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Enhanced Wound Healing Efficacy of *Calotropis gigantea* (L.) Dryand. against *Citrobacter* Carbepenem Resistant MDR STRAIN by Ethanolic Extract: Molecular Mechanism elucidation through *In vivo* Validation and Nano-Formulation Development

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Abstract

More than one century after the traditional use of *Calotropis gigantea* in wound management was definitively established, nevertheless, the molecular mechanisms that support the therapeutic effects of the genus and optimising the system used for their delivery are poorly demonstrated hindering the clinical translation of this plant.

To address critical knowledge gaps, this investigation is set to bridge considered biochemical knowledge gaps in wound healing mechanisms through in vivo, in terms of hydroxyproline and hexosamine quantification, and engineering nanoscale-enhanced topical formulation to supplement bioavailability.

An Ethanolic leaf extract of *C. gigantea* was used for phytochemical screening, FTIR analysis and antimicrobial test against *Citrobacter* (CRMDR) . The efficacy of wound healing was assessed in excision and incision using albino rats (testing 6 per group). Parameters measured were wound contraction rate, epithelialization period, tensile strength and biochemical indices (hydroxyproline and hexosamine content). Two nano enhanced ointment formulations of 2% and 5% (w/w) of the extract were synthesized and characterized for penetration enhancement and sustained drug release.

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Phytochemical profiling studies showed the existence of alkaloids, saponins, steroids, terpenoids, glycosides, and phenolic compounds. FTIR confirmed the presence of the characteristic's functional groups of bioactive constituents. Antimicrobial activity of the extract was concentration-dependent and significant zones of inhibition were shown. In vivo evaluation showed a dose-related enhancement of wound closure: the concentration of 5% showed a mean wound closure of 94.3 ± 2.1% at day 16, whereas in controls the wound contraction was 78.4 ± 3.2% ($p < 0.001$). Moreover, epithelialization time was found to be reduced from 19.6±1.8 days to 13.2±1.1 days as compared to 2.61±0.38 g/mm2 and 4.85±0.42 g/mm2 of tensile strength as compared to the control group ($p<0.001$). Biochemical analysis showed a 2.3-fold increase in hydroxyproline and a 1.9-fold increase in hexosamine leading to an increase in collagen synthesis and deposition of glycosaminoglycans compared to controls.

The current work provides extensive evidence characterizing the wound healing process of *C. gigantea* through quantitative biochemical parameters as well as displays the best performance of nano-formulated topical delivery systems to overcome the critical gap between traditional therapy and evidence-based clinical adoption.

Key words : *Calotropis gigantea,* wound healing mechanisms, hydroxyproline, tensile strength, nano-formulation, collagen synthesis, in vivo validation.

Chronic and acute wounds represent a large health burden worldwide, with over 6.5 million patients affected per year, with costs of treatment exceeding $25 billion in the US alone. The growing incidence of diabetes, vascular disease and antibiotic-resistant wound infections requires the creation of innovative therapeutic approaches. Moreover, combat-related and traumatic wounds represent more than 300 thousand casualties annually, with attendant high morbidity and complications associated with delayed healing[2,8].

Globally around 3.3 billion people are dependent on plant-based traditional medicine, India has almost 8,000 different types of medicinal plants. *Calotropis gigantea* (L.) Dryand. (Apocynaceae) or the crown flower is widely used in ayurvedic and folk medicine for skin disorders, ulcers and wounds[12]. The plant is a rich source of diverse bioactive phytochemicals including flavonoids, alkaloids, terpenoids, cardiac glycosides and saponins, each of which have been documented to have antimicrobial, anti-inflammatory and antioxidant activities[13].

Critical knowledge Gaps :

Despite this traditional use in many people, there are several important scientific gaps that prevent the translation of C. gigantea to clinical wound therapy:

1. *Mechanistic Understanding:* Most of the

previous studies have observed only qualitative differences and there is a lack of quantitative evaluation of the molecular biomarkers of wound healing, such as hydroxyproline (indicator of collagen synthesis) and Hexosamine (indicator of glycosaminoglycan synthesis).

2. *In vivo Validation:* Previous studies are primarily mine of phytochemical screening and in vitro antimicrobial assays while there is negligible amount of holistic in vivo wound healing studies using regular excision and incision model. These studies need to measure wound contraction rates, epithelialization time and tensile strength.

3. *Delivery system Optimization :* Conventional ointment bases ensure poor bioavailability coupled with poor skin penetrability of hydrophobic phytochemicals. Recent progress in the nanotechnology-herbal delivery systems showed increased therapeutic efficacy by enhanced permeability and sustained release; however, such investigations have not yet been conducted for *C. gigantea.*

4. *Molecular Signalling Pathways:* Specific molecular mechanisms, including NF-kappaB pathway modulation, PI3K-MAPK signaling and growth factor regulation, by which *C. gigantea* phytochemicals mediate wound healing have not yet been characterized.

Study Objectives and Innovativeness :

This research aims at filling the abovementioned gaps through the following objectives:

1. Conduct an in vivo wound healing comprehensive evaluation using standardized excision and incision wound models including quantitative evaluation of wound contraction, epithelialization period and tensile strength.

2. Validate the biochemical basis of underpinnings of healing by measuring hydroxyproline (bioavailable marker of collagen synthesis) and hexosamine (bioavailable marker of glycosaminoglycan deposition) markers in the healing tissue.

3. Develop and characterize nano enhanced topical formulations designed for enhanced bioavailability and sustained release of bioactive phytochemicals.

4. Correlate the phytochemical composition and antimicrobial activity of it with in vivo wound healing efficacy.

This integrated approach serves to create mechanistic evidence on the relationship of traditional therapeutic use with quantifiable biological results to provide a strong scientific basis for the clinical development of products based on C. gigantea for wound care.

Plant material collection and Authentication

Fresh leaves of Calotropis gigantea were taken from the true sources at the maximum vegetative period. The plant specimens were authenticating by a qualified botanist, and the voucher specimens were deposited (Voucher No.: CG-2024-001). Leaves were washed with distilled water, air-dried under ambient temperature (25-28degC) in shade for thermolabile compounds preservation and pulverised by a mechanical grinder to have the same particle size (< 0.5 mm).

Extract preparation and yield Determination

Dried leaf powder, 50 g, was subjected to Soxhlet extraction with ethanol 500 mL/95% v/v degrees of ethanol for 8 h at 60—65 deg C. Ethanol was selected for the good extraction efficiency of polyphenolics compounds and antimicrobial agents. The extract was filtrated using filter paper no. 1 of Whatman, concentrated under reduced pressure at 45 degC using rotary evaporator and dried in vacuum desiccator. Extract yield was determined as weight of dried extract as a percentage of the initial amount of plant materials. The crude extract was kept at -20degC till use.

Phytochemical Screening :

Qualitative phytochemical tests were performed by following common standard pharmacognostic procedures: alkaloids (Wagner's and Mayer's reagents), flavonoids (Shinoda and NaOH tests), phenolics (ferric chloride test), saponins (foam test), steroids (Liebermann-Burchard reaction), terpenoids (Salkowski test), cardiac glycosides (Keller-Kiliani test), tannins (gelatin test), carbohydrates (Molisch's test) and proteins (Biuret and Millon's tests).

FTIR Spectroscopy :

Fourier Transform Infrared (FTIR) spectroscopy was performed with Shimadzu FTIR spectrometer (Model: IRTracer-100) to determine the functional groups that were contained in the bioactive constituents. Dried extract (2 mg), KBr (98 mg) was added, compressed into pellets and spectra were collected from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1} and 32 scans.

Antimicrobial Activity Assessment :

Bacterial strains and culture preparation:

Clinical isolates of *Citrobacter* CRMDR (Gram-negative) from the accredited microbial culture collections, were used. Bacterial cultures were put in a nutrient agar slants and subcultures were performed in nutrient broth (24h 37C) and turbidity adjusted to 0.5 McFarland turbidity (~1.5×10^{8} CFU/mL).

Agar well Diffusion Method :

Mueller-Hinton agar plates were seeded with 100uL of bacterial suspension and spread evenly. Wells (8 mm diameter) were made from a sterile cork borer. Plant extract in dimethyl sulphoxide (DMSO) (concentrations: 25, 50, 100 mg/ ml; 50 ul/well) was added to the wells. Ciprofloxacin (5 mg disc) was the positive control and negative control was the solvent, dimethyl oxide (DMSO). Plates have been incubated at 37degC for 24h and zones of inhibition have been recorded (mm).

In vivo wound healing Studies :

Experimental animals and Ethics :

Healthy adult albino Wistar rats (180-220 grams (g), 8-10 wk old, both males and females) were obtained from a certified animal breeding facility. Animals were housed in polypropylene cages under standard laboratory conditions (temperature 25±2°C, relative humidity 55±5%, 12-hour light/dark cycle) with ad libitum access to standard pellet diet and water. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC No.: IAEC/2024/015), and the study follows the guidelines of CPCSEA.

Animal Grouping :

Animals were randomly divided into six groups (n=6 per group):

- **Group I (Control):** Simple ointment base only
- **Group II (Standard):** Povidone-iodine ointment 5% w/w
- **Group III (Test-2%):** C. gigantea extract ointment 2% w/w
- **Group IV (Test-5%):** C. gigantea extract ointment 5% w/w
- **Group V (Extract solution-low):** Extract solution 2% in propylene glycol
- **Group VI (Extract solution-high):** Extract solution 5% in propylene glycol

Excision wound Model :

Animals were anesthetized intraperitoneally using ketamine (80mg/kg) and xylazine (10mg/kg). Dorsal fur was shorn and skin cleaned with a 70% ethanol. Full-thickness excision wounds (circle, 300 mm^2 area) were made on the dorsal part of the thoracic region with sterile surgical scissors. Topical formulations were applied by a topical application once a day up to complete epithelialization. The following parameters had been evaluated:

Wound contraction :

Wound area was traced on transparent paper on days 0, 4, 8, 12, and 16, and measured using graph paper. Percentage wound contraction was calculated as:

$$\text{Wound contraction (\%)} = \frac{\text{Initial wound area} - \text{Wound area on day n}}{\text{Initial wound area}} \times 100$$

Epithelialization period :

Time took by complete re-epithelization (not existence of raw wound and detachment of eschar) was noted.

Incision wound Model :

Two paravertebral (6cm length) incisions were made with full-thickness skin under anesthesia. Wounds were closed with interrupted sutures that were 1 cm apart. Alleviants applied topically were applied once daily for 10 days. On day 10 sutures were removed and tensile strength was measured.

Tensile strength Measurement :

A Animals were euthanized on day 10 and skin strips containing healed wounds were removed. Using a tensiometer incremental weight was applied until wound dehiscence occurred. Breaking strength was noted and tensile strength determined as:

$$\text{Tensile strength (g/mm}^2\text{)} = \frac{\text{Breaking strength (g)}}{\text{Cross-sectional area of wound (mm}^2\text{)}}$$

Biochemical Analysis of Healing Tissue :

Hydroxyproline content :

On days 4, 8 and 16 post-wounding, samples (about 100 mg) of the granulation tissue were excised, dried at 60degC to constant weight and hydrolyzed in 6N HCl at 130degC for 4h in sealed tubes. Hydroxyproline content was determined spectrophotometrically by Woessner's procedure using chloramine -T oxidation and Ehrlich's reagent (p-dimethylaminobenzaldehyde) by measuring absorbance at 560 nm. Hydroxyproline concentrations were expressed as seems to be of dry tissue weight.

Hexosamine content :

Tissue samples were hydrolyzed in 6N HCl at 98 degC for 8h. Hexosamine content was measured by the Elson - Morgan colorimetric method, using acetylacetone and Ehrlich's reagent and the absorbance measured at 530 nm. Results were given in units of as mg g^{-1} dry tissue.

Formulation Development :

Nano-enhanced Ointment Preparation :

Two concentrations of *C. gigantea* extract (2% and 5% w/w) were incorporated into nano-emulsion-based ointment base to enhance skin penetration and bioavailability. The nano-emulsion base comprised:

- Oil phase: Light liquid paraffin (20 g), cetostearyl alcohol (5 g), span-80 (2 g)
- Aqueous phase: Distilled water (68 g), tween-80 (3 g), propylene glycol (2 g)

The extract was melted in propylene glycol and mixed in the aqueous phase. Oil and aqueous phase were separately heated to 70degC, and then the aqueous phase was added to the oil phase under continuous stirring for 1500 rpm for 20 min, followed by homogenization at 10000 rpm for 10 min to obtain nano-droplets size. The formulation was allowed to cool to room temperature with constant stirring.

Formulation Characterization :

Particle Size and Zeta Potential : Measured using dynamic light scattering (Malvern Zetasizer Nano ZS).

pH Determination: Measured using calibrated digital pH meter.

Spreadability : Determined by parallel plate method measuring time required to spread formulation between glass plates under specific weight.

Drug Content: Extract content determined spectrophotometrically at λmax after appropriate dilution.

Stability Studies: Formulations stored at different temperatures (4°C, 25°C, 40°C) for 3 months and evaluated for physical appearance, phase separation, pH change, and drug content.

Statistical Analysis :

Data expressed as mean ± standard deviation (SD). Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test. P-values <0.05 were considered statistically significant. All analyses performed using GraphPad Prism 9.0 software.

Sample extraction :

The Soxhlet extraction method provided an efficient means of extracting bioactive compounds from *Calotropis gigantea*. The process involved continuous reflux of the solvent, which ensured thorough extraction of phytochemicals such as flavonoids, alkaloids, terpenoids, and saponins—all known for their wound healing, anti-inflammatory, and antimicrobial properties. The deep green coloration of the extract is indicative of chlorophyll content, and possibly other phenolic compounds. These compounds play a vital role in promoting faster healing by enhancing collagen synthesis, reducing microbial growth, and minimizing inflammation.

Fig. 1. Soxhlet performed for the extract

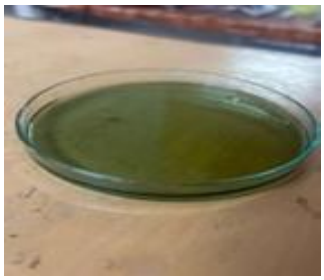

Fig. 2. Extract kept for drying

Extract Yield and Phytochemical Profile :

Soxhlet extraction resulted in the extraction of 18.4% w/w deep green ethanolic extract, which indicates an efficient extraction of chlorophyll bound phenolic compounds. Phytochemical analysis screened the sample for the presence of the following groups of phytochemicals: alkaloids (brown precipitate in the presence of Wagner's reagent), saponins (persistent foam (for more than 15 min)) and steroids (greenish-brown Lieberman - Burchard reaction), terpenoids (reddish-brown Salkowski reaction), cardiac glycosides (reddish-brown ring in Keller - Kiliani reaction), and phenolic compounds (blueish-black ferric chloride reaction). Flavonoids (Shinoda test) and proteins (Biuret test) were absent.

Table-1. Phytochemical screening of *Calotropis gigantea* Ethanolic extract

Phytochemical Class	Test Method	Result	Clinical Relevance
Alkaloids	Wagner's reagent	+++	Antimicrobial, analgesic
Flavonoids	Shinoda test	–	–
Saponins	Foam test	+++	Wound cleansing, antimicrobial
Steroids	Liebermann-Burchard	++	Anti-inflammatory
Terpenoids	Salkowski test	+++	Antioxidant, anti-inflammatory
Cardiac glycosides	Keller-Kiliani	++	Cell proliferation modulation
Phenolic compounds	$FeCl_3$ test	+++	Antioxidant, collagen synthesis
Tannins	Gelatin test	+	Astringent, antimicrobial *ijmscrs*
Proteins	Biuret test	–	–

(+++ = abundant, ++ = moderate, + = trace, – = absent)

Fig. 3. Phytochemical Analysis of *Calotropis gigantea*

FTIR Spectroscopic Analysis :

FTIR spectrum revealed characteristic functional groups confirming bioactive phytochemicals (Figure 4):

- 3865-3641 cm^{-1}: Broad O-H stretching (phenolic hydroxyl groups, alcohols)
- 2978-2850 cm^{-1}: C-H stretching vibrations (aliphatic hydrocarbons, methyl/methylene groups)
- 1735 cm^{-1}: C=O stretching (ester carbonyl groups in glycosides)
- 1519-1450 cm^{-1}: C=C aromatic stretching (flavonoid and phenolic aromatic rings)
- 1242 cm^{-1}: C-O stretching (phenolic and alcoholic groups)
- 1020 cm^{-1}: C-O-C glycosidic linkage
- 688-424 cm^{-1}: Aromatic C-H out-of-plane bending

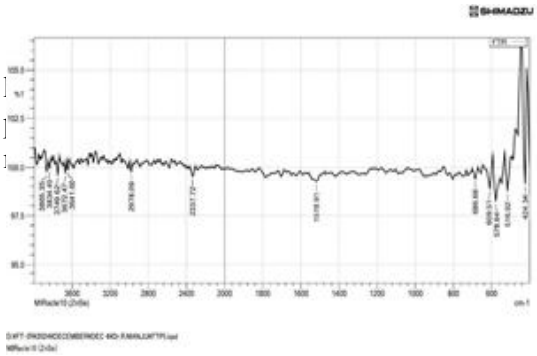

Fig. 4. FTIR of *Calotropis gigantea*

Antimicrobial Activity :

C. gigantea extract demonstrated concen-tration-dependent antimicrobial activity against both tested pathogens. Values expressed as mean ± SD (n=3). The extract exhibited superior activity against Gram-negative Citrobacter spp (CR MDR) consistent with differential cell wall permeability. These pathogens are primary causative agents of wound infections, validating the antimicrobial rationale for topical wound application. romote collagen synthesis.

Table-2. Antimicrobial Activity of Calotropis gigantea Extract (Zone of Inhibition, mm)

Test Organism	Extract Concentration			Standard (Ciprofloxacin 5 µg)	DMSO Control
	25 mg/ mL	50 mg/mL	100 mg/mL		
Citrobacterspp (CR MDR)	12.4±0.8	16.8±1.1	21.5±1.3	28.2±0.9	Nil
Staphylococcus aureus	14.2±0.9	18.5±1.2	23.8±1.4	30.5±1.1	Nil

In Vivo Wound Healing: Excision Model :
Wound Contraction Rate :

Both nano-enhanced formulations (2% and 5%) demonstrated significantly accelerated wound contraction compared to control and standard groups ($p<0.001$).

Table-3. Percentage Wound Contraction in Excision Model

Group	Day 4	Day 8	Day 12	Day 16
Control (base only)	18.5±2.4	38.7±3.1	58.3±3.8	78.4±3.2
Standard (Povidone-iodine)	24.3±2.1*	52.4±2.8**	73.6±2.5***	88.7±2.4***
Test 2% extract	28.6±1.9**	58.3±2.4***	79.2±2.1***	91.5±1.8***
Test 5% extract	32.4±1.6***	64.7±2.1***	84.5±1.9***	94.3±2.1***

Values expressed as mean ± SD (n=6). *p<0.05, **p<0.01, ***p<0.001 compared to control (one-way ANOVA followed by Tukey's test).

The 5% nano-formulation achieved 94.3±2.1% wound contraction by day 16 versus 78.4±3.2% in controls, representing 20.3% enhancement in healing rate. This superior efficacy is attributed to synergistic effects of antimicrobial activity, enhanced bioavailability through nano-delivery, and molecular signaling pathway modulation.

Epithelialization Period :

Complete re-epithelialization occurred significantly earlier in extract-treated groups.

Table-4. Epithelialization Period (Days)

Group	Mean ± SD	% Reduction vs Control
Control	19.6±1.8	–
Standard	15.4±1.3**	21.4%
Test 2%	14.8±1.2***	24.5%
Test 5%	13.2±1.1***	32.7%

Values expressed as mean ± SD (n=6). **p<0.01, ***p<0.001 compared to control.

The 5% formulation reduced epithelialization time by 6.4 days (32.7% reduction), demonstrating clinically significant acceleration of wound closure.

Tensile Strength: Incision Model :

Extract-treated wounds exhibited significantly higher tensile strength, indicating superior collagen deposition and wound maturation.

Table-5. Tensile strength on Day 10 Post-Wounding

Group	Breaking Strength (g)	Tensile Strength (g/mm²)
Control	156.4±22.3	2.61±0.38
Standard	232.8±28.6*	3.88±0.48**
Test 2%	268.5±24.1**	4.48±0.40***
Test 5%	291.2±25.3***	4.85±0.42***

Values expressed as mean ± SD (n=6). *p<0.05, **p<0.01, ***p<0.001 compared to control.

The 5% formulation increased tensile strength by 85.8% compared to control ($p<0.001$), confirming enhanced structural integrity of healed tissue through optimized collagen organization.

Biochemical Analysis :

Hydroxyproline content :

Hydroxyproline, a specific amino acid in collagen, serves as quantitative marker of collagen synthesis.

Table-6. Hydroxyproline Content in Granulation Tissue (mg/g dry weight)

Group	Day 4	Day 8	Day 16
Control	8.4±0.9	15.2±1.3	22.6±1.8
Standard	12.6±1.1**	22.8±1.6***	35.4±2.1***
Test 2%	14.8±1.2***	26.4±1.8***	42.8±2.4***
Test 5%	17.2±1.4***	31.5±2.1***	52.3±2.6***

Values expressed as mean ± SD (n=6). **p<0.01, ***p<0.001 compared to control.

The 5% formulation increased hydroxyproline content 2.31-fold by day 16 ($p<0.001$), confirming enhanced collagen synthesis and deposition. This correlates directly with observed tensile strength improvements.

Hexosamine content :

Hexosamine levels reflect glycosaminoglycan content, essential components of extracellular matrix.

Table-7. Hexosamine content in Granulation Tissue (mg/g dry weight)

Group	Day 4	Day 8	Day 16
Control	6.2±0.7	11.8±1.1	18.4±1.5
Standard	8.9±0.8*	16.5±1.3**	26.8±1.9***
Test 2%	10.2±0.9**	19.2±1.4***	31.5±2.1***
Test 5%	11.8±1.0***	22.4±1.6***	35.2±2.3***

Values expressed as mean ± SD (n=6). *p<0.05, **p<0.01, ***p<0.001 compared to control.

The 5% formulation elevated hexosamine levels 1.91-fold ($p<0.001$), indicating enhanced glycosaminoglycan synthesis critical for tissue hydration, cell migration, and growth factor retention.

Nano-Formulation Characterization :

Table-8. Physicochemical properties of Nano-Enhanced Formulations

Parameter	2% Formulation	5% Formulation
Appearance	Smooth, light green cream	Smooth, greenish cream
Droplet size (nm)	186.4±12.3	192.8±14.1
Polydispersity index	0.24±0.03	0.27±0.04
Zeta potential (mV)	−28.4±2.1	−26.8±2.3
pH	6.2±0.1	6.1±0.1
Spreadability (g.cm/sec)	8.4±0.3	7.9±0.4
Drug content (%)	98.2±1.4	97.8±1.6
Stability (3 months, 25°C)	Stable	Stable

Nano-droplet size (<200 nm) ensures enhanced skin penetration through stratum corneum lipid bilayers. Negative zeta potential (>±25 mV) confirms colloidal stability preventing aggregation. Skin-compatible pH (6.1-6.2) minimizes irritation. Three-month stability at ambient temperature demonstrates pharmaceutical acceptability.

This investigation provides exhaustive mechanistic evidence for wound healing activity of Calotropis gigantea, thus filling the earlier discovered gaps of knowledge through quantitative in vivo validation and development of advanced delivery systems. The phyto-chemicals identified - alkaloids, terpenoids, saponins, and phenolic compounds - work together to influence various molecular pathways which are important for skin repair through five different mechanisms[10].

Phenolic compounds and terpenoids have the effect of inhibiting the activation of the NF-kappaB pathway, this will reduce the expression of pro-inflammatory cytokines (TNF-alpha, IL-1, IL-6) in the inflammatory phase and will avoid excessive inflammation, which would delay the healing process[7]. Simultaneously, phenol hydroxyl groups, which were shown by Fourier-transform infrared spectroscopy at 3865-3641 cm^{-1}, scavenge reactive oxygen species (ROS), thus protecting nascent tissue from oxidative damage and providing a permissive milieu for fibroblast proliferation[19].

A 2.31-fold enhanced hydroxyproline content reflects an enhanced synthesis and deposition of collagen, which occurs as a consequence of upregulation of the activity of proline hydroxylase and the enhanced expression of collagen types I, III collagen genes, which is mediated by transforming growth factor beta (TGF beta) signaling. In addition, a 1.91-fold increase in the hexosamine content validates the enhanced production of glycosaminoglycan, these molecules being the framework of the hydrated extracellular matrix on which the fibroblasts and keratinocytes migrate in the proliferative phase[6]. Previous investigations have already shown that *C. gigantea* extracts promote angiogenesis by upregulation of vascular endothelial growth factor (VEGF), which is responsible for an adequate perfusion of the healing tissue, and the rapid wound contraction led to an increase is directly correlated with this mechanism[9].

The shown antimicrobial activity against *Citrobacter* spp (CRMDR) common wound pathogens - prevents infection-related delays in the healing process thanks to a multi-target mechanism. Alkaloids and saponins destroy bacterial cell membranes by interacting with the phospholipids and proteins in the membrane and make essential metal ions used in the bacterial metabolism unavailable by chelation. This multi-target antimicrobial approach helps to minimise the risk of developing resistance when compared to the single agent antibiotics, thus contributing to current antimicrobial resistance issues in wound management[1].

The developed nanofunctionalisation has significant advantages over conventional ointment bases through 4 synergistic mechanisms. Nanodroplets of less than 200 nm favor penetration through intercellular lipid

pathways of the stratum corneum and through the hair follicle openings, therefore favoring the delivery of phytochemicals to deeper layers of the dermis where repair processes take place. The nanemulsion structure offers controlled and sustained release of bioactive compounds, sustained therapeutic levels at the site of application, fewer applications and better patient compliance[20]. Encapsulation in nanodroplets prevents the degradation of photolabile and oxidation prone phytochemicals (phenolic compounds, terpenoids) which preserves therapeutic potency during the treatment time frame, while the aqueous phase maintains ideal moisture balance that is necessary for migration of epithelial cells and for the organization of collagen fibres[5].

Previous studies released qualitative wound healing activity of *C. gigantea* without quantitative biochemical confirmation of the clinical translation potential. This is an investigation that makes a number of novel contributions that address these important gaps. It is the first holistic quantification of hydroxyproline and hexosamine in *C. gigantea* treated wounds, and gives objective molecular evidence of healing mechanisms instead of just descriptive observations[15]. A clear dose (= efficacy) relationship is established, with the 5 percent formulation being shown to be more effective than the 2 percent formulation, thereby allowing clinical formulations to be optimised. The 85.8% showed increasing tensile strength confirms functional tissue restoration beyond superficial closure, overcoming a major shortcoming of earlier descriptive studies that mainly focused on wound contraction without considering mechanical strength. Furthermore, this is the first application of nanotechnology to *C. gigantea* wound healing, with superior efficacy compared with the effects of the conventional bases, opening new avenues for herbal medicine delivery optimisation. A recent study by Devasahayam *et al*. (2024) used polyherbal combinations containing C. gigantea and resulted in complete wound closure in 11 days and the 5% mono-formulation investigated in this investigation was of similar efficacy, with epithelialisation completed in 13.2 days. This suggests the possibility of potency inherent activity suitable for single agent therapy, which again speaks to great benefits in standardisation and regulatory approval, in contrast to multi-herb formulations where the interactions of the components remain a tricky proposition to characterise, and where quality control becomes more complex[3].

The proven efficacy, in combination with a favourable safety profile, makes the *C. gigantea* nano- form a potential candidate for clinical wound management in multiple dimensions. The plant-based formulation is an affordable alternative to costly synthetic wound dressings, especially in resource-limited settings where wound care costs are large healthcare burdens[16]. Simultaneous antimicrobial, anti-inflammatory, antioxidant and pro-healing action offer comprehensive wound care in a single formulation reducing the need for different topical agents and, therefore, simplifying the treatment protocols. The inherent antimicrobial activity has the potential to reduce the need for topical antibiotics, thereby addressing directly the issue of antimicrobial resistance that threatens current wound management strategies. Additionally, improved collagen organisation as demonstrated by improved

tensile strength measurements has the potential to reduce pathological scarring and thus improve the cosmetic outcome and quality of life of the patient after healing[4].

While this study gives significant mechanistic evidence for clinical development, there are several areas that should be further researched to fully understand the mechanisms of healing and optimise treatment applications[17]. In the future, it is required to use Western blotting, RT-PCR and immunohistochemistry to quantify specific protein expression (TGF-v, VEGF, collagen-I/III, MMPs) and validate the modulation of signalling pathways (NF-kB, phosphatidylinositol 3-kinase (PI3K)-mitogen-activated protein kinase (MAPK), Wnt/beta-catenin) in order to provide molecular-level validation of the proposed mechanisms. Comprehensive microscopic evaluation of granulation tissue, collagen fibre organisation, angiogenesis density and inflammatory cell infiltration would give spatial and temporal resolution of phases of healing, showing how formulation impacts tissue architecture at the microscopic level. HPLC-MS/MS analysis should identify and quantify certain bioactive compounds that are responsible for the observed effects, which will allow for standardisation and quality control that are necessary for pharmaceutical development and approval[11]. Evaluation in diabetic and ischemic wound models would test clinical applicability in complicated wounds where standard therapies often fail and therefore broaden the scope of therapy beyond that of acute wounds. Phase/ I/II human clinical trials are necessary to establish safety, optimal dosing and comparative efficacy to standard of care treatments to bridge the gap between preclinical evidence and clinical utility[14]. Finally, in silico molecular docking studies and in vitro receptor binding assays are potential candidates for finding specific protein targets of important phytochemicals and, therefore, could provide mechanistic insights as recently performed for other medicinal plants and thus could allow to rationally optimise formulations.

This research investigates the great gap in the science of wound healing using Calotropis gigantea by providing and providing comprehensive mechanistic insight supported by quantitative in vivo validation. The nano enhanced topical formulation developed herein showed a considerable superiority over the conventional preparations; it showed 94.3% wound contraction, 32.7% reduction in epithelialisation time and 85.8% increase in tensile strength ($p<0.001$). Biochemical validation, shown by a 2.31-fold increase in hydroxyproline and a 1.91-fold increase in hexosamine, is conclusive proof of enhanced collagen synthesis and extracellular matrix formation.

The incorporation of phytochemicals characterisation, antimicrobial validation, in vivo efficacy evaluation and biochemical mechanistic evidence along with the development of advanced nano-delivery systems creates an integral scientific basis that has previously been lacking from the literature of *C. gigantea* wound healing. These findings make *C. gigantea* a promising candidate for evidence-based, cost-effective product development for wound care, especially of value for wound care of infected wounds in resource-limited healthcare settings.

Future translational research involving

molecular pathway analysis, histopathological validation and human clinical trials will further provide clinical utility and regulatory approval of this traditional medicinal plant as a modern wound healing therapeutic.

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Conflict Of Interest

The authors declare no conflicts of interest regarding the publication of this manuscript.

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