammatory cells in the histological study and contraction values that were closer to fibrinase at this point, the CaE 1.0 percent group performed better.

It's important to remember that the extract concentrations were chosen based on previous research in which the 0.4 percent extract showed significant healing activity, but it also showed inflammatory processes similar to those shown in the NC 0.5% group, which did not hinder healing. On day 14, throughout the remodelling process, all groups—aside from

the CV group displayed excellent cicatricial technique. The groups treated with extract exhibited inflammatory cells, suggesting that even in cases when the leukocyte count was in the inflammatory phase, the debridement process would still occur, albeit with improved cicatricial quality and contraction rates compared to the control group. better than the formulation in wound models made of rat skin. The formulation that was being tested shown significant healing activity in addition to being safe to add NC extract to. All organoleptic alterations in formulations stored at room temperature.

Although the pH decreased, it was still within the slightly acidic physiological pH range (4.6–5.8) of the epidermis. This not only helped to inhibit bacteria and fungi from growing on the skin's surface but also protected the skin from reacting badly to the formulation, including exasperation, redness, pruritus, and other side effects. The results of the experiment showed that the NCME extract shrunk the wound enhanced contraction of the wound and faster healing of the wound, most likely through altering the immune response. These results validate the species' traditional use in wound healing.

CONCLUSION

As per the studies and reports MRSA is multi resistant and difficult to treat as to date there are no satisfactory antimicrobial drugs. Therefore, comparatively, the NCME seems to have a potential efficacy to combat MRSA. Conclusively NCME results showed antimicrobial, anti-inflammatory, and antioxidant activity as an important target for the formulation of a new novel drug against many microbial infections with minimal side effects and could be a future role model for wound healing medications as its potency to accelerate wound healing by promoting myofibroblast activity, an increase of hydroxyproline and collagen deposition is rapid and superior, also its regulation of MPO and MMP-8 enzymatic activities is quite satisfactory required for wound healing.

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