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Phytochemical Profiling, Antioxidant, Antibacterial, and Wound Healing Effects of Rosemary Oil Hydrogel

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Abstract

Rosmarinus officinalis L., a Mediterranean perennial herb, is well known for its antibacterial, antioxidant, anti-inflammatory, and wound-healing properties. This study investigates the phytochemical profile of *R. officinalis*, including glycosides, terpenoids, phenols, flavonoids, saponins, alkaloids, and tannins, along with its biological activities. Quantitative analysis revealed high levels of terpenoids (71.15 ± 0.18 mg/mL), tannins (26.32 ± 0.16 mg TTA/mL), phenolics (8.20 ± 0.001 mg GAE/mL), and flavonoids (0.1874 ± 0.002 mg QE/mL). Gas chromatography–mass spectrometry (GC-MS) identified a chemotype with low 1,8-cineole (1.77%) and a high concentration of 1,2-hexanediol (67.34%), which may enhance the formulation's stability and suitability for medicinal and cosmetic applications due to its antimicrobial and humectant properties. The DPPH assay demonstrated strong antioxidant activity with an IC_{50} value of 21.80 ± 0.56 µg/mL, which is more potent than ascorbic acid (IC_{50} = 33.79 ± 0.33 µg/mL). Antimicrobial testing using the disc diffusion method showed moderate activity against *Staphylococcus aureus* (17.33 ± 0.45 mm at 50% concentration) and limited activity against *Escherichia coli* (12 mm at 50%) compared to gentamicin (24.3 mm and 23.1 mm, respectively). The rosemary oil hydrogel demonstrated a statistically significant improvement in wound contraction (66.87% ± 2.15% by Day 16, $p < 0.05$) in a *Staphylococcus aureus*-infected mouse model when compared to negative, placebo, and standard treatment groups. These results support the therapeutic potential of *R. officinalis* oil hydrogel for topical wound care, combining antimicrobial, antioxidant, and healing effects in a biocompatible formulation.

Keywords: Rosemary oil, Phytochemical analysis, Total phenolic content, Total flavonoid content, Total tannin content, Antioxidant properties, Antibacterial activity, GC-MS, Animal studies.

Introduction

Herbal medicines and natural ingredients have been widely used for centuries due to their therapeutic benefits and minimal adverse effects [1]. In recent years, scientific interest in medicinal plants has grown significantly, driven by the need for alternative treatments with fewer side effects [2]. *Rosmarinus officinalis* L. (Lamiaceae), commonly known as rosemary, is a Mediterranean evergreen shrub that thrives in warm, sunny regions with well-drained soil. It is naturally widespread and cultivated across Southern Europe (Spain, Italy, and Greece), North Africa (Morocco, Tunisia, and Egypt), and parts of Asia (India and China), and is commercially grown in the United States and Australia for the extraction of its essential oils used in pharmaceutical and industrial applications [3]. The

pharmacological importance of rosemary lies primarily in its leaves, which are rich in essential oils and bioactive c225ompounds such as monoterpenes, diterpenes, and phenolic acids. Among these, carnosic acid, carnosol, and rosmarinic acid are well-recognized for their potent antioxidant, anti-inflammatory, and antimicrobial effects [4]. Traditionally, rosemary has been used to enhance memory, relieve muscular discomfort, aid digestion, and purify the air [5]. In modern formulations, rosemary extracts and oils are widely used in dermatological applications, hair growth serums, wound healing agents, and as preservatives in food and cosmetics due to their bioactive profile [6].

The essential oil of rosemary contains a wide spectrum of phytochemicals, including terpenoids, phenolics, flavonoids, saponins, tannins, and alkaloids, all of which contribute to its broad therapeutic activity [5]. Key constituents such as 1,8-cineole (eucalyptol), camphor, α-pinene, borneol, and linalool are responsible for its antimicrobial and antioxidant properties. However, the exact composition of rosemary oil can vary based on geographical origin, extraction method, and climatic factors, resulting in distinct chemotypes with unique biological activities [6].

Hydrogels have recently emerged as promising biomaterials for wound management due to their ability to maintain a moist environment, enhance tissue regeneration, and provide controlled drug delivery [7]. The integration of essential oils into hydrogels has been shown to significantly enhance antimicrobial efficacy and biocompatibility [8]. For instance, studies have demonstrated that hydrogels incorporating tea tree or eucalyptus oils outperformed conventional wound dressings in antimicrobial action [9]. Despite the established medicinal properties of rosemary oil, there is limited research on its incorporation into hydrogel systems, particularly for the treatment of infected wounds [10]. Therefore, this study aims to evaluate the phytochemical composition of *R. officinalis* essential oil and investigate its antibacterial, antioxidant, and wound-healing properties when formulated into a hydrogel [11]. By addressing this gap, the study offers a natural, biocompatible alternative for managing infected wounds, potentially reducing dependence on synthetic antimicrobials and accelerating tissue repair through a synergistic blend of traditional plant-based therapy and modern drug delivery technology [12].

Materials and Methods

Chemicals, Reagents, and Samples

The rosemary (*Rosmarinus officinalis*) essential oil utilized in this study was commercially obtained from Green Herbology, France. Various chemicals and reagents were utilized, including Bromine water (70%), glacial acetic acid (99.7%), and tannic acid (85%), that were purchased from Bendosen (Malaysia). Ferric chloride (98%) and iodine solution (96%) were obtained from Alpha Chemika (India). Concentrated sulfuric acid (95–98%), ammonium hydroxide (28–30%), hydrochloric acid (37%), and chloroform (99%) were acquired from LOBA Chemie (India). Potassium iodide (99.5%), iodine (99.8%), and Folin–Ciocalteu reagent (FCR) were purchased from R&M Chemicals (Malaysia). Gallic acid (98%) was obtained from SRL (India). Sodium carbonate (99.5%) and aluminum chloride (98%) were supplied by Progressive Scientific (Malaysia). HPLC-grade methanol (99.9%) and ascorbic acid (98%) were procured from HmbG Chemicals (Malaysia). Sodium hydroxide (98%) was obtained from ESTB (Malaysia), while sodium nitrite (97%) was sourced from

Chemiz. Quercetin (≥95%), Tween 80 (99%), Carbomer 940 (≥98%), glycerin (99.5%), and triethanolamine (99%) were supplied by Sigma-Aldrich Chemie (Germany). DPPH (purity ≥95%) was obtained from Cool Chemical (China). All solutions were freshly prepared prior to use, and distilled water was used throughout the investigation for reagent preparation, sample dilution, and hydrogel formulation.

Instrumentation

An electronic analytical balance (Model: PE600, Premier Calibration, Selangor, Malaysia) was utilized to precisely weigh all reagents and samples. A UV-visible spectrophotometer (Model: UV-1800, Shimadzu Corporation, Kyoto, Japan) was used to perform spectroscopic measurements of phytochemical quantifications such as total phenolic content (TPC), total flavonoid content (TFC), total terpenoid content (TTpC), and total tannin content (TTC). The Shimadzu GCMS-QP2020 NX (Shimadzu Corporation, Kyoto, Japan) was used for the identification and quantification of key bioactive components of rosemary oil.

Phytochemical Identification Tests

The preliminary phytochemical screening of rosemary oil includes the quantitative determination of key bioactive groups using standard colorimetric and gravimetric methods. TPC was evaluated using the Folin-Ciocalteu reagent method, which uses electron transfer to produce a blue complex detectable at 765 nm [12]. TFC content was determined using the aluminium chloride colorimetric technique, which produces a flavonoid-aluminium combination with a distinctive absorbance of 415 nm [13]. The vanillin-HCl method, which estimates condensed tannins via red complex production [14] was used to determine TTC. The TTpC was determined by ethanol extraction, followed by colour development with sulfuric acid [15]. Total Saponin Content (TSC) was determined using a gravimetric method involving ethanol extraction and drying of the residue to constant weight [16]. Total Alkaloids Content (TAC) was measured based on acid–base extraction followed by precipitation with ammonium hydroxide [17]. All assays were conducted in triplicate, and results were expressed in their respective equivalents (e.g., mg GAE/mL for phenolics).

Determination of Total Phenolic Content

The TPC of rosemary oil was assessed using the FCR. A standard calibration curve was prepared using gallic acid solutions (25, 50, 75, and 100 µg/mL) dissolved in methanol. Each concentration was mixed with 5 mL of FCRand 5 mL of 7.5% Na_2CO_3, incubated at 40 °C for 30 min, and measured at 760 nm using a UV-Vis spectrophotometer. A blank containing methanol and reagents without extract was used for correction. For sample analysis, 1 mL of rosemary oil was diluted in 10 mL methanol and treated similarly to the standard [14]. The mean absorbance values were used to calculate TPC in milligrams of gallic acid equivalents (GAE) per gram of dry extractusing the formula:

$$C = (c \times V) / m$$

Where:

- C = Total phenolic content (mg GAE/g)
- c = Gallic acid concentration (mg/mL)
- V = Extract volume (mL)
- m = Extract mass (g)

Determinationof Total Flavonoid Content

The TFC of rosemary oil was determined using the aluminium chloride method. A quercetin stock solution (4 mg/mL) was prepared in methanol and diluted to concentrations ranging from 0.25 to 1 mg/mL. The reagents used included 5% $NaNO_2$, 10%

$AlCl_3$, and 1 M NaOH. For the reaction, 4 mL of distilled water was mixed with 1 mL of quercetin solution. After 5 min, 0.3 mL of 5% $NaNO_2$ was added, followed by 0.3 mL of 10% $AlCl_3$. After 6 min, 2 mL of 1 M NaOH was added, and the final volume was adjusted to 10 mL. The absorbance was measured at 510 nm using a UV-vis spectrophotometer A blank containing reagents without extract was used for correction. Rosemary oil extracts (4 mg/mL in methanol) were prepared and diluted accordingly. A reaction mixture consisting of 2 mL of oil extract, 4 mL of distilled water, 0.5 mL of 5% $NaNO_2$, 0.5 mL of 10% $AlCl_3$, and 2 mL of 1 M NaOH was incubated for 10 min before measuring absorbance at 510 nm.TFCwas expressed in quercetin equivalents (QE) per gram of dry extract [14].

$$C = (c \times V) / m$$

Where:

- C = Total flavonoid content (mg QE/g)
- c = Quercetin concentration (mg/mL)
- V = Extract volume (mL)
- m = Extract mass (g)

Determinationof Total Tannin Content

The TTC was determined using the FCR. The FCR was prepared by diluting 2 mL of FCR with distilled water to 20 mL. A 3.5% sodium carbonate solution was prepared by dissolving 3.5 g of Na_2CO_3 in 100 mL of distilled water. A standard gallic acid solution (1 mg/mL) was prepared by dissolving 10 mg of gallic acid in 10 mL of methanol, followed by serial dilutions to obtain concentrations of 25, 50, 75, and 100 µg/mL. To each concentration, 5 mL FCR and 5 mL of Na_2CO_3 solution were added. The mixture was incubated at 40 °C for 30 min, and absorbance was measured at 760 nm. A blank containing methanol and reagents without extract was used for correction. For rosemary oil extracts, 1 mL of the sample was dissolved in 7.5 mL of distilled water, followed by the addition of 0.5 mL of FCR and 1 mL of Na_2CO_3 solution. After incubation at 40 °C for 30 min, 2 mL of the sample solution was diluted with 5 mL of distilled water, and absorbance was measured at 700 nm [14]. The mean absorbance values were used to calculate TTC in milligrams of total tannic acid (TTA) equivalents per gram of dry extractusing the formula:

$$C = (c \times V) / m$$

Where:

- C = Total tannin content (mg TTA/g)
- c = Tannic acid concentration (mg/mL)
- V = Extract volume (mL)
- m = Extract mass (g)

Determination of Total Terpenoid Content

The TTpC was determined using the pomelo oil method. A stock solution was prepared by dissolving 1 mg of pomelo oil (PO) in 10 mL of ethanol, followed by serial dilutions to obtain concentrations of 0.5, 1, 1.5, 2, 2.5, and 3 µL. For the assay, 1 mL of extract was mixed with 2 mL of chloroform, vortexed, and left for 3 min. Then, 200 µL of concentrated sulfuric acid was added, and the mixture was incubated at room temperature in the dark for 1.5–2 hrs, resulting in a reddish-brown precipitate. The supernatant was carefully decanted, and 3 mL of 100% methanol was added, vertexing until complete dissolution. Absorbance was measured at 538 nm using a spectrometer. A blank containing reagent without extract was used for correction. For rosemary oil, the same protocol was followed. The absorbance values of the triplicate samples were averaged and used to generate a calibration curve for terpenoid quantification [15]. TTpC was calculated in PO equivalents per gram of dry extract using the formula:

$$C = (c \times V) / m$$

Where:

- C = Total terpenoid content (mg PO/g)
- c = Pomelo oil concentration (mg/mL)
- V = Extract volume (mL)
- m = Extract mass (g)

Gas-chromatography Mass spectrometer

GC-MS analysis was conducted to identify and quantify key bioactive constituents in rosemary oil using a Shimadzu GCMS-QP2020 NX system (Shimadzu Corporation, Kyoto, Japan), equipped with an AOC-20i autosampler and a Restek Rxi-5Sil MS capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness; Restek, USA). The gas chromatograph operated in split injection mode with a split ratio of 1:50. The oven temperature was initially held at 60 °C for 2 min, increased to 200 °C at a rate of 3 °C/min and held for 5 min, followed by a ramp to 280 °C at 10 °C/min and held for 10 min. The injector and interface temperatures were maintained at 250 °C and 280 °C, respectively. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min, with a total flow of 54.1 mL/min, carrier pressure of 57.5 mL/min, and purge flow of 3.0 mL/min. Mass spectrometric detection was performed in electron impact (EI) ionization mode at 70 eV. Data acquisition was carried out in full scan mode across a mass range of m/z 50–500 with an event time of 0.30 sec. The ion source temperature was set at 230 °C, and a solvent delay of 4.0 min was applied to reduce background interference. Identification of volatile compounds was performed by comparing the obtained spectra with entries in the NIST 14 mass spectral library.

Sample Preparation

To prevent contamination, the syringe was pre-washed with solvent three times**,** followed by three additional rinses with fresh solvent**,** and then rinsed once with the sample. With a viscosity compensation time of 0.2 sec, the plunger speed was set to high for both injection and suction. Following five pumping cycles, the wash volume was changed to 8 µL, and the injection port was set at a dwell time of 0.3 sec [14].

Antioxidant activity determination (DPPH assay)

The antioxidant activity of rosemary oil was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. A DPPH solution was made by dissolving 3.943 mg in 100 mL of methanol. A stock solution of ascorbic acid (10 mg/10 mL in methanol) was produced and then diluted (1 mL in 10 mL methanol) to serve as a standard. Rosemary oil was also diluted following the same technique. Various sample concentrations (0.5-5 mL) were combined with 3 mL of DPPH solution in a 96-well plate, and the final volume was adjusted to 10 mL with methanol. The mixture was incubated in the dark at room temperature for 30 min. The absorbance was measured at 517 nm with a UV-Vis spectrophotometer. A blank containing methanol and DPPH without extract was used for correction Radical inhibition (%) was calculated using the formula:

Where:

- A_0 = Absorbance of control
- A_1 = Absorbance of sample

IC_{50} values were determined via regression analysis [16]. The IC_{50} value for rosemary oil was found to be 21.80 µg/mL, indicating strong antioxidant activity compared to ascorbic acid (IC_{50} = 33.79 µg/mL).

Antimicrobial activity

The antimicrobial properties of the essential oil were evaluated using the disk diffusion method. The assay was conducted using an 18-hour culture incubated at 37 °C in

10 mL of McFarland medium. To ensure equal microbial growth (*E. coli* and *S. aureus*), the solutions were evenly dispersed on McFarland agar plates using a sterile cotton swab. McFarland medium was first prepared with the microorganism. The essential oil was diluted using an appropriate amount of ethyl acetate. The nutrient agar plates were divided into four different concentrations, and *E. coli* and *S. aureus* were spread using a cotton swab. Sterilized filter paper was punched into 6 mm discs, impregnated with 10 µL of test samples (four different concentrations), and placed on the inoculated agar surface. Additionally, four sides were marked, and antibiotic standards (gentamicin and ampicillin) were applied along with two blanks (ethyl acetate). The plates were sealed with sterile laboratory parafilm to prevent evaporation of the test samples. They were left at room temperature for 30 min to allow for oil diffusion before being incubated at 37 °C for 18 hrs. After incubation, the inhibitory zone in millimetreswas measured using a Vernier calliper. All experiments were performed in triplicate, and mean values were calculated. The interpretation of inhibition zone diameters was done based on the Clinical and Laboratory Standards Institute (CLSI) guidelines, M100 (2023 edition), where applicable, particularly for standard antibiotics [17].

Preparation of Plain Gel and Rosemary oil Hydrogel

Preparation of Simple Gel: To prepare a simple gel, 50 mL of purified water was taken, and 5 mL of glycerine was added. Then, 0.5 g of carbomer was gradually added while stirring at 500 rpm for 10 min. If no clumps were detected, a few drops of triethanolamine were added to finalize the gel.

Preparation of Rosemary Oil Hydrogel: For rosemary oil hydrogel 5 mL of rosemary oil was mixed with 2 mL of Tween 80. This mixture was added to 100 mL of distilled water without creating bubbles and stirred at 500 rpm for 10 min. The solution was then sonicated for 10 min. In the next step, 50 mL of distilled water was taken, and 5 mL of glycerine was added. Gradually, 0.5 g of carbomer was incorporated while stirring at 500 rpm. After proper mixing, 50 mL from the previous mixture was slowly added to the current mixture, followed by stirring at 500 rpm for 10 min. If no clumps were detected, a few drops of triethanolamine were added to produce a stable hydrogel [17].

Wound Creation Followed by Staphylococcus aureus Inoculation

Mice weighing 25–45 g and aged 12 weeks underwent surgical excision of a full-thickness skin patch in the dorsal region under brief chloroform anaesthesia. Following the procedure, the animals were assigned to four experimental groups and housed individually based on their respective treatments. Group 1 served as the negative control (no treatment), Group 2 received a standard antibacterial treatment (Gentamicin cream 0.3%), Group 3 was treated with a placebo hydrogel (lacking rosemary oil), and Group 4 received a rosemary oil-infused hydrogel. Each cage contained three mice, which were designated alphabetically within their respective groups: Group 1 (A1, A2, A3), Group 2 (B1, B2, B3), Group 3 (C1, C2, C3), and Group 4 (D1, D2, D3). Prior to wound creation, the dorsal fur was shaved, and the exposed skin was disinfected using 70% ethanol. The animals were positioned laterally, and a circular full-thickness wound (9–10 mm in diameter) was excised from the dorsocervical region using sterile straight surgical scissors, tissue forceps, a scalpel blade, and a ruler for precise measurement. A depilatory cream was applied to ensure complete hair removal. Following excision, the wound area was cleaned with 70% ethanol. To induce infection, *Staphylococcus aureus* was prepared and

applied to the wound using a sterile cotton swab. The inoculated wounds were left undisturbed for 24 hrs to allow bacterial colonization. This inoculation period was designated as day 0. After 24 hrs, the respective treatments were administered to each group, marking the beginning of the experimental treatment phase (day 1) [18].

Microbial Growth Test

In the microbial growth test, two nutrient agar Petri plates were taken, and the wound area was swabbed with a sterile cotton swab. The Petri plates were then placed in a 37 °C incubator for 24 hrs to confirm bacterial growth. This procedure was performed after 24 hrs of infection, or on day 1.

Infected Wound Closure Test

Using a ruler, the wound closure was measured on Days 1, 3, 6, 9, 12, and 16. The wound's thickness, which was 9 to 10 mm, was measured, and the gel was applied once a day. Group 1 (A1, A2, A3) was the negative group, meaning they did not receive any treatment; Group 2 (B1, B2, B3) was the positive group, meaning they received standard Gentamicin cream at a 0.3% concentration; Group 3 (C1, C2, C3) was the placebo hydrogel (without rosemary oil); and Group 4 (D1, D2, D3) was the rosemary oil hydrogel to gauge the wound's healing.

Skin Irritation Test

Prior to applying the gel, a picture was taken of the mice in the area of the wound for the skin irritation test. Images were taken again after the gel had been applied for 6 and 12 hrs in mice A1, B1, C1 and D1.

Inflammation Test

Before applying the gel for the inflammation test, an image of the mice at the wound site was taken. Afterward, images were taken again after the gel had been applied for 6 and 12 hrs for mice A1, B1, C1, and D1.

Ethical Approval

This study was conducted following the guidelines for animal research ethics and was approved by the Institutional Animal Care and Use Committee (IACUC) at Lincoln University College Malaysia (Approval No: LUC/IACUC/2024/001). All animal handling procedures complied with the National Institute of Health (NIH) guidelines for the care and use of laboratory animals.

Statistical Analysis

All experiments were performed in triplicate and were expressed as mean ± standard deviation (SD). Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. A p-value of <0.05 was considered statistically significant. Data analysis was performed using SPSS software version 25.

Results and Discussion

Phytochemical Composition and Bioactive Profiling

Terpenoids, phenolics, flavonoids, tannins, alkaloids, glycosides, and saponins were among the many secondary metabolites identified by the phytochemical examination of rosemary oil. Characteristic reactions, such as the reddish-brown interface seen when terpenoids reacted with sulfuric acid and the continuous foam development suggestive of saponins, were used in qualitative testing to establish their presence. In accordance with findings from Ioniță et al. [19], who reported terpenoid concentrations ranging from 40 to 60 PO mg/mL depending on extraction methods [20], here this study shows

quantitative assessments revealed terpenoids to be the most abundant constituents at 71.15 ± 0.18 PO mg/mL. They also highlighted the high antioxidant activity associated with terpenoids, though they did not specify exact concentrations [20]. Several rosemary cultivars had terpenoid levels ranging from 60 to 75 PO mg/mL [19], which supports the high terpenoid content in this study [21]. The 8.1984 ± 0.00135 GAE mg/ mL TPC is in line with readings for commercial rosemary oils. While Teixeira et al. [22] reported a TPC of 8.90 ± 0.05GAE mg/ mL in commercially available samples, whereas TPC values ranging from 10.50 to 15.30 GAE mg/ml in hydro-distilled rosemary oils, supporting the idea that commercial oils typically have lower phenolic concentrations than freshly prepared extracts. In terms of flavonoid content, the 0.1874 ± 0.00195 QE mg/mL concentration is consistent with results from Nieto et al. [23], who reported a flavonoid concentration of 0.180 ± 0.0021 QE mg/mL in commercial rosemary oil [22]. However, slightly higher values of 0.192 ± 0.0018 QE mg/mL were found, suggesting that the amount of flavonoid in rosemary oil can vary depending on the extraction process and the plant's origin [24]. The detected tannin concentration of 26.320 ± 0.16 TTA mg /mL is in line with values found in published research. Tannin concentrations of 26.8 ± 0.17 TTA mg/mL were found in a study reported by Bakkali et al. [25], while significantly higher levels (27.1 ± 0.18 TTA mg/mL) were found in other research, indicating that particular extraction techniques or plant sources may affect tannin retention [24]. These quantitative findings are summarized in Table 1. These results highlight how the phytochemical makeup of rosemary oil is influenced by factors such as extraction methods, ambient factors, and geographic origin. The production of essential oils must be standardized in order to guarantee constant therapeutic efficacy, especially for medical purposes.

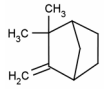
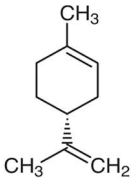
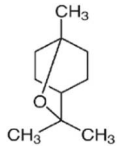
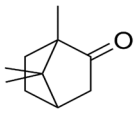
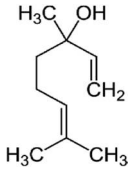
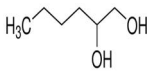
Table 1. **Phytochemical Composition of Rosemary Oil.**

Parameters	Value (Mean ± SD)
Total phenolic content GAE mg/mL	8.1983 ± 0.00135
Total Flavonoid Content QE mg/mL	0.1874 ± 0.00195
Total Tannic content TTAmg/mL	26.320 ± 0.16
Total Terpenoid Content PO mg/mL	71.15 ± 0.18

Gas-chromatography Mass spectrometer Rosemary oil.

GC-MS analysis conducted in this study identified several key bioactive constituents in rosemary oil, including 1,2-hexanediol (67.34%), α-pinene (8.0–10.5%), camphor (7.0–9.5%), camphene (5.0–6.8%), limonene (2.0–4.5%), linalool (1.5–3.5%), and 1,8-cineole (1.77%) as shown in Table 2. Among these, 1,2-hexanediol was the most abundant, indicating a distinct chemotype with enhanced antimicrobial and stabilizing properties [26]. This unique composition may increase the oil's shelf life and therapeutic effectiveness, particularly in topical or cosmetic formulations [27,28]. These compounds are well-documented for their anti-inflammatory, antibacterial, and antioxidant activities. The presence of 1,8-cineole, even at 1.77%, supports rosemary oil's applications in respiratory and neurological care. This increased quantity points to a unique chemotype that may improve the oil's stability and antimicrobial qualities, making it especially promising for use in skincare products [28]. In some cases, lower detection of these essential components might indicate limitations in the extraction or analytical procedures. According to these results, there is a considerable amount of variation in the content of rosemary oil, which can be attributed to several factors such the plant's origin, extraction methods, and possible adulteration.

Table 2. **GC-MS Composition of Rosemary Oil with Retention Time and Concentration.**

Compounds	Retention Time (min)	Concentration (%)	Chemical Structures
Camphene	5.00 - 5.60	5.0 - 6.8	
α-Pinene	5.30 – 6.00	8.0 - 10.5	
Limonene	6.50 – 7.20	2.0 - 4.5	
1,8-Cineol (Eucalyptol)	7.50 - 8.20	1.77	
Camphor	9.10 - 9.80	7.0 - 9.5	
Linalool	10.00 - 11.00	1.5 - 3.5	
1,2-Hexanediol	14.00 - 15.00	67.34	

Assessment of DPPH Free Radical Scavenging Activity

The antioxidant capacity of rosemary oil was assessed using the DPPH test. The results showed a concentration-dependent increase in free radical scavenging activity. The IC_{50} value of rosemary oil was found to be 21.80 µg/mL, showing significant antioxidant activity. This value was lower than the IC_{50} of the reference ascorbic acid (33.79 µg/mL), indicating that rosemary oil is a stronger antioxidant in this assay technique. The free radical scavenging activity (FRSA) was 67.61% at 10 µg/mL and dropped to 50.78% at 20 µg/mL. FRSA was measured at 35.88% as the concentration rose to 30 µg/mL and then dropped to 27.70% at 40 µg/mL. With an FRSA of 24.57%, the lowest activity was noted at 50 µg/mL. For instance, reported an IC_{50} value of 23.4 µg/mL, reflecting stronger antioxidant capability, while found an IC_{50} of 21.5 µg/mL, indicating more potent activity than my study, observed an IC_{50} of 27.2 µg/mL, which is similar to my result but still suggests higher antioxidant efficacy. In contrast, Ibupoto et al. [29] reported a greater IC_{50} of 35.6 µg/mL, slightly weaker, demonstrating variability in outcomes.

These findings show that rosemary oil has a considerable amount of antioxidant activity, albeit a little less than that of ascorbic acid. In contrast to earlier research, the IC_{50} values of rosemary oil differ based on the plant's origin and extraction methods. The results of this investigation are consistent with the reported IC_{50} values for rosemary oil, which vary from 19.4 µg/mL to 24.3 µg/mL. Differences in the quantities of bioactive compounds, particularly flavonoids and phenolics, which are essential for neutralizing free radicals, are the cause of the variance in antioxidant potential. The findings also demonstrate that rosemary oil is a potent antioxidant and a potential natural source for medicinal uses. The information indicates that although rosemary oil has a significant ability to scavenge free radicals, its potency might be increased when mixed with other substances that are high in antioxidants.

Antibacterial activities of the Rosemary oil

In comparison, the findings indicate that rosemary oil demonstrates significant antibacterial action against *Staphylococcus aureus*, consistent with existing research, but shows minimal efficacy against *Escherichia coli*. This supports the notion that rosemary oil

may be more effective against Gram-positive bacteria than Gram-negative pathogens, highlighting the variability in bacterial susceptibility. The antibacterial activity of rosemary oil against *S. aureus* and *E. coli* showed intermediate susceptibility in *S. aureus* at lower concentrations, while *E. coli* exhibited resistance at all tested concentrations. The interpretation of the zone diameters, particularly for the standard antibiotics (gentamicin and ampicillin), was based on the CLSI guidelines (m100, 2023). At 25%, rosemary oil inhibited *S. aureus* by 15.67 mm and at 50% by 17.33 mm, aligning with [30], who reported limited activity against Gram-positive bacteria. For *E. coli*, inhibition zones were 11.33 mm at 25% and 12 mm at 50%, consistent with findings by Ansari et al. [31], who observed limited efficacy against Gram-negative bacteria due to the protective outer membrane of *E. coli*. Gentamicin demonstrated inhibition zones of 23.1 mm for *E. coli* and 24.3 mm for *S. aureus*, confirming the susceptibility of both strains to this antibiotic, as reported by previous studies. Ampicillin showed minimal inhibition against *E. coli*, confirming the expected resistance of this strain, a typical observation for this antibiotic. However, it demonstrated significant activity against *S. aureus*, with a zone of 36.7 mm, which aligns with the findings of Vaou et al. [32]. The solvent control (ethyl acetate) showed a 10 mm inhibition for both bacterial strains, confirming that the observed antibacterial effects were due to rosemary oil. This aligns with previous findings that emphasize the importance including solvent controls in essential oil studies [32]. Additionally examined the effect of solvents on bioactive extractions, confirming the low activity of ethyl acetate on its own. As shown in Table 3, rosemary oil demonstrated notable antibacterial activity against *Staphylococcus aureus*, while its effect on *Escherichia coli* was minimal.

The main components of rosemary essential oil, including α-pinene, 1,8-cineole, and camphor, are mostly responsible for its antibacterial and antioxidant properties. These substances are very bioactive, with camphor and α-pinene showing strong antibacterial action against *Staphylococcus aureus* and other Gram-positive bacteria. They can integrate with bacterial cell membranes due to their lipophilic nature, which compromises the integrity of the membrane and increases permeability, allowing intracellular contents to flow out. The protective outer barrier that prevents hydrophobic substances from penetrating is probably the cause of the moderate effectiveness against Gram-negative bacteria like *Escherichia coli* [32]. The antioxidant properties of 1,8-cineole and α-pinene help to neutralize free radicals. In the DPPH assay, studies have demonstrated that rosemary essential oil has a free radical-scavenging activity of roughly 62.45%, which is more than the combined activities of 1,8-cineole (42.7%) and α-pinene (45.61%). According to this, the oil's constituents work in concert to increase its total antioxidant capacity. These results highlight rosemary essential oil's medicinal potential, especially in compositions meant to encourage wound healing by utilizing its antibacterial and antioxidant qualities [29].

Table 3. **Antibacterial Activity of Rosemary Oil Against *Staphylococcus aureus* and *Escherichia coli*.**

Microorganism	Gentamicin (10 µg/disc)	Ampicillin (10 µg/disc)	Concentration 25%	Concentration 50%	Concentration 75%	Ethyl Acetate (Solvent Control)
Escherichia coli	23.1 mm	0 mm	11.33 mm	12 mm	7 mm	10 mm
Staphylococcus aureus	24.3 mm	36.7mm	15.67 mm	17.33 mm	13.67 mm	10 mm

Microbial Growth Test of Rosemary Oil Hydrogel

The findings of this investigation revealed variable degrees of antimicrobial activity among treatment groups. The gentamicin group demonstrated a clear inhibition zone on day one, which was sustained until the final day, demonstrating significant and prolonged antimicrobial activity, consistent with earlier studies highlighting the potent antibacterial capabilities of gentamicin against a wide range of pathogens [33]. In contrast, the rosemary oil hydrogel group showed a considerable reduction in bacterial colonies on day 1 and a minor drop in bacterial growth over time, indicating limited antibacterial action. While rosemary oil is known for its antioxidant and anti-inflammatory properties, it has been found to have antibacterial benefits [34].

Figure 1. **Antibacterial Activity of Rosemary Oil Hydrogel Against Bacterial Growth**

In this trial, its efficacy was lower than that of gentamicin, which is consistent with the fact that natural oils normally have lower potency than conventional antibiotics [35]. Throughout the investigation, both the negative control and placebo hydrogel groups showed significant bacterial growth but no antimicrobial impact, highlighting their absence of antibacterial capabilities. These data support the hypothesis that while essential oils like rosemary oil may provide adjunct antimicrobial effects, they do not replace the efficacy of antibiotics like gentamicin. The microbial growth test demonstrated the antibacterial effect of rosemary oil hydrogel. As shown in Fig. 1, bacterial colonies were significantly reduced in the hydrogel-treated group compared to the control, confirming its antimicrobial potential.

Infected Wound Healing Monitoring of Rosemary Oil Hydrogel

The rosemary oil hydrogel was found to dramatically speed wound contraction when compared to the negative control and placebo gel groups. This discovery is consistent with previous research that has emphasized rosemary oil's wound-healing qualities [36] and found that essential oils, such as rosemary, promote collagen production and reduce inflammation, which is crucial for wound healing. Similarly, found that bioactive hydrogels loaded with plant-derived chemicals create a moist environment that promotes epithelialization and fibroblast proliferation, both of which are necessary for quicker wound closure. Furthermore, the antibacterial and antioxidant characteristics of rosemary oil may have helped to reduce the microbial burden, preventing wound infection and promoting healing. The 66.87% contraction rate seen in this investigation is comparable to contraction rates reported in earlier studies using plant-based hydrogels, where wound closure exceeded 60% in a similar timeframe

[37]. These findings indicate the potential use of rosemary oil hydrogels in wound care, particularly for infections where natural antibacterial agents are advantageous. This study demonstrates that including rosemary oil in hydrogels can improve wound healing via a synergistic effect of antioxidant and moisture-retentive qualities. The effectiveness of rosemary oil hydrogel in wound healing was assessed by measuring wound contraction over time. As shown in Fig. 2, Fig. 3 and Table 4, the hydrogel-treated group demonstrated a significantly higher wound closure rate compared to the control groups, indicating its potential to accelerate the healing process.

Figure 2. **Wound contraction percentage over time for different treatment groups**

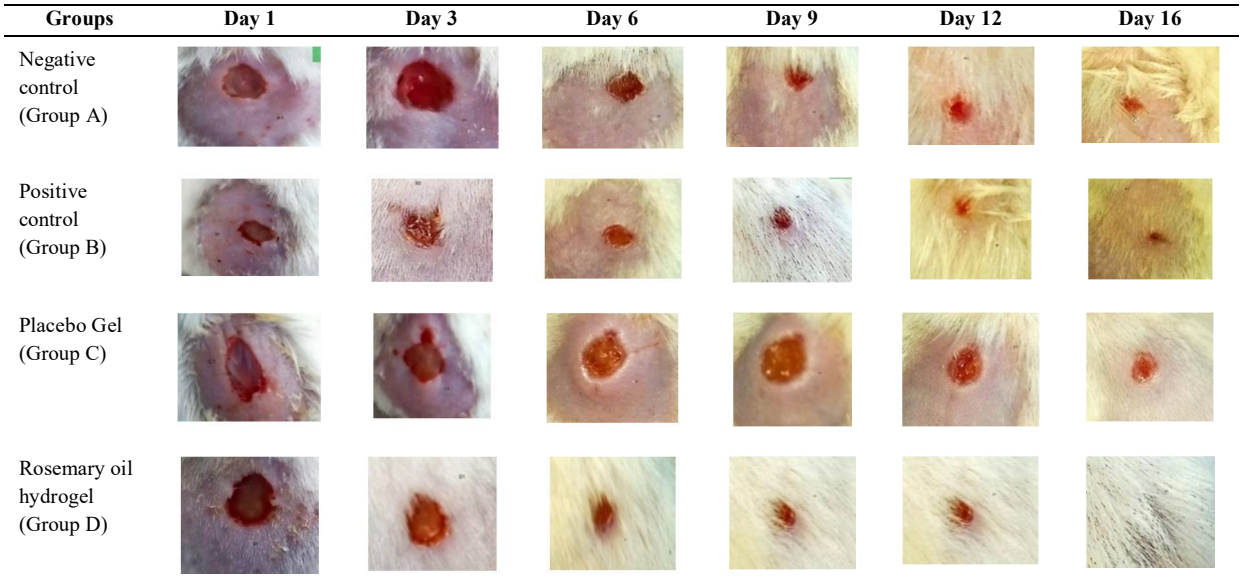


Figure 3. **Wound healing over time in 4 groups: untreated, gentamicin, placebo gel, and rosemary oil hydrogel**

Table 4. **Comparative Analysis of Wound Contraction Across Treatment Groups.**

Groups	Average (mm)						Contraction (%)			Percentage (%)
	Day 1			Day 16						
	1	2	3	1	2	3	1	2	3	
Negative control (Group A)	7	6.66	7	3.06	2.73	2.66	56.28	59	62	40.49
Positive control (Group B)	5.66	6.66	7	2.3	2.4	2.6	59.36	63.96	62.85	61.05
Placebo Gel (Group C)	6.86	7.4	7.26	4.63	4.46	4.2	32.5	39.72	42.14	38.12
Rosemary oil hydrogel (Group D)	7.5	8.46	8.83	3.26	2.43	2.4	56.53	71.27	72.81	66.87

Skin Irritation Test of Rosemary Oil Hydrogel

The skin irritation test demonstrated the unique benefits of rosemary oil hydrogel. Mice D1 treated with rosemary oil hydrogel exhibited no irritation, redness, or discomfort, indicating high skin tolerance and easy recovery. This outperformed the placebo hydrogel (Mice C1), which caused minor discomfort and mild redness, indicating slower recovery. Mice A1 (negative control) showed chronic irritation and pain, indicating a lack of healing assistance. Mice B1 (gentamicin cream) demonstrated no irritation or redness, which is consistent with its long-standing use in aiding healing without causing adverse skin reactions. These findings are consistent with previous research in which rosemary oil's anti-inflammatory and antioxidant capabilities aided tissue regeneration while minimizing discomfort [38]. However, our findings show that rosemary oil hydrogel has similar skin compatibility to gentamicin while remaining natural and multifunctional. Comparable results with Manuka honey hydrogels show effective skin tolerance and healing [39].

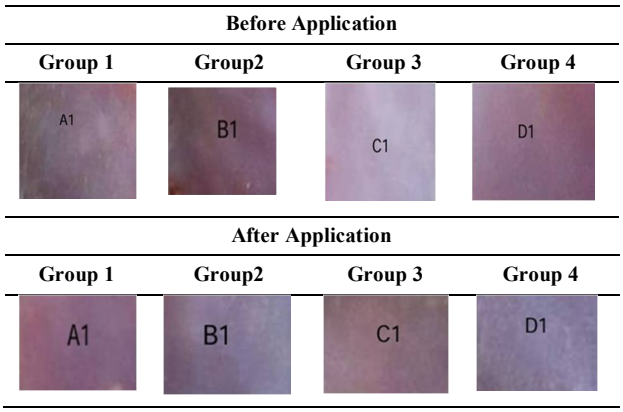


Figure 4. **Skin Irritation Test Results for Rosemary Oil Hydrogel and Control Groups**

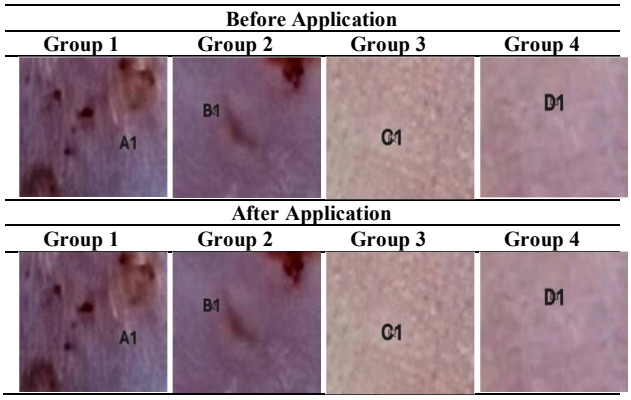
These findings highlight the potential of rosemary oil hydrogels as a promising natural option in wound treatment, calling for further development and clinical testing. The skin irritation test was conducted to evaluate the biocompatibility of rosemary oil hydrogel. As shown in Fig. 4, the hydrogel-treated group exhibited no visible signs of irritation, redness, or discomfort, indicating its suitability for topical application.

Inflammation test of Rosemary oil hydrogel

The inflammation test results show that rosemary oil hydrogel (Mice D1) had the greatest results, with faster healing and less inflammation than the other groups. This demonstrates its promise as an excellent wound care treatment, thanks to its anti-inflammatory and antioxidant characteristics. In contrast, the negative control group (Mice A1) showed poor recovery with persistent inflammation, and the placebo hydrogel (Mice C1) resulted in slower healing and sustained inflammation, indicating limited efficacy. The positive control group (Mice B1) treated with gentamicin cream demonstrated modest inflammation reduction and successful healing, confirming its recognized antibacterial characteristics but indicating a potential for improvement when compared to rosemary oil hydrogel. These findings are consistent with and expand on previous research. Rosemary oil's ability to reduce inflammation and improve healing has been related to its bioactive components, such as carnosic acid and rosmarinic acid, which inhibit pro-inflammatory cytokines and stimulate tissue regeneration [40]. Other hydrogels infused with natural agents, such as turmeric or aloe vera, have exhibited similar reductions in inflammation but slower healing rates than rosemary oil formulations. Our findings highlight that rosemary oil hydrogel not only promotes faster healing but also significantly reduces inflammation, making it a promising option for advanced wound care. Future research could refine dosage and investigate long-term usage to ensure clinical viability. The anti-inflammatory effect of rosemary oil hydrogel

was assessed through the inflammation test. As shown in Fig. 5, the hydrogel-treated group exhibited a noticeable reduction in inflammation compared to the control groups, demonstrating its potential for wound healing and skin recovery.

Figure 5. **Inflammation Test Results for Rosemary Oil Hydrogel and Control Groups.**



Conclusion

The current study confirmed the therapeutic potential of rosemary oil through detailed phytochemical and biological evaluations. Quantitative analysis revealed a TPC of 8.20 ± 0.001 mg GAE/mL, TFC of 0.1874 ± 0.002 mg QE/mL TTC of 26.32 ± 0.16 mg GAE/mL, and TTpC of 71.15 ± 0.18 mg/mL. Although slightly lower than fresh extracts, these values are consistent with commercial oils and support the presence of significant bioactive compounds. GC-MS analysis further validated this by identifying 1,2-Hexanediol (67.34%) and 1,8-Cineole (1.77%) as major constituents, both known for their antioxidant and antimicrobial properties. The findings demonstrated that rosemary oil possesses strong antioxidant activity and mild antimicrobial efficacy, particularly against *Staphylococcus aureus*. In vivo, the rosemary oil hydrogel accelerated wound contraction (66.87% by Day 16), exhibited excellent skin compatibility, and reduced inflammation effectively. These results collectively indicate that rosemary oil is a suitable candidate for both medicinal and cosmetic applications, offering multifunctional benefits in wound healing, infection management, and antioxidant support.

Conflicts of Interest

The authors declare no conflict of interest.

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