# Wound Healing Activity of Formulated NipaAlco Gel on Chronic-Binge Ethanol-Fed Male ICR Mice (*Mus musculus* L.)

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## ABSTRACT

Chronic binge alcohol consumption impairs immune function and delays tissue repair, thereby increasing the risk of infection and prolonging wound healing. The present study evaluated the efficacy of a bio-based NipaAlco Gel, formulated with *Citrus reticulata* peel extract, as a potential alternative treatment to enhance wound healing under chronic-binge exposure through continuous feeding and oral gavage for 30 days. The wound-healing efficacy of the formulated NipaAlco Gel was evaluated through in vitro and in vivo assays using 12 mice divided into four groups (n = 3 per group), with the data analyzed using analysis of variance (ANOVA). Phytochemical analysis of *C. reticulata* peel extract confirmed the presence of alkaloids, flavonoids, phenols, saponins, and tannins. Gel formulations, NipaAlcoMHX and NipaAlcoCitro, were prepared at varying concentrations. Antimicrobial testing showed that AlcoMHX Gel 3% was the best-performing formulation, producing the largest inhibition zones against *Staphylococcus aureus* (25.10 mm), *Escherichia coli* (31.33 mm), and *Candida albicans* (20.33 mm). The 3% gel formulations exhibited optimal bactericidal and fungicidal effects, as indicated by Minimum Inhibitory Concentration, Minimum Bactericidal Concentration, and Minimum Fungicidal Concentration results. In vivo qualitative and quantitative assessments showed that all treatment groups exhibited progressive wound healing during the 30-day observation period. Although the negative control also showed wound closure by Day 30, healing was slower and less pronounced compared with the treated groups. This indicates that the treatment effect was modest but still observable, as treated wounds demonstrated earlier improvement, faster reduction in wound area, and better healing progression than the untreated control. Histological analysis showed that skin treated with NipaAlcoCitro and NipaAlcoMHX gels achieved fair to good wound healing, comparable to the positive control, Povidone-Iodine Ointment in this study. These findings suggest that the formulated gels may possess wound-healing properties comparable to those observed with the positive control under the conditions tested.

***Keywords***: *Citrus reticulata, Nypa fruticans, phytochemical, antimicrobial, wound-healing, chronic-binge*

## Introduction

Alcohol use, especially chronic binge drinking, is a major public health issue because it increases the risk for many health problems. According to the World Health Organization's International Classification of Diseases, 10th Edition, alcohol is a necessary cause for over 30 conditions and contributes to many more (Rehm, 2011). Health problems linked to alcohol include infectious diseases, cancer, diabetes, mental health disorders, heart disease, liver and pancreas disease, and both accidental and intentional injuries. One way alcohol raises disease risk is by harming the immune system, especially with heavy drinking. Alcoholism makes it harder for the body to repair tissue by increasing the risk of infections and lowering the number of cells in both the innate and adaptive immune systems. Chronic binge drinking causes tissue damage with serious medical effects, and the amount and length of exposure affect how severe the injury is and how long recovery takes (Morguette et al., 2023). While researchers have learned a lot about how alcohol disrupts molecular processes, the exact ways alcohol causes tissue injury are still being studied (Jung et al., 2011; Kumar et al., 2013).

Alcohol affects nearly every organ and tissue in the body through multifactorial effects on cellular and molecular functions (Greiffenstein and Molina, 2008). Consequently, it targets a wide range of physiological processes. Some mechanisms underlying alcohol-induced tissue damage are common across multiple tissue types, while others are tissue-specific. The present study evaluates a product with the potential to facilitate and accelerate skin tissue repair (Deng et al., 2022) in chronic-binge ethanol-fed Institute of Cancer Research (ICR) mice (*Mus musculus* L.). Mammalian skin consists of three layers: the epidermis, which forms the surface; the dermis; and the deep hypodermis (Albahri et al., 2023).

As the largest organ of the body, the skin serves as the outer covering and comprises multiple layers of ectodermal tissue that protect underlying muscles, bones, ligaments, and internal organs (Edwards and Harding, 2004; Menke et al., 2007). It also provides defense against microbes, heat, light, and injury. "Tissue repair" refers to the complex, multifactorial process that occurs in response to disruption of the normal anatomical structure and function of skin tissue, typically described as a succession of three overlapping phases: inflammation, tissue formation (or proliferation), and tissue remodeling (Gosain and DiPietro, 2004; Keylock et al., 2008).

The inflammation phase is sometimes subdivided into a vascular response (hemostasis) and a cellular response (inflammation). This phase involves platelet activation, leading to fibrin clot formation and secretion of chemotactic agents, followed by recruitment of neutrophils to cleanse the wound of foreign particles, including bacteria, T-cells to sustain inflammation and recruit monocytes, and activation of macrophages. While these innate physiological wound healing mechanisms can occur without exogenous treatments (Dehdashtian et al., 2018), the completion of full-thickness chronic wound healing may require extended periods, increasing susceptibility to infection due to open wounds and potentially disrupting timely healing processes, thereby extending tissue damage (Su and Richmond, 2015). Moreover, bio-based product derived from local beneficial fruit, which may have a potential anti-inflammatory, antioxidant properties, antimicrobial activities against common pathogens, and pro-collagen synthesis properties that may enhance the skin tissue repair process (Bilal et al., 2024).

Plants and their extracts have been utilized as traditional medicine throughout

history due to their diverse chemical constituents (Taamalli et al., 2019; Cedillo-Cortezano et al., 2024). Plant-derived extracts serve as sources of biologically active compounds and have been employed in the treatment of various diseases (Ennajar et al., 2009; Okon et al., 2015). The World Health Organization (WHO) estimates that 80% of the global population relies on traditional herbal medicines as a primary form of health care. Citrus fruits are primarily consumed as juice or in desserts, and citrus processing industries generate substantial quantities of by-products (50% of the fruit), including peels, seeds, and pulp (Dahmani et al., 2020). Citrus peels are rich in components such as flavonoids, vitamin C, carotenoids, dietary fibers, and essential oils, which are recognized for their pharmacological properties and health benefits (Barros et al., 2007). Wound healing is promoted by bioactive compounds such as triterpenoids, alkaloids, and biomolecules found in many plant natural products, which typically influence one or more phases of the tissue repair process (Ishfaq et al., 2020).

In response to the alcohol shortage during the COVID-19 pandemic, Mariano Marcos State University (MMSU) developed an alternative disinfectant, Nipa bioethanol (Nipahol), by distilling sap from *Nypa fruticans*. This initiative, conducted through the Saranay Against COVID-19 project at the National Bioenergy Research and Innovation Center (NBERIC), demonstrated the antimicrobial potential of nipa-derived ethanol. Building on this innovation, the present study explores a bio-based formulation combining Nipa bioethanol with peel extract from *C. reticulata* and MMSU Herbal X. This combination is hypothesized to enhance wound healing through synergistic antimicrobial and bioactive effects.

Accordingly, this study aimed to evaluate the wound healing potential of the formulated NipaAlco Gel. Specifically, the objectives were to: (1) identify the bioactive compounds present in *C. reticulata* peel extract; (2) develop gel formulations incorporating Nipa bioethanol, citrus peel extract, and MMSU Herbal X; (3) assess antimicrobial activity using standard microbiological assays; and (4) evaluate wound healing efficacy through morphological, quantitative, and histological analyses in chronic-binge ethanol-fed Institute of Cancer Research (ICR) mice (*Mus musculus* L.).

The findings provide preliminary evidence suggesting the potential of NipaAlco Gel as a cost-effective, plant-based wound-healing formulation for alcohol-impaired healing conditions. Considering the exploratory nature of the study, the limited sample size, and the absence of a vehicle-only gel base control, further studies with larger animal groups and appropriate control formulations are recommended. Furthermore, this research provides a scientific basis for the utilization of agricultural by-products such as citrus peels and supports future investigations into natural product-based interventions for tissue repair.

## Methods

### Locale of the Study

The formulation of NipaAlcoCitro Gel and in vitro assessments were conducted at Mariano Marcos State University-National BioEnergy Research Innovation Center. The in vivo experimental setup was conducted at the MMSU Laboratory Animal Care Facility (LACF). Histological tissue preparation was performed at the MMSU-College of Arts and Sciences (MMSU-CAS) BioComputing Laboratory. Histological analysis and slide interpretation were completed at the Mariano Marcos Memorial Hospital & Medical Center (MMMH & MC), Department of Pathology and Laboratories, from October 2021 to March 2022.

### *Design of the Study*

A Completely Randomized Design (CRD) with four groups was employed in this study. Each group consisted of three male ICR mice and received different treatments: Control (negative) - untreated; Treatment II (T2) - topical application of NipaAlcoCitro Gel for 30 days; Treatment III (T3) - topical application of NipaAlcoMHX Gel for 30 days; and Control (positive) – topical application of Povidone-Iodine Ointment for 30 days.

**Table1.** Composition of NipaAlcoCitro and NipaAlcoMHX Gel Formulations

| Formulation | Active component | Active concentration | Estimated active ingredient content per gram of gel | Gel base/excipients |
|---|---|---|---|---|
| NipaAlcoCitro Gel 1% | *C. reticulata* peel extract | 1% w/w | 10 mg/g gel | Carbomer, distilled water, triethanolamine, glycerin/fixative/fragrance |
| NipaAlcoCitro Gel 2% | | 2% w/w | 20 mg/g gel | |
| NipaAlcoCitro Gel 3% | | 3% w/w | 30 mg/g gel | |
| NipaAlcoMHX Gel 1% | MMSU Herbal X | 1% w/w | 10 mg/g gel | |
| NipaAlcoMHX Gel 2% | | 2% w/w | 20 mg/g gel | |
| NipaAlcoMHX Gel 3% | | 3% w/w | 30 mg/g gel | |

Histomorphological changes, including wound contraction diameter and histological analysis, were assessed in the wounds of binge ethanol-fed ICR mice treated with the formulated NipaAlcoCitro Gel presented in Table 1. Data were compared to results from chronic-binge ethanol-fed ICR mice that were untreated, treated with NipaAlcoMHX Gel, or treated with the commercially available drug Povidone-Iodine Ointment. Twelve male ICR mice were obtained from LACF. The study was conducted in accordance with the university's Institutional Animal Care and Use Committee (IACUC) and the Bureau of Animal Industry (BAI) with a Ref No. *AR-2022-003*.

### *Data collection & analysis*

For phytochemical analysis, Fresh *Citrus reticulata* fruits of good quality were thoroughly washed with tap water to remove dirt and foreign particles. The peels were manually separated using a sterile knife through vertical cutting motions, rinsed again with tap water, and drained to remove excess moisture. The collected peels were oven-dried for two (2) days until completely dry and subsequently ground using a blender to obtain a coarse powder. Soxhlet extraction was then performed using 500 g of the dried peel powder placed in a glass thimble and extracted with ethanol as the solvent in a Soxhlet apparatus at the MMSU Crop Research Laboratory-Tuklas Lunas Development Center. The extraction temperature was maintained just below the boiling point of ethanol (78.37 °C), and the process was carried out for several cycles until exhaustive extraction was achieved. The resulting extract was concentrated using a rotary evaporator and prepared for subsequent phytochemical screening and formulation of the NipaAlcoCitro gel.

For in vitro, Nipa bioethanol was readily obtained from NBERIC, and *Citrus reticulata* fruits in good condition were acquired from the local market of San Nicolas, Ilocos Norte, Philippines. The pre-screened MMSU Herbal X was procured from the MMSU Tuklas Lunas Development Center. The *C. reticulata* ethanolic extract and MMSU Herbal X were infused at different concentrations (Misra et al., 2018). Using a precision balance, 2.34g of thickening agent Carbomer was weighed and placed into a beaker. It was mixed with 229 mL of distilled water and stirred on a magnetic stirrer at room temperature. Once dissolved, 1 mL of Triethanolamine, a stabilizer, was added to neutralize the gel

mixture. The desired amount of Nipa was carefully measured and thoroughly mixed with the ethanolic extract of *C. reticulata*. The gel was stirred until the mixture became homogenous. It was checked for undissolved particles before adding 10ml of fixative agent, fragrance, and glycerin. After which, the mixture was divided into 100ml bottles for packaging. The same procedure was used to prepare NipaAlcoMHX Gel.

The antimicrobial potency of the formulated NipaAlcoMHX Gel, NipaAlcoCitro Gel, and their individual components was tested against clinically isolated and relevant microorganisms commonly identified in wounds, including *Staphylococcus aureus, Escherichia coli,* and *Candida albicans.* Using a digital balance (Aryal, 2015), 38 g of Mueller-Hinton Agar (MHA, CM0337B) was weighed and added to a sterile 1000 ml beaker. One liter of distilled water was added to dissolve the mixture, which was then transferred to a sterile flask and autoclaved at 121°C. After autoclaving, the agar was poured into sixty Petri plates, each with a depth of 4 to 5 mm, and allowed to solidify. For fungal assays, 39 g of Potato Dextrose Agar (PDA) was weighed and placed into another sterile 1000 ml beaker, followed by the addition of 1 liter of distilled water. The solution was heated and mixed until fully dissolved, transferred to a sterile flask, and autoclaved at 121°C. The agar was then plated into thirty Petri plates. The prepared MHA and PDA plates were inoculated with the test microorganisms. A sterile borer was used to create cavities with a 6.5 mm in diameter in each agar plate, which were then filled with formulated NipaAlcoCitro Gel at different concentrations, *C. reticulata* ethanolic extract, MMSU Herbal X, or Nipa bioethanol. Plates were sealed, labeled according to the test microorganism and content, and incubated for 24 hours. The diameter of the zone of inhibition (ZOI) was measured using a digital caliper (mm).

Subsequently, a minimum inhibitory concentration (MIC) assay was conducted. Microbial inocula were standardized prior to testing to ensure uniformity across assays. Turbidity of bacterial suspensions was adjusted to approximately 0.5 McFarland standard, corresponding to an estimated concentration of $1.5 \times 10^8$ CFU/mL. This standardization ensured comparability of antimicrobial activity across treatments. Two milliliters of each formulated concentration of NipaAlcoCitro Gel and NipaAlcoMHX Gel were introduced into sterile test tubes containing Nutrient Broth (NB) for bacteria and Potato Dextrose Broth (PDB) for fungi. One tube containing only broth and the microorganism served as the negative control, while another tube containing broth and each sample served as the positive control. Tubes were incubated at 37°C for 24 hours and examined for visible growth or turbidity. All tubes were compared qualitatively with the positive and negative controls. For the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) assays, a portion from each MIC test tube was plated on prepared Nutrient Agar (NA) and PDA plates, incubated, and inspected for colonies after 24 hours, where it was observed aseptically.

For the in vivo phase, 12 male ICR mice of the same age were used and housed at LACF. Prior to the study, monitoring review and approval were obtained from the university's IACUC , and all necessary parameters for study completion were discussed with BAI ().

After a 5-day acclimatization period having 1%, 2%, 3% and 4%, respectively, the ICR mice were introduced to chronic-binge ethanol feeding and were ready for the administration of different treatments. Mice were provided with 5% ethanol in drinking bottles for one month. Food, in the form of 40g Bio feeds/rodent (GMP3) consisting of crude protein, crude fat, calcium and phosphorus which was administered to the

mice once a day in each meal. Meanwhile, binged ethanol-feeding was administered early in the morning every 7 days of the month. 5% ethanol was administered to ICR mice through oral gavage with a ball-ended feeding needle. The size of the oral gavage was carefully selected in consideration of the ICR mice's body. The chronic-binge ethanol feeding model used in this study was adapted from the National Institute on Alcohol Abuse and Alcoholism (NIAAA) model (Bertola et al., 2013), with minor modifications, as illustrated in Figure 1. In the figure, the vertical lines represent the schedule of ethanol administration via oral gavage, which was carried out once weekly (7:00–9:00 AM) throughout the experimental period. Although five ethanol-fed phases are depicted to illustrate continuous exposure, only four binge ethanol administrations were performed within the one-month duration of the study. After completion of the ethanol feeding protocol, full-thickness wounds (approximately 1 cm in diameter) were created on the dorsal-lumbar region of the mice to simulate alcohol-impaired wound conditions. Wound healing assessment, along with topical treatment application, was initiated immediately after wound induction and continued over the 30-day observation period.

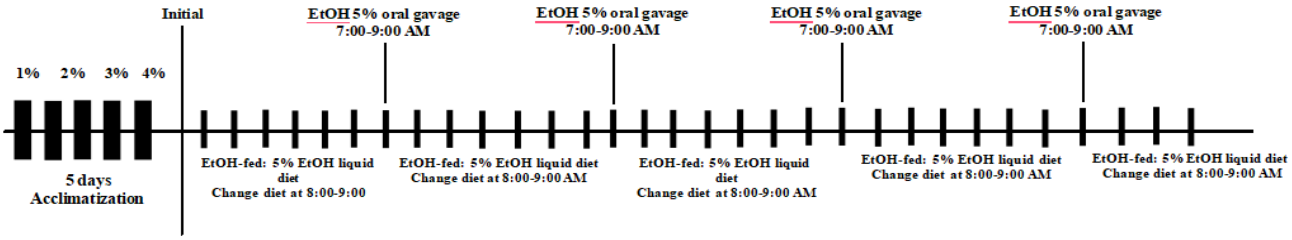



**Figure 1.** Modified protocol of the National Institute on Alcohol Abuse and Alcoholism (NIAAA) chronic-binge ethanol feeding model. Source: Adapted from Bertola et al. (2013).

All experimental animals in every group were locally anesthetized with Lidocaine. Hair clipping and disinfection in the target area using cotton soaked in alcohol were performed prior to excision of the skin, 1cm in diameter, with uniform depth using a digital caliper. In accordance with IACUC guidelines, an incision was made on the dorsal-lumbar region of the animal using curved iris scissors.

Twelve test animals were randomly assigned to four groups, each with three replicates. The concentrations of NipaAlcoCitro Gel and NipaAlcoMHX Gel from Phase I that demonstrated the most promising antimicrobial properties were used in this phase. One gram of gel (pH 6.0–6.5) was applied once daily following treatments for smooth application and safer for skin irritation: Control (negative) – untreated; Group II – topical application of NipaAlcoCitro Gel for 30 days; Group III – topical application of NipaAlcoMHX Gel for 30 days; and Control (positive) – topical application of Povidone-Iodine Ointment for 30 days.

A Vernier caliper was used to measure the wound contraction every 2 days of the observation period. The reduction of the wound area was calculated using the formula by Rabanal (2007):

$$\%\ Healing = \frac{Initial\ wound\ diameter\ (mm) - Final\ wound\ diameter}{Initial\ wound\ diameter\ (mm)} \times 100$$

The dynamic process of wound healing has various phases, knowledge of which is essential for identifying the pathology involved in a chronic, intractable wound. Various instruments for assessing wound healing have been described, primarily for clinical evaluation of the wound. However, very few instruments are currently available

for histological grading of wounds (Gupta, A. & Kumar, P., 2015). Qualitative assessment of the wound-healing activity of the formulated NipaAlcoCitro Gel was conducted using the wound-healing morphological criteria described by Domingo et al. (2024), which classify wound progression based on visible changes such as redness, swelling, moisture, pus formation, scar development, dryness, hair regrowth, and complete wound closure. Wound descriptions ranged from very red, very swollen and moist; red, swollen, moist, presence of pus; red, slightly swollen and moist; swollen, dry surface; scar formation, dry surface; to presence of hair, wound healed completely. The initial state of the wounds was recorded as baseline shown in Table 2 (Kumar and Gupta, 2015).

**Table 2.** Individual Criteria for Healing Scoring of Cutaneous Tissue.

| Number | Histological Parameter |
|---|---|
| 1 | Amount of granulation tissue (profound-1, moderate-2, scanty-3, absent-4) |
| 2 | Inflammatory infiltrate (plenty-1, moderate-2, a few-3) |
| 3 | Collagen fiber orientation (vertical-1, mixed-2, horizontal-3) |
| 4 | Pattern of collagen (reticular-1, mixed-2, fascicle-3) |
| 5 | Amount of early collagen (profound-1, moderate-2, minimal-3, absent-4) |
| 6 | Amount of mature collagen (profound-1, moderate-2, minimal-3) |

All 12 ICR mice were dissected after one month of treatment. One skin tissue section was collected from each mouse, resulting in a total of 12 skin tissue sections for histological evaluation, with three sections representing each treatment group, then placed and preserved in a 10% Neutral Buffered Formalin for histological examination. The skin tissue was fixed overnight at room temperature. Upon completion, the sections were thoroughly washed in water to remove the fixative and subjected to 70% ETOH for 15 min, 90% ETOH for 15 min, 95% ETOH for 15 min, followed by three changes of absolute ETOH for 15 min, 30 min, and 45 min, respectively, before proceeding to the clearing method. Since xylene was used as the clearing agent, the procedure was performed in a fume hood. Specimen-filled cassettes were subjected to a series of xylene changes, immersed in molten paraffin wax for two consecutive 20-min changes, and followed by a final 45 min infiltration. Sections were transferred from the cassettes and oriented in an embedding block using paraffin wax. The skin tissue was molded in a sterile molder. Labelled cassettes were placed on top of the molder it corresponds to, topped with more wax, and allowed to solidify. Once embedded, the tissues were sectioned using a rotary microtome. The ribbons were transferred into a 60°C to 70°C water bath. Once the tissues floated to the surface, rough-edged frozen slides were used to scoop them up. The slides were placed on top of a digital hotplate at 80°C for 1h to remove the paraffin wax. Thereafter, they were soaked in xylene for 25 min, absolute ETOH for 3 min, 90% ETOH for 3 min, 80% for 3 min, 70% ETOH for 3 min, and finally distilled water for 3 min. Excess water was wiped before staining. During staining and mounting, haematoxylin was applied to the tissue sections and allowed to stand for 5 min, followed by the bluing reagent for 10 to 15 sec, and rinsing with distilled water. The slides were dipped in absolute ETOH. Excess alcohol was blotted dry. Subsequently, eosin was dropped on the tissue slide and allowed to settle for 2 to 3 min. Finally, the slides were immersed in three batches of absolute ETOH and blotted dry before a mount was applied to the tissue slide and coverslip. All slides were kept in

serial order throughout the process. These procedures were performed at the MMSU-CAS Bio-Computing Laboratory and the MMMH & MC Department of Pathology and Laboratories for histological blinded scoring.

Two-way analysis of variance (two-way ANOVA) was employed to analyze differences in the ZOI of samples against clinically important microorganisms, the initial and final weights (g) of the experimental animals, and the data obtained across the different treatments in the in vivo phase.

## Results and Discussion

Preliminary tests were done before proceeding to the *in-vivo* phase of the study to evaluate various biological phenomena in specific environments and specific cells without the distractions and potential confounding variables present in the whole organism, in this context, ICR Mice. Part of the Phase I of the study is the phytochemical analysis of the *Citrus reticulata* ethanolic peel extract derived from Soxhlet extraction. The results are presented in Table 3.

**Table 3**. Qualitative and Quantitative Phytochemical Analyses of *Citrus reticulata* peel Ethanolic extract

| Target Phytochemical Compound | Method | Result | |
|---|---|---|---|
| | | **Qualitative (+/-)** | **Quantitative (+/-)** (based on the slope of the standard calibration at y=mx+b) |
| **Alkaloids** | Dragendroff's, Wagner's, and Mayer's Tests | + | Undetermined |
| **Flavonoids** | Shinoda Method | + | + Absorbance: 0.500 TFC (µg/ml): 77.18 |
| **Saponins** | Sodium Bicarbonate Test | + | Undetermined |
| **Tannins** | Ferric Chloride Test | + | Undetermined |
| **Phenols** | Folin-Ciocalteau and Aluminum Chloride Method | + | + Absorbance: 2.442 TPC (µg/ml): 304.10 |

Legend:
Positive/Present +
Negative/Absent -

Preliminary qualitative and quantitative phytochemical analyses of the ethanolic peel extract of *Citrus reticulata* (Table 3) confirmed the presence of alkaloids, flavonoids, saponins, tannins, and phenols, consistent with findings reported by Okon et al. (2015). Quantitative determination of total flavonoid content (TFC) and total phenolic content (TPC) was performed using quercetin and gallic acid standard calibration curves, respectively, following established protocols (Mahboubi et al., 2012). These phytochemical groups have been widely reported in the literature as potential sources of natural antioxidants and bioactive compounds (Saxena et al., 2013); however, it is important to note that the present study only confirmed their presence through screening and did not directly evaluate their individual contributions to the

observed antimicrobial and wound healing activities. The use of ethanol as the extraction solvent was based on its polarity and efficiency in extracting a broad range of plant constituents, which may contribute to higher extraction yield compared to less polar solvents (Das et al., 2010). These findings are consistent with previous reports indicating that organic solvent extracts often exhibit more consistent antimicrobial activity than aqueous extracts (Parekh et al., 2005). Nonetheless, the observed bioactivity in this study may be attributed to the combined or synergistic effects of the formulation components rather than to any single class of secondary metabolites. Therefore, the role of these phytochemicals should be interpreted as a potential contributing factor rather than definitive evidence of causation.

***Inhibitory effect of individual components used in NipaAlco gel formulation***

Table 4 presents the inhibitory effects of the individual components used in the formulation of NipaAlcoCitro Gel and NipaAlcoMHX Gel against clinically relevant microorganisms commonly found in mammalian cutaneous tissue. These microorganisms include *S. aureus, E. coli*, and the fungus *C. albicans*. CrPE and MHX did not exhibit inhibitory effects *on S. aureus* and *E. coli*, respectively, but both demonstrated inhibitory activity against *C. albicans*.

**Table 4.** Zone of Inhibition (mm) of the individual components against selected microorganisms

| Sample being tested | *S. aureus* | *E. coli* | *C. albicans* |
|---|---|---|---|
| **CrPE** | $0.0^{b}$ | $17.43^{a}$ | $14.53^{a}$ |
| **MHX** | $15.70^{a}$ | $0.00^{c}$ | $28.63^{b}$ |
| **Nipa Bioethanol** | $18.67^{a}$ | $8.00^{b}$ | $17.9^{a}$ |
| **Significance** | * | * | * |

Legend:
** - highly significant at 1% level of significance
* - significant at 5% level of significance
ns - not significant

Table 5 presents the inhibitory effects of various concentrations of NipaAlcoCitro Gel and NipaAlcoMHX Gel against clinically relevant microorganisms commonly found in mammalian cutaneous tissue, including *S. aureus*, *E. coli*, and the fungus *C. albicans*. Among the AlcoCitro Gel formulations, the 2% concentration exhibited the highest zone of inhibition (ZOI) against *S. aureus* at 24.97 mm. Similarly, AlcoMHX Gel at 3% demonstrated the highest ZOI among its formulations, measuring 25.10 mm. For *E. coli*, AlcoCitro Gel at 1% showed the highest ZOI of 25.83 mm, while AlcoMHX Gel at 2% produced a ZOI of 21.90 mm. Against *C. albicans*, AlcoCitro Gel at 2% achieved the highest ZOI of 13.20 mm, and AlcoMHX Gel at 3% reached 20.33 mm. Data from Table 1 indicate that the primary individual components, such as pure *C. reticulata* peel extract and pure MMSU Herbal X, did not exhibit inhibitory effects against *S. aureus* and *E. coli*, respectively. In contrast, antimicrobial activity was observed when these components were formulated as gels, likely due to the inclusion of Nipa bioethanol, which demonstrated significant inhibitory effects against the tested microorganisms. Two-way analysis of variance (two-way ANOVA) revealed that the inhibitory effects of different concentrations of both AlcoCitro Gel and AlcoMHX Gel were significantly different for *E. coli*, but not for *S. aureus* and *C. albicans*. These findings suggest that each formulation exerts a distinct effect on the bacterial species tested. Therefore, the formulations exhibiting the highest ZOI were selected for subsequent microbiological assays in the study. Sotirus (2016) reported

that nipa underwent phytochemical screening and demonstrated antibacterial activity against *S. aureus* and *E. coli.* The study identified triterpenoids, flavonoids, glycosides, saponins, and tannins as compounds contributing to the antibacterial properties of nipa. Consequently, the inclusion of nipa bioethanol as a primary component in the formulated gel products likely contributed to their observed inhibitory effects against these bacterial species.

**Table 5.** Zone of Inhibition (mm) of the different formulations of Alco Gel against selected microorganisms.

| Sample being tested | *S.aureus* | *E. coli* | *C. albicans* |
|---|---|---|---|
| **AlcoCitro Gel (1%)** | 23.17$^{a}$ | 25.83$^{ab}$ | 12.27$^{a}$ |
| **AlcoCitro Gel (2%)** | 24.97$^{a}$ | 24.07$^{ab}$ | 13.2$^{a}$ |
| **AlcoCitro Gel (3%)** | 19.97$^{a}$ | 18.07$^{abc}$ | 12.7$^{a}$ |
| **AlcoMHX Gel (1%)** | 20.27$^{a}$ | 21.00$^{ab}$ | 13.53$^{a}$ |
| **AlcoMHX Gel (2%)** | 19.57$^{a}$ | 21.90$^{ab}$ | 9.87$^{a}$ |
| **AlcoMHX Gel (3%)** | 25.10$^{a}$ | 31.33$^{a}$ | 20.33$^{a}$ |
| **Significance** | ns | ** | ns |

Legend:
** - highly significant at 1% level of significance
* - significant at 5% level of significance
ns - not significant

This result suggests that the antimicrobial activity of the formulated gels was not solely dependent on the individual plant-derived components, but may have resulted from the combined action of the formulation matrix. In Table 3, CrPE showed no inhibition against *S. aureus*, while MHX showed no inhibition against *E. coli*; however, Nipa bioethanol itself produced measurable inhibition against all tested microorganisms. When incorporated into the gel system, Nipa bioethanol may have acted not only as an antimicrobial component but also as a solvent or carrier that improved the dispersion, release, and possible penetration of bioactive compounds from CrPE and MHX. Thus, the observed activity of the formulated gels may reflect an additive or synergistic interaction among Nipa bioethanol, the plant extract, and the gel base rather than the activity of a single component alone. However, this interpretation remains preliminary because the present study did not include a gel-base-only control or formal synergy testing; therefore, future studies should include checkerboard assays, fractional inhibitory concentration index analysis, and vehicle controls to confirm synergistic effects.

### *Minimum Inhibitory Concentration (MIC) of the formulated NipaAlco gel*

Table 5 shows that after 24 hours of incubation under aerobic conditions at 37°C, turbidity was present in test tubes containing bacterial and fungal species with CrPE at 1% and 2%, indicating microbial growth. In contrast, test tubes containing bacterial species with CrPE at 3% exhibited no turbidity, demonstrating inhibition of these microorganisms. However, turbidity remained in the fungal test tube with CrPE at 3%, suggesting that *C. albicans* requires higher concentrations of *C. reticulata* ethanolic peel extract for in vitro inhibition.

*S. aureus, E. coli, and C. albicans* were selected for this study because they are among the most common microorganisms found in mammalian wounds and can develop resistance to antimicrobial agents. The antibacterial effects of *Citrus reticulata* are primarily attributed to phytochemicals, specifically alkaloids, saponins, tannins, and flavonoids present in the peel extract, as reported by Wintola & Afolayan (2015).

**Table 6.** Test for Minimum Inhibitory Concentration (MIC) of the formulated NipaAlcoCitro Gel and NipaAlcoMHX Gel.

| Formulations of NipaAlcoCitro Gel | Qualitative Assessment on the MIC of the Gel | | |
|---|---|---|---|
| | ***S. aureus*** | ***E. coli*** | ***C. albicans*** |
| **CrPE 1%** | Turbid | Turbid | Turbid |
| **CrPE 2%** | Turbid | Turbid | Turbid |
| **CrPE 3%** | Not turbid | Not turbid | Turbid |
| **MHX 1%** | Turbid | Turbid | Turbid |
| **MHX 2%** | Turbid | Turbid | Turbid |
| **MHX 3%** | Not turbid | Turbid | Not turbid |

Table 6 shows that turbidity developed in test tubes containing bacterial and fungal species with MHX at 1% and 2% concentrations after 24 hours of aerobic incubation at 37°C, indicating microbial growth. In contrast, no turbidity was observed in test tubes containing *S. aureus* and *C. albicans* with CrPE at 3%, demonstrating inhibition of these microorganisms. However, turbidity was present in the *E. coli* test tube with CrPE at 3%, suggesting that *E. coli* requires higher concentrations of MMSU Herbal X extract for inhibition in vitro. Plant extracts are generally considered more effective against gram-positive bacteria such as *S. aureus*, as their cell walls are more easily penetrated than those of gram-negative bacteria like *E. coli*, which possess an outer membrane with a lipopolysaccharide layer that restricts the entry of certain antimicrobial agents. These findings suggest that some indigenous plants from Ilocos Norte may serve as potential sources of antimicrobial agents. The activity observed in this study reflects the response of the selected test organisms (*Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*) under the conditions tested, while reports on activity against resistant strains are drawn from existing literature rather than directly assessed in this work (Parekh et al., 2005; Wintola & Afolayan, 2015). Previous studies have indicated that plant-derived extracts may act through mechanisms such as disruption of microbial cell structures, including the lipopolysaccharide layer in Gram-negative bacteria, which can enhance microbial susceptibility.

***Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) of the formulated Alco gel***

Following MIC determination, aliquots from all tubes containing 2% and 3% formulations of both gel products were inoculated onto NA and PDA plates and incubated for 24 hours at 37°C and 35°C, respectively. The MBC endpoint is defined as the lowest concentration of an antimicrobial agent at which 99.9% of the bacterial population is eliminated. This endpoint was determined by assessing the presence or absence of bacterial and fungal growth on the agar plates after incubation. As shown in Table 7, suspensions from CrPE 3% tubes exhibited no bacterial growth, confirming bactericidal activity. However, six *C. albicans* colonies were observed on the PDA plate. In contrast, the MHX 3% suspension produced a single *E. coli* colony, while no visible colony growth was detected on the PDA plate or the *S. aureus* NA plate. The bactericidal and fungicidal activities of plant extracts are attributed to the presence of secondary metabolites, which protect plants against bacterial, fungal, and viral infections. The results from these tests indicate that the effective bactericidal and fungicidal concentrations of NipaAlcoCitro Gel and NipaAlcoMHX Gel exceed 3%, achieving both MBC and MFC endpoints.

**Table 7.** Test for Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) of the formulated Alco Gel through colony count.

| Formulations of Alco Gel | Qualitative Assessment of MBC | | |
|---|---|---|---|
| | *S. aureus* | *E. coli* | *C. albicans* |
| **CrPE 2%** | Confluent | Eleven colonies | Confluent |
| **CrPE 3%** | No colony | No colony | Six colonies |
| **MHX 2%** | No colony | Confluent | No colony |
| **MHX 3%** | No colony | One colony | No colony |

***Morphological changes on the wound of male ICR mice treated with NipaAlco gel***

Morphological changes in the wounds of male ICR mice treated with NipaAlcoCitro Gel 3% and NipaAlcoMHX Gel 3% over a 30-day observation period are summarized in Table 8. The initial wound state at Day 0 served as the baseline for assessment. Sterile topical applications of NipaAlcoCitro Gel, NipaAlcoMHX Gel, and Povidone-Iodine Ointment were administered every two days to their respective treatment groups. On Day 2, wounds in the negative control and Treatment Group 2 (T2-CrPE) were described as very red, very swollen, and moist. In contrast, wounds in Treatment Group 3 (T3-MHX) and the positive control were red, swollen, and moist, with evident pus. The presence of pus is a recognizable indicator of localized inflammation or possible infection during the wound-healing period. By Day 8, the pus had completely disappeared, suggesting improvement in wound condition and a reduction in inflammatory response. Wounds treated in T2-CrPE and T3-MHX groups developed a swollen but dry surface by Day 14, followed by scar formation attributed to fibroblast activity in adjacent skin areas. This process results in the production of fibrous connective tissue composed of collagen. Notably, the positive control group achieved a dry wound surface by Day 10.

Advanced healing characterized by wound closure and hair regrowth, was observed in the T2-CrPE group on Day 28 and in the T3-MHX group on Day 26. The positive control group achieved complete healing by Day 22. The negative control group did not exhibit thorough tissue repair, likely due to the absence of topical treatment. Furthermore, Loyola University Health System (2014) reports that chronic-binge alcoholism impairs the production of macrophage inflammatory protein-1 alpha (MIP-1α), which recruits macrophages to the wound site. Binge alcohol also reduces levels of cathelicidin-related antimicrobial peptide (CRAMP), a key immune component present in the epidermis. These antimicrobial peptides kill bacteria and recruit macrophages and other immune cells to the wound site. Such effects likely contribute to delayed wound closure and increased infection severity in intoxicated individuals.

**Table 8.** Morphological Assessment on the Wound of the Experimental ICR Mice

| Treatment Groups | Observation Period (in days) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 24 | 26 | 28 | 30 |
| **Control (-)** | A | A | A | B | B | B | C | C | C | C | D | D | D | D | E | E |
| **Group 2 (T2 – CrPE)** | A | A | B | B | B | C | C | D | D | E | E | E | E | E | F | F |
| **Group 3 (T3 – MHX)** | A | B | B | B | C | C | C | D | D | E | E | E | E | E | F | F |



Legend:

A – Very red; very swollen and moist
B – Red; swollen; moist; presence of pus
C – Red; slightly swollen and moist
D – Swollen; dry surface
E – Scar formation; dry surface
F – Presence of hair; wound healed completely

| Control (+) | A | B | B | C | C | D | D | D | E | E | E | E | F | F | F | F |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

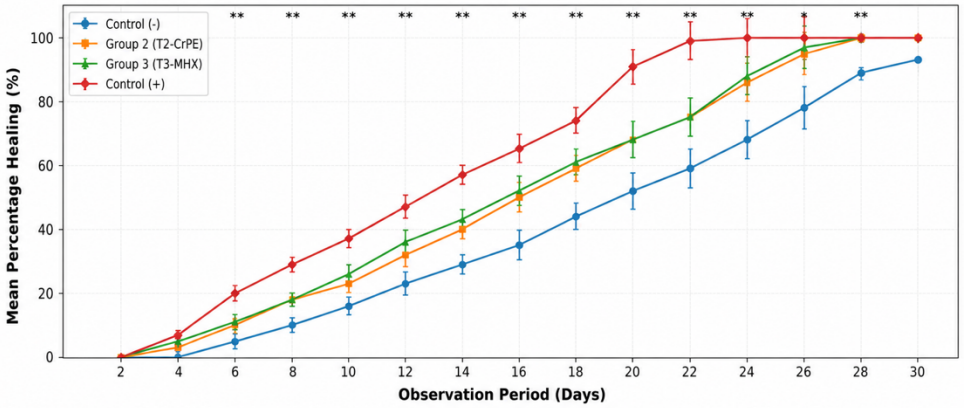
***Healing percentage of different ICR Mice treatments within the 30d observation period***

Figure 2 presents the healing percentages for four treatments over a 30-day observation period. Topical application began following the induction of a 1 cm wound at the dorso-lumbar region of ICR mice. No evidence of cutaneous healing was observed from Day 0 to Day 4 in the negative control group. In contrast, the positive control group exhibited the highest healing percentage on Day 4, followed by T3-MHX and T2-CrPE.

All treatments demonstrated an increasing trend in healing percentage through Day 30. The positive control consistently achieved the highest mean percentage. Complete healing, indicated by hair regrowth, was observed in the T2-CrPE and T3-MHX consistently achieved the highest mean percentage. Complete healing, indicated by hair regrowth, was observed in the T2-CrPE and T3-MHX groups by Day 28.

Statistical analysis indicated that healing percentages from Day 4 to Day 24 were highly significant at the 1% level. Healing percentages from Day 26 to Day 28 were significant at the 5% level. These results suggest that all treatments, except the negative control, significantly promoted healing in the experimental animals.

As shown in Figure 2, all the treatments induced their healing activity towards the ICR mice, manifested by the increasing trend in their percentage healing. The negative control group did not achieve complete healing during the 30-day observation period.



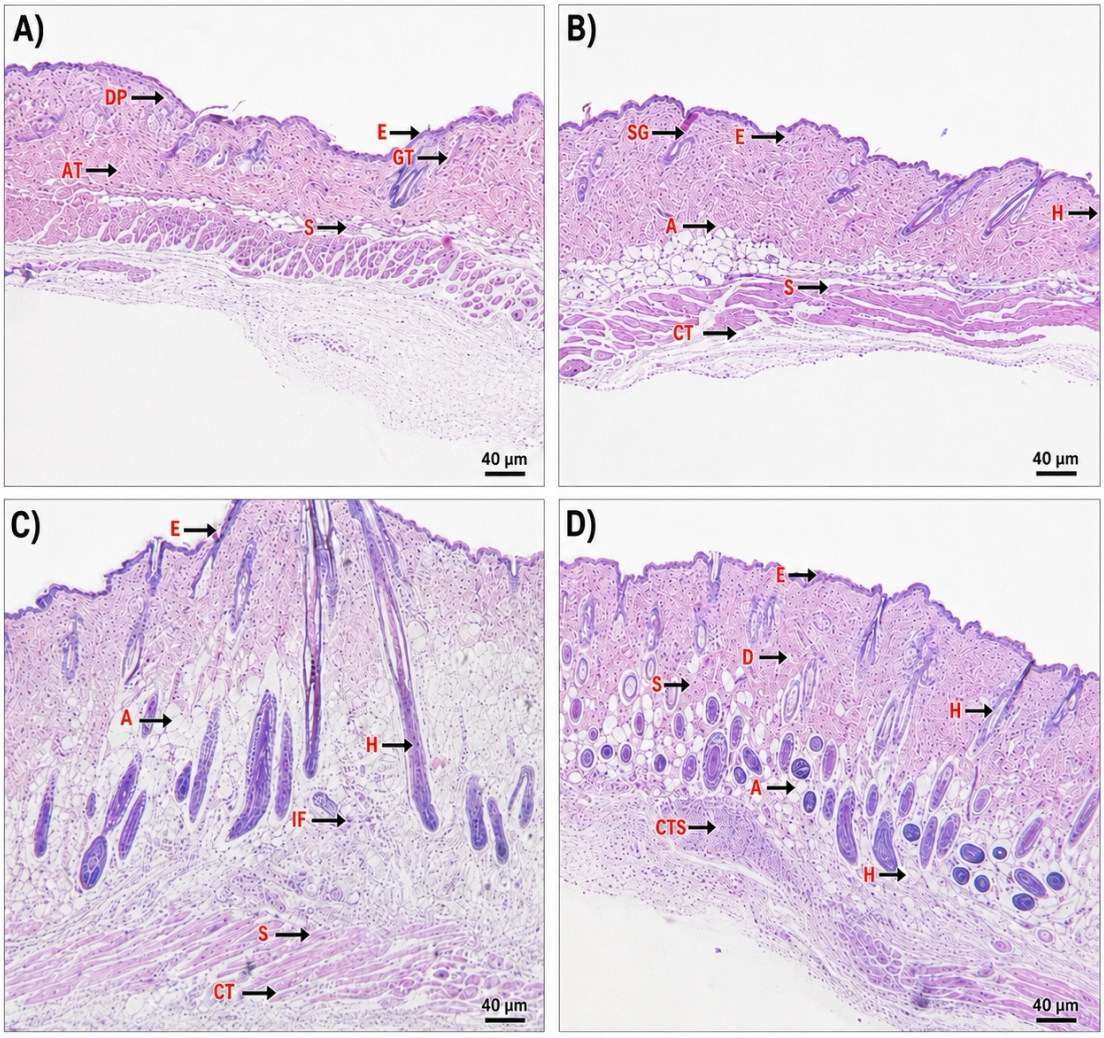

| | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 24 | 26 | 28 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Exact p-value | — | 0.0043 | 0.0015 | 0.0002 | 0.0006 | 0.0014 | 0.0002 | 0.0014 | 0.0007 | 0.0058 | 0.0012 | 0.0034 | 0.0261 | 0.0001 | — |
| Significance | NA | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | * | ** | NA |

**Figure 2.** Treatment-Dependent Changes in Mean Percentage Healing of ICR Mice Over the 30-Day Observation Period

***Comparison of the wound healing property of NipaAlcoCitro Gel and NipaAlcoMHX Gel through histological examination***

In histological examination of skin tissue under varied treatments, histological parameters such as the amount of granulation tissue, inflammatory infiltrate, collagen fiber orientation, collagen pattern, amount of early

collagen, and amount of mature collagen were quantified. These parameters were recommended by Kumar & Gupta (2015). Good healing status ranges from 16 to 19, fair ranges from 12 to 15, and poor ranges from 8 to 11, and lower scores indicate poorer wound-healing outcomes. In the study, the amount of early- and mature-type collagen was not evaluated because trichome staining was not performed.




*Legend:*
**E** - Epidermis
**D** - Dermis
**H** - Hypodermis
**DP** - Papillary Dermis
**S** - Subcutis
**A** - Adipose tissue
**GT** - Granulated Tissue
**SG** - Sebaceous gland
**CT/CTS** - Connective tissue
**IF** - Inflammatory Infiltrate

**Figure 3.** Photomicrograph of H&E-stained section of skin tissue of chronic-binge ethanol-fed male ICR Mice under varied treatment groups with a scale bar = 40*um*. (*A. Control (-), B. T2-CrPE, C. T3-MHX, D. Control (+)*)

Figure 3 presents the photomicrographs of skin tissue sections stained with hematoxylin and eosin (H&E), a standard histological technique in which hematoxylin stains cell nuclei blue-purple, while eosin stains cytoplasmic and extracellular matrix components pink (Gupta & Kumar, 2015). The images illustrate the histological features of chronic-binge ethanol-fed ICR mice under different treatment conditions. In the untreated group (Figure 3A), the tissue exhibits characteristics of poor wound healing, including disrupted epithelial continuity, presence of inflammatory infiltrates, and

limited organization of dermal structures, consistent with its lower healing score (8-11) (Gupta & Kumar, 2015). Wound healing is a complex process involving overlapping phases of inflammation, proliferation, and remodeling, and disruption in these processes-such as those associated with alcohol exposure can delay tissue repair (Gosain & DiPietro, 2004; Jung et al., 2011). In contrast, the treated groups (Figures 3B and 3C) show improvements in tissue organization, including partial re-epithelialization, reduced inflammatory cell presence, and more defined dermal layers. Collagen fibers appear more aligned and compact, suggesting progression toward tissue remodeling, which is an essential phase in restoring tissue integrity (Gosain & DiPietro, 2004). The positive control group (Figure 3D) demonstrates more advanced structural restoration, with a more continuous epithelial layer, reduced inflammation, and clearer organization of connective tissue components. Additionally, structures such as developing hair follicles and glandular elements are more evident in treated and control (+) groups, indicating ongoing tissue regeneration and epithelial differentiation (Menke et al., 2007). These observations correspond with the histological scoring results, where treated groups fall within the fair to good healing range (12–19) (Gupta & Kumar, 2015). Overall, the findings suggest that the formulated gels support tissue repair processes under alcohol-impaired conditions, showing comparable trends to the positive control, although full restoration of normal tissue architecture was not completely achieved within the observation period.

**Table 9.** Histological scoring of stained skin tissue

| | A. (-) Control | B.T2-CrPE | C. T3-MHX | D. (+) Control |
|---|---|---|---|---|
| **Amount of granulation tissue** | Moderate: 2 | Absent: 4 | Absent: 4 | Absent: 4 |
| **Inflammatory infiltrate** | Few: 3 | Few: 3 | Few: 3 | Few: 3 |
| **Collagen fiber orientation** | Horizontal: 3 | Horizontal: 3 | Horizontal: 3 | Horizontal: 3 |
| **Pattern of Collagen** | Fascicle: 3 | Fascicle: 3 | Fascicle: 3 | Fascicle: 3 |

Figure 3.b presents the skin tissue of mice treated with NipaAlcoCitro Gel. Figure 3.c shows the skin tissue treated with NipaAlcoMHX Gel. The scores of the replicates from the two treatments fell under fair to good wound healing, in accordance with the bracket set by Kumar & Gupta (2015) in their paper entitled “Assessment of the histological state of the healing wound” presented in Table 9. The flavonoid content of *C. reticulata* peel extract accounts for the wide range of biological activities reported for the plant, as evidenced by its antimicrobial activity. Numerous studies have also been carried out on the antioxidant activities of flavonoids and their potential to treat several diseases, including inflammatory diseases, oxidative stress, acute and chronic wounds, liver dysfunction, microbial infections, cancer, and cardiovascular diseases. Moreover, other identified phytochemicals in the peel extract, such as phenols, alkaloids, saponins, and tannins, are responsible for numerous pharmacological properties and are known to exhibit inhibitory effects on inflammation. During tissue repair, many secondary metabolites influence inflammatory cells, promoting the migration and proliferation of endothelial cells and leading to neovascularisation of connective tissue. These synthesize extracellular matrices such as collagen and keratin, resulting in re-epithelialization of the wounded tissue and, hence, scar formation; this is evident in the replicates under these treatments.

Given the scores obtained for the histological parameters, it can be inferred that the healing activities of T2-CrPE and T3-MHX are comparable to those of the commercially available product, Povidone-Iodine ointment.

## Conclusion

The present study demonstrated that NipaAlcoCitro Gel 3% and NipaAlcoMHX Gel 3% exhibited measurable antimicrobial activity and supported wound healing in chronic-binge ethanol-fed ICR mice. Both formulations showed progressive increases in wound contraction over the 30-day observation period and histological features corresponding to fair to good healing based on the scoring criteria used. Compared with the untreated group, treated groups exhibited improved tissue organization, including partial re-epithelialization and reduced inflammatory features, while showing healing patterns that were comparable in trend to the positive control.

Phytochemical screening confirmed the presence of selected secondary metabolite groups, including flavonoids and phenolic compounds; however, their specific contributions to the observed biological effects were not directly evaluated in this study. As such, the antimicrobial and wound healing responses observed may be associated with the combined effects of the formulation components rather than a single class of compounds. These findings suggest the potential of the formulated gels as alternative topical agents under alcohol-impaired conditions, although further investigation is required to confirm their efficacy and to clarify the mechanisms involved.

Considering the limitations of the present study, including the small sample size, absence of a vehicle-only gel base control, endpoint-only histological evaluation, and limited 30-day observation period, future studies are recommended to further validate the wound-healing potential of the formulated gels. Subsequent investigations should employ larger animal groups, include appropriate vehicle controls, and extend the monitoring period to better assess long-term tissue remodeling and scar maturation. In addition, future research should determine the minimum inhibitory concentration using microdilution methods, include additional clinically relevant wound-associated microorganisms such as *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*, and evaluate antioxidant activity through assays such as DPPH and HPLC. Special staining techniques, particularly trichrome staining, should also be applied to assess collagen deposition and maturation, while biochemical markers such as AST and ALT should be included to further characterize alcohol-induced physiological effects in the experimental model.

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## Biodata

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