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"ANTI-INFLAMMATORY EFFICACY AND EMBRYOTOXICITY ASSESSMENT OF A NOVEL AZADIRACHTA INDICA–LIGNOCAINE GEL USING IN VITRO AND ZEBRAFISH MODELS FOR WOUND MANAGEMENT"

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Abstract

Background: Effective wound healing requires a balance of inflammation and oxidative stress control. Conventional wound-healing agents are often limited by cytotoxicity and teratogenicity, underscoring the need for safer alternatives. Azadirachta indica (neem) possesses antimicrobial and anti-inflammatory properties, while lignocaine provides local analgesia and anti-inflammatory effects. This study evaluated the anti-inflammatory efficacy and embryotoxic safety of a novel neem–lignocaine gel.

Methods: Neem extract was prepared and formulated with lignocaine into a gel. Anti-inflammatory activity was assessed in vitro using bovine serum albumin and egg albumin denaturation assays, along with human erythrocyte membrane stabilization, at concentrations of 10–50 µg/mL. Embryotoxicity was evaluated in zebrafish (Danio rerio) embryos exposed to 5–80 µg/mL, with survival, hatching, and morphological outcomes recorded. Cytotoxicity was preliminarily screened using the brine shrimp lethality assay.

Results: The neem–lignocaine gel exhibited significant dose-dependent inhibition of protein denaturation, reaching maximal effect at 50 µg/mL, and was comparable to standard anti-inflammatory agents. Membrane stabilization also matched the reference drug at higher concentrations. Brine shrimp lethality was minimal except at 80 µg/mL, indicating an acceptable safety margin. In zebrafish embryos, low concentrations (≤5 µg/mL) maintained >75% hatching and viability with normal development. However, higher concentrations (>40 µg/mL) produced reduced hatching rates (<40%) and morphological abnormalities, establishing a distinct therapeutic versus toxic dose range.

Conclusion: Neem–lignocaine gel demonstrated potent anti-inflammatory activity with acceptable safety at lower concentrations. Dose optimization is crucial, as embryotoxicity becomes significant at higher levels, supporting its therapeutic potential in wound healing.

Keywords: Neem, Lignocaine, Anti-inflammatory, Embryotoxicity, Zebrafish, Wound healing

INTRODUCTION

Wound infections continue to represent a major global health challenge due to the increasing burden of multidrug-resistant (MDR) bacteria and the formation of biofilms that limit the effectiveness of conventional antibiotics [1,2]. Topical antimicrobial formulations are preferred for wound care because they provide localized delivery, enhance drug concentration at the site of infection, and minimize systemic side effects [3].

Azadirachta indica (neem) has long been used in traditional medicine for its antimicrobial, anti-inflammatory, and antioxidant properties [4,5]. Neem-derived compounds such as azadirachtin, nimbidin, and quercetin have been shown to inhibit bacterial growth, modulate inflammatory responses, and accelerate wound healing [6]. Lignocaine, a widely used local anesthetic, not only provides analgesia but also possesses anti-inflammatory activity through stabilization of cell membranes and inhibition of pro-inflammatory mediator release [7].

The combination of neem and lignocaine in a gel formulation offers a potentially synergistic approach to wound management by addressing infection, inflammation, and pain simultaneously. However, the embryotoxicological safety of such herbal–synthetic combinations has not been adequately studied. Zebrafish (Danio rerio) embryos provide an ideal model for evaluating developmental toxicity, as they are genetically similar to humans, transparent, and suitable for high-throughput screening in compliance with OECD guidelines [8,9].

Therefore, the present study was designed to formulate a neem–lignocaine gel, evaluate its anti-inflammatory activity using established in vitro assays, and assess cytotoxicity and embryotoxicity profiles to determine its potential for safe wound-healing applications.

MATERIALS AND METHODS

Study Design

This experimental and comparative study was conducted over 6–8 months across laboratory, preclinical, and clinical settings. The investigation aimed to evaluate the antimicrobial, anti-inflammatory, antioxidant, and wound-healing properties of a novel Azadirachta indica–lignocaine gel and to compare its safety and efficacy with conventional lignocaine formulations.

Preparation of Neem Extract

Five grams of Azadirachta indica (neem) powder were weighed and mixed with 100 mL of distilled water. The mixture was heated at 50 °C for 20 minutes to facilitate phytochemical extraction, followed by filtration through muslin cloth. The filtrate was concentrated by gentle heating to a final volume of 10 mL and incorporated into the gel formulation with lignocaine.

In Vitro Anti-inflammatory Assays

Anti-inflammatory activity was assessed using three established assays:

- ✓ **Bovine Serum Albumin (BSA) Denaturation Assay**
- ✓ **Egg Albumin Denaturation Assay**
- ✓ **Human Red Blood Cell (RBC) Membrane Stabilisation Assay**

For protein denaturation assays, test concentrations (10–50 µg/mL) were prepared and mixed with protein solutions. After adjusting pH to 6.3, samples were incubated at room temperature for 10 minutes and heated at 55 °C for 30 minutes. Diclofenac sodium and DMSO served as positive and negative controls, respectively. Absorbance was measured at 660 nm, and percentage inhibition was calculated.

For the RBC membrane stabilisation assay, a 10% human RBC suspension was incubated with the test gel (10–50 µg/mL) at 37 °C for 30 minutes. Following centrifugation, haemolysis was quantified spectrophotometrically at 560 nm. All assays were conducted in triplicate.

Embryotoxicity Assay

Embryotoxicity was assessed using zebrafish (Danio rerio) embryos. Fertilised embryos (20 per well) were distributed in 6-, 12-, and 24-well plates containing E3 medium. Test concentrations (5, 10, 20, 40, 80 µg/mL) were dispersed by sonication. Embryos were incubated at 28 °C under a 14:10 h light/dark cycle, with medium renewed every 12 h and dead embryos removed. Endpoints included survival, hatching rate, and developmental abnormalities (yolk-sac oedema, spinal curvature) observed up to 96 h post-fertilisation and photographed.

Statistical Analysis

In vitro data were analysed using one-way ANOVA with Tukey's post hoc test. Embryotoxicity data were analysed using the Kruskal–Wallis test followed by Dunn's multiple comparison test. A p-value < 0.05 was considered statistically significant.

RESULTS

The Neem–Lignocaine gel was assessed for its anti-inflammatory and cytotoxic properties through a series of in vitro and in vivo assays.

In the BSA and Egg Albumin denaturation assays, the gel demonstrated a clear, concentration-dependent inhibition of protein denaturation. At the lower concentration of 10 µg/mL, inhibition rates were comparable to the standard reference drug, while maximum inhibition was observed at 50 µg/mL. These findings confirm the gel's strong anti-inflammatory effect, with efficacy on par with established therapeutic agents in preventing protein denaturation.

The membrane stabilisation assay further supported these observations. The gel exhibited a significant, dose-dependent stabilising effect on erythrocyte membranes within the 10–50 µg/mL range. At higher concentrations, inhibition values were nearly equivalent to those of the standard, underscoring the gel's ability to maintain membrane integrity and prevent haemolysis associated with inflammatory processes.

The Brine Shrimp Lethality Assay revealed that the gel possessed mild, concentration-dependent cytotoxicity. Nauplii survival was largely preserved at lower concentrations, with a gradual decline in viability at higher doses. Importantly, toxicity levels remained within acceptable biomedical safety margins, indicating that the formulation is biocompatible and suitable for further therapeutic evaluation. In zebrafish embryonic assays, the gel produced concentration-related developmental effects. At lower doses, embryos developed normally and progressed through standard stages without abnormalities. In contrast, higher concentrations were associated with delayed hatching, pericardial oedema, and spinal deformities, suggesting potential teratogenic effects at elevated levels. Embryo hatching and viability both declined in a dose-dependent fashion, with minimal toxicity observed below 10 µg/mL, whereas doses ≥40 µg/mL resulted in marked embryotoxicity.
Collectively, these findings highlight the Neem–Lignocaine gel as a promising formulation with strong anti-inflammatory potential and acceptable biocompatibility, while emphasising the importance of dose optimisation to mitigate developmental toxicity.

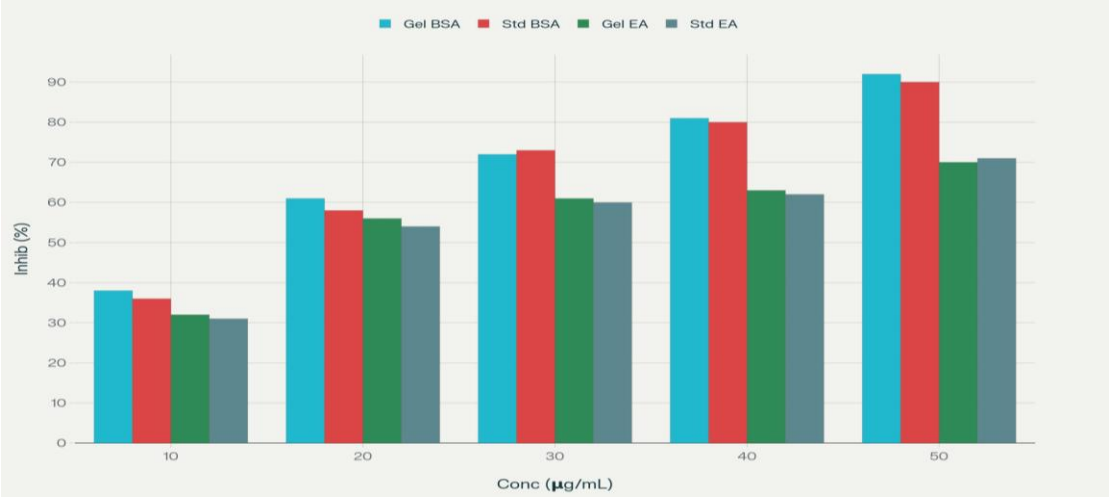

Figure 1.Inhibition of Protien denaturation across various concentrations

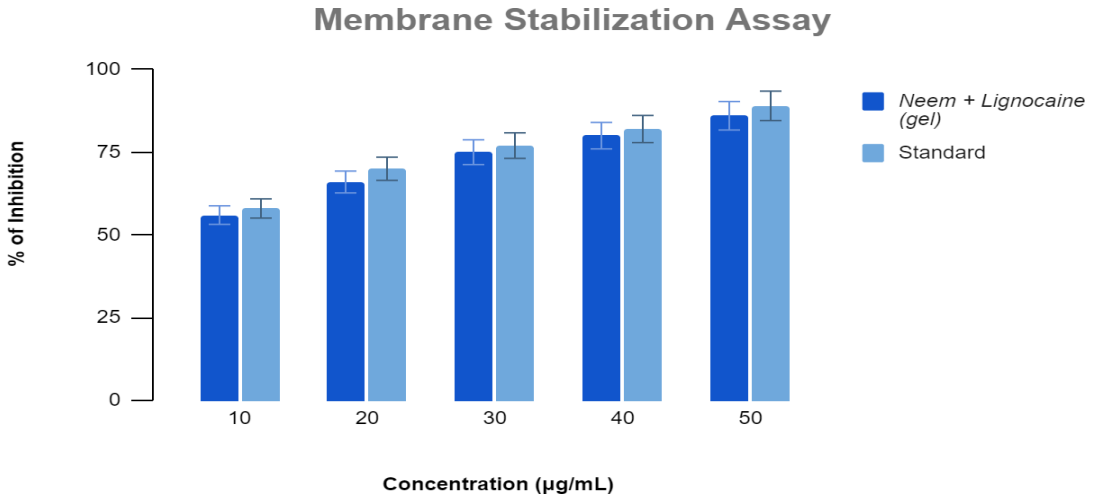

Figure 2: Membrane stabilisation Assay showing the percentage inhibition of protein denaturation

DISCUSSION

This study evaluated the anti-inflammatory efficacy and safety profile of a novel Neem (Azadirachta indica)–Lignocaine gel formulation, highlighting its potential as a dual-purpose topical agent for wound management. Anti-inflammatory activity was assessed using Bovine Serum Albumin (BSA) and Egg Albumin denaturation assays, which model the protein denaturation processes occurring during inflammation [12,13]. Protein denaturation is a hallmark of inflammatory conditions, as it disrupts normal cellular function and increases vascular permeability.

The results demonstrated dose-dependent inhibition of protein denaturation, with maximal effect at 50 µg/mL, matching or exceeding the standard drug control. The Egg Albumin assay supported these findings, showing minimal activity at 10 µg/mL that progressively increased at 20–50 µg/mL, achieving equivalence with the reference drug at 50 µg/mL. These results suggest that the bioactive compounds in Neem—such as nimbidin, nimbin, and quercetin—combined with lignocaine, act synergistically to stabilise proteins and exert anti-inflammatory effects comparable to conventional drugs [14–16].

The membrane stabilisation assay confirmed that the gel effectively inhibited heat-induced erythrocyte haemolysis in a dose-dependent manner, similar to the standard. This membrane-protective effect is advantageous for wound healing, as it may reduce tissue damage and support cellular repair processes [17,18].

Cytotoxicity evaluation using the Brine Shrimp Lethality Assay indicated that the gel is largely biocompatible. While nauplii survival declined at higher concentrations, only the 80 µg/mL dose on day 2 showed mild cytotoxicity, whereas lower concentrations maintained high viability. This aligns with established correlations between brine shrimp and mammalian toxicity, validating the assay as an initial screening tool [19,20].

Zebrafish embryos were employed for detailed toxicological assessment due to their transparency and developmental similarity to higher vertebrates [21]. At concentrations ≤5 µg/mL, embryos developed normally with proper segmentation and organogenesis. Higher doses caused teratogenic effects, including delayed hatching, pericardial oedema, spinal deformities, and reduced motility, with hatching and viability rates declining sharply at concentrations ≥20 µg/mL and severe toxicity at 80 µg/mL [21,22]. The embryotoxicity is likely mediated by Neem bioactives such as azadirachtin and nimbin, while overdoses of lignocaine may further contribute to developmental disruption.

These findings establish a therapeutic window of 10–50 µg/mL, with minimal toxicity observed at ≤20 µg/mL, supporting safe application in wound healing. Overall, Neem–Lignocaine gel exhibits promising anti-inflammatory activity while demonstrating acceptable biocompatibility, though careful dose optimisation is crucial to avoid potential embryotoxic effects in vulnerable populations.

CONCLUSION

Neem–Lignocaine gel demonstrated potent anti-inflammatory activity across multiple in vitro assays, effectively stabilising proteins and cell membranes in a dose-dependent manner. Cytotoxicity evaluation using brine shrimp and zebrafish embryos indicated that the formulation is biocompatible at lower concentrations (≤20 µg/mL), while higher doses may induce embryotoxic effects, including delayed hatching and morphological abnormalities. These findings define a clear therapeutic window, supporting the gel's potential as a safe and effective topical agent for wound healing. Careful dose optimisation is essential, especially for use in sensitive populations, to balance efficacy with safety. Overall, this novel Neem–Lignocaine formulation offers a promising alternative to conventional synthetic agents, combining natural anti-inflammatory properties with analgesic benefits for enhanced wound care.

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