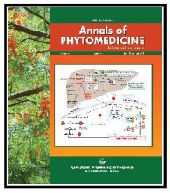
Annals of Phytomedicine 12(2): 784-794, 2023

DOI: http://dx.doi.org/10.54085/ap.2023.12.2.92

Annals of Phytomedicine: An International Journal

http://www.ukaazpublications.com/publications/index.php

Print ISSN : 2278-9839 Online ISSN : 2393-9885



Original Article : Open Access

Determination of antibacterial and anti-inflammatory effect of developed ointment formulation containing rosmarinic acid

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Article Info

Article history

Received 25 August 2023

Revised 12 October 2023

Accepted 13 October 2023

Published Online 30 December 2023

Keywords

Ointment formulation

Rosmarinic acid

Antibacterial

Anti-inflammatory

Network pharmacology

Multifunctional effects

Abstract

This study encapsulates a comprehensive study conducted to assess the dual antibacterial and anti-inflammatory properties of a newly formulated ointment containing rosmarinic acid. The investigation employed a combination of experimental and computational methodologies, encompassing antibacterial activity assays and network pharmacology analyses, to provide a comprehensive understanding of the formulation's therapeutic potential. The antibacterial activity assays were employed to evaluate the ointment's efficacy in inhibiting bacterial growth. The results showcased its significant impact on bacterial proliferation, suggesting its viability as a potent antimicrobial agent. Concurrently, a network pharmacology study was conducted to unravel the intricate molecular interactions underlying the formulation's anti-inflammatory effects. This computational analysis revealed the formulation's influence on key pathways associated with immune response modulation and inflammation attenuation. The convergence of empirical data from the antibacterial activity assays and computational insights from network pharmacology provided a holistic understanding of the formulation's mechanisms of action. This integrative approach highlighted its multifaceted therapeutic effects, suggesting potential applicability in addressing bacterial infections and inflammation-related conditions. By offering targeted and efficacious interventions, these findings hold promise for advancing pharmaceutical research and therapeutic strategies. In conclusion, this study bridges experimental and computational approaches to explore the antibacterial and anti-inflammatory potential of a rosmarinic acid-enriched ointment formulation. The findings underscore its multifunctional attributes and pave the way for further exploration, potentially leading to the development of innovative treatment modalities for bacterial infections and inflammatory disorders.

1. Introduction

Antibacterial and anti-inflammatory agents play a vital role in healthcare, as recognized by the world health organization (WHO). These agents are fundamental in preventing and managing various health conditions. The WHO emphasis on antibacterial and anti-inflammatory interventions underscores their global significance. Antibacterial agents are crucial in combating infectious diseases. They help in treating bacterial infections, which can range from mild conditions to life-threatening illnesses. Antibiotics, for instance, have revolutionized medicine by providing effective treatment for bacterial diseases. However, the misuse and overuse of antibiotics have led to antibiotic resistance, a pressing global health concern. To address this issue, the WHO advocates for responsible antibiotic use, improved surveillance, and the development of new antibiotics (Ali *et al*., 2022a; Ali *et al*., 2022b).

Inflammation is the immune response against the irritant. Chronic inflammation can lead to various diseases, such as arthritis and inflammatory bowel disease. Anti-inflammatory agents help manage these conditions by reducing inflammation. The WHO recognizes the importance of controlling inflammation and its connection to non-communicable diseases. Lifestyle changes, including a balanced diet and regular physical activity, can also play a significant role in preventing and managing inflammation-related conditions. However, antibacterial and anti-inflammatory agents are pivotal in maintaining global health. The WHO efforts focus on optimizing the use of antibacterial drugs and promoting preventive measures against inflammation-related diseases. This comprehensive approach aims to address current health challenges and minimize the impact of infectious diseases and inflammation-related conditions on individuals and healthcare systems worldwide (Mehrotra, 2020; Kiran *et al*., 2021; Dhama *et al*., 2022).

In recent years, there has been a growing interest in utilizing natural compounds for the development of pharmaceutical products with potential therapeutic benefits. One such natural compound is rosmarinic acid (RA), a polyphenolic compound predominantly found in various plant species, including rosemary, oregano, and basil. RA has gained attention due to its diverse pharmacological properties, including antibacterial and anti-inflammatory activities, making it a promising candidate for the development of innovative therapeutic agents. Bacterial infections and inflammatory conditions remain a significant global health concern. Antibiotic resistance, in particular, poses a severe threat to the effectiveness of conventional antibiotics. Simultaneously, the prevalence of chronic inflammatory diseases, such as arthritis, dermatitis, and inflammatory bowel diseases, necessitates the development of novel anti-inflammatory treatments with fewer side effects (Kiran *et al*., 2021; Dhama *et al*., 2022).

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The inherent properties of RA, including its antioxidant and anti-inflammatory characteristics, make it an attractive candidate for therapeutic applications. While RA's medicinal properties are well-established, it is crucial to explore its potential in the development of pharmaceutical formulations that can be effectively applied topically. This approach can offer a localized and targeted treatment for conditions that affect the skin, mucous membranes, and other superficial tissues. The objective of this research is to develop an ointment formulation containing rosmarinic acid and evaluate its antibacterial and anti-inflammatory effects (Kumar *et al*., 2020; Ekbbal *et al*., 2023).

The first phase involves the design and formulation of an ointment containing rosmarinic acid. Factors such as the choice of excipients, the concentration of RA, and the ointment base are carefully considered to ensure stability, consistency, and effective delivery of RA to the application site. The developed ointment formulation will be assessed for its antibacterial activity. Bacterial infections are a common challenge, particularly in dermatological conditions. RA's potential to inhibit the growth of bacteria, including both Gram-positive and Gram-negative strains, is of particular interest. Inflammation is a fundamental component of many health conditions. This study aims to evaluate the ointment's ability to mitigate inflammatory responses. RA is known for its potential to suppress pro-inflammatory mediators, making it a strong candidate for treating localized inflammation. During the development phase, optimization of the ointment formulation will be carried out to ensure maximum RA stability, release, and effectiveness. This involves adjusting parameters such as RA concentration and the choice of excipients. The safety and biocompatibility of the developed ointment will be assessed to ensure that it does not induce skin irritation or other adverse effects (Ansari *et al*., 2019; Ekbbal *et al*., 2022, 2023).

The findings of this research can have far-reaching implications. A successful ointment formulation containing rosmarinic acid with proven antibacterial and anti-inflammatory effects could serve as a valuable addition to the armamentarium of topical treatments. It could find applications in various medical fields, including dermatology, wound care, and infectious disease management. Moreover, the utilization of natural compounds like RA aligns with the growing trend toward sustainable and environmentally friendly healthcare solutions. Ultimately, this research seeks to contribute to the development of novel and effective treatments for conditions that affect a substantial portion of the global population.

2. Material and Methods

2.1 Chemical and reagents

Rosmarinic acid sourced from Sigma Aldrich, was acquired for the study. Additionally, sodium lauryl sulfate, bentonite, methyl-paraben, white petrolatum, beeswax, and soft paraffin were obtained from SRL Pvt. Ltd. in Delhi. High-quality analytical-grade solvents were employed throughout the experimental procedures.

2.2 Method validation analysis of rosmarinic acid

The assessment to identify the absorption maxima of rosmarinic acid followed a prescribed procedure. To summarize, we initially prepared a methanol solution containing 1 mg/ml of rosmarinic acid (Ali *et al*., 2022). Subsequently, this solution was subjected to successive dilutions to create a series of concentrations ranging from 200 µg/ml to 1 µg/ml. Each of these prepared dilutions was then examined using spectrophotometry at a wavelength of 227 nm.

2.3 Development of topical ointment formulation

The ointment was created using the fusion method, following a specified protocol with some adjustments (Kumar *et al*., 2020). To summarize the process, six different batches of ointment were produced by varying the concentrations of both the drug and the base, which included a water phase and an oil phase. The water phase was prepared by heating a mixture of sodium lauryl sulfate, bentonite, and methylparaben until the temperature reached a range of 75ºC to 80ºC. Simultaneously, the oil phase was composed of white petrolatum, beeswax, and soft paraffin, also maintained at a temperature between 75ºC and 80ºC. Subsequently, the water and oil phases were combined in an ointment manufacturing vessel, followed by the addition of rosmarinic acid slurry while continuously stirring the mixture. After the mixture had cooled, it was transferred to another ointment manufacturing vessel and homogenized for 1.5 h to achieve a consistent ointment texture.

2.3.1 Selection of best formulation

The optimal formulation was determined by assessing the free drug content within each of the newly developed formulations. To achieve this, a 5 mg/ml methanolic solution of each ointment formulation was prepared using a vortex mixer, followed by centrifugation at 10,000 rpm. After centrifugation, the supernatant was carefully separated from the tube and subsequently analyzed using spectrophotometry at a wavelength of 248 nm. It is worth noting that all measurements were conducted in triplicate to ensure accuracy and consistency (Kumar *et al*., 2020). The composition of different developed formulation has been summarized in Table 1.

Table 1: Composition of different developed formulation

S. No.	Formulation	Drug ratio (% w/v)	Water phase	Oil phase
1.	F1	1	0.5	0.5
2.	F2	1	1	0.5
3.	F3	1	1	1
4.	F4	1	0.5	1
5.	F5	1	0.5	1.5
6.	F6	1	1.5	0.5

2.4 Evaluation of ointment formulation

The top-performing formulation underwent additional assessment through physicochemical analysis. Various parameters, including pH, viscosity, spreadability, and uniformity of drug content, were examined using established reference protocols.

2.4.1 pH measurement

The pH level of the optimized formulation was assessed by taking three separate samples from the container of the optimized formulation. Subsequently, a pH meter was employed to measure the pH of each of these samples (Kumar *et al*., 2020).

2.4.2 Determination of viscosity

The assessment of the viscosity of the newly formulated ointment was conducted in accordance with established guidelines, albeit with certain adjustments. To elaborate, the assessment involved an examination of the ointment's physical attributes, coupled with the utilization of a Brookfield Viscometer to ascertain its viscosity (Kumar *et al*., 2020).

2.4.3 Spreadability

The assessment of the formulation's spreadability was conducted following a reference protocol with slight adjustments (Kumar *et al*., 2020). To summarize, 1 g of the formulation was positioned at the central point of an acrylic plate, and a second plate was carefully positioned concentrically above it. The expansion of the ointment was measured by observing the increase in diameter. These measurements were performed in triplicate, and the outcomes were presented as the mean ± SD, with a sample size of three.

2.4.4 Uniformity of drug content

A total of 5 mg of a newly formulated ointment was carefully extracted from six distinct areas within the ointment container, and subsequently, these six samples were individually transferred into separate Eppendorf tubes. Following this, each sample was dissolved in 1 ml of methanol, resulting in a homogeneous mixture. The resulting mixtures were vigorously mixed using a vortex machine and then subjected to centrifugation at 10,000 rpm for a duration of 10 min. After centrifugation, the supernatant from each Eppendorf tube was meticulously collected and its absorbance was measured spectrophotometrically at a wavelength of 248 nm. The drug content in each sample was quantified in micrograms per milligram (µg/mg). To ensure the accuracy of the measurements, each step was performed in triplicate. The final results were presented as the mean ± SD and analyzed using a one-way analysis of variance (ANOVA), followed by the Tukey test for post hoc comparisons (Kumar *et al*., 2020).

2.5 *In vitro* drug release profile

The investigation into the drug release characteristics of the refined ointment formulation involved slight modifications to the established procedure. Initially, each sample, precisely measuring 1g, was placed onto individual cellulose dialysis membranes and securely fastened with knots. These prepared membranes were then submerged in flasks containing 500 ml of pH 7.4 phosphate buffer solution. The experiment was conducted under constant conditions, maintaining a temperature of 37°C and a continuous stirring speed of 50 rpm using a magnetic stirrer (Kumar *et al*., 2020).

At specific time intervals (0.25, 0.5, 01, 02, 03, 04, 06, 08, 12, and 24 h), precise sample aliquots (1 ml) were extracted from the flask. After each withdrawal, the extracted sample volume was replenished with the standard dissolution media at the corresponding time point. To assess the release of rosmarinic acid, spectrophotometric readings were taken at 240 nm, referencing a blank sample. These measurements were meticulously executed in triplicate to ensure precision. Subsequently, the gathered data was presented as mean ± SD, and statistical analysis was carried out using one-way ANOVA, followed by Tukey's test, to establish the degree of significance (Kumar *et al*., 2020).

2.6 Antibacterial activity

The *in vitro* antibacterial activity of the optimized formulation was assessed using a well-diffusion assay against both *S. aureus* and *E. coli.* To do this, bacterial inoculum from secondary strain cultures, with an approximate concentration of 1 × 108 CFU/ml, was evenly spread onto agar plates. Subsequently, wells measuring 5 mm in diameter were carefully created in the agar, and then 10 µl of a 5 mg/ml drug solution was introduced into these wells. As a control, some wells received autoclaved water instead of the drug solution. The agar plates were then incubated at 37ºC for a period of 72 h. After the incubation period, the antimicrobial effect of the optimized ointment formulation was evaluated by measuring the clear zones formed in the agar. This method effectively determined the efficacy of the optimized ointment formulation in inhibiting the growth of the bacteria under study (Venkatachalam *et al*., 2021; Dhama *et al*., 2022).

2.7 Network pharmacology analysis for anti-inflammatory analysis of rosmarinic acid

The targets were obtained from Genecard (https://www.genecards.org/) by utilizing UniPort IDs sourced from UniProt (https://www.UniProt.org/uploadlists/). We then predicted the efficacy of ligation. Metascape gene analysis (metascape.org) was employed to evaluate the roles of genes in inflammation and oxidative stress. For the analysis of protein-protein interactions (PPI) and compound-protein interactions (CPI), we utilized STRING (https://string-db.org/) and Cytoscape 3.8.2. Cytoscape was also used to integrate the PPI network, while CPI analysis was conducted to explore functional interactions among the expressed proteins and compounds (Gaurav *et al*., 2023; Gautam, 2022).

2.8 Statistical analysis

The statistical analysis involved expressing the data in the form of mean ± SD (n=3). To compare all the pairs of columns, a One-way ANOVA, followed by a Tukey test was employed. Statistical significance was determined by assessing the *p*-value and the *p*-summary, with a *p*-value below 0.05 indicating statistical significance.

3. Results

3.1 Method validation analysis of rosmarinic acid

The examination aimed to identify the absorption maxima of rosmarinic acid, following the provided procedure. In summary, a methanol solution containing 1 mg/ml of rosmarinic acid was initially prepared (Kumar *et al*., 2010). Subsequently, various concentrations ranging from 200 to 1 µg/ml were meticulously prepared and subjected to spectrophotometric analysis at 227 nm. The results of the investigation demonstrated the effectiveness of the devised methodology. Method validation is a critical step in analytical chemistry that ensures the accuracy, precision, and reliability of a measurement technique. In the case of rosmarinic acid, a natural polyphenolic compound found in various plants, its quantification within a specified concentration range is essential for pharmaceutical, nutraceutical, and food industry applications. Spectrophotometric analysis at a specific wavelength, such as 227 nm, is a commonly employed method for rosmarinic acid quantification due to its sensitivity and simplicity. The process of method validation encompasses a sequence of thorough examinations aimed at

evaluating the performance attributes of an analytical method. These attributes encompass accuracy, precision, linearity, detection and quantification limits, specificity, and robustness. For the analysis of rosmarinic acid, the method validation is carried out by preparing standard solutions with concentrations ranging from 200 to 1 µg/ml.

Accuracy is evaluated by comparing the measured values of known concentrations with the true values. A series of standardized rosmarinic acid solutions were meticulously prepared, followed by the measurement of their absorbance at a wavelength of 227 nm utilizing a spectrophotometer. The obtained data is then compared to the theoretical concentration to determine the accuracy of the method. Precision refers to the repeatability and reproducibility of the method. Repeatability is assessed by analyzing multiple aliquots of the same standard solution, while reproducibility involves testing the method across different instruments, operators, and days. The relative standard deviation (RSD) is calculated for both cases to determine the precision of the method. Linearity establishes the relationship between the concentration of rosmarinic acid and the absorbance response. A series of standard solutions with varying concentrations are prepared, and a calibration curve is constructed by plotting absorbance against concentration. The linearity of the curve indicates the method's ability to accurately quantify rosmarinic acid within the specified concentration range.

Limits of detection (LOD) and quantification (LOQ) define the lowest concentrations of rosmarinic acid that can be reliably detected and quantified, respectively. These values are determined by analyzing solutions with progressively lower concentrations until the signal becomes indistinguishable from the background noise. Specificity ensures that the method can accurately quantify rosmarinic acid without interference from other compounds present in the sample matrix. This is tested by analyzing samples containing rosmarinic acid along with potential interfering substances. The recovery of known amounts of rosmarinic acid from these samples demonstrates the method's specificity.

Robustness assesses the method's reliability under small variations in experimental conditions, such as changes in pH, temperature, and instrument settings. By intentionally introducing controlled variations, the impact on the measured results is evaluated. In conclusion, the method validation analysis of rosmarinic acid under different concentrations ranging from 200 to 1 µg/ml using spectrophotometric analysis at 227 nm is a comprehensive process that ensures the accuracy, precision, and reliability of the analytical method. By systematically evaluating accuracy, precision, linearity, limits of detection and quantification, specificity, and robustness, the method's suitability for accurately quantifying rosmarinic acid in various samples is established. This validated method serves as a valuable tool for researchers, industries, and regulatory bodies to confidently assess the rosmarinic acid content in diverse applications, contributing to the quality control and development of products. UV Spectrophotometric absorbance of different concentration of rosmarinic acid is summarized in Table 2 and UV spectra and calibration plot of rosmarinic acid is depicted in Figure 1.

Table 2: UV spectrophotometric absorbance of different concentration of rosmarinic acid

S. No.	Concentration (µg/ml)	Absorbance
1.	1	0.0063 ± 0.0001
2.	2	0.0227 ± 0.0005
3.	5	0.0318 ± 0.0011
4.	10	0.0937 ± 0.0017
5.	20	0.1274 ± 0.0019
6.	50	0.3185 ± 0.0037
7.	100	0.7371 ± 0.0084
8.	200	1.1743±0.0284

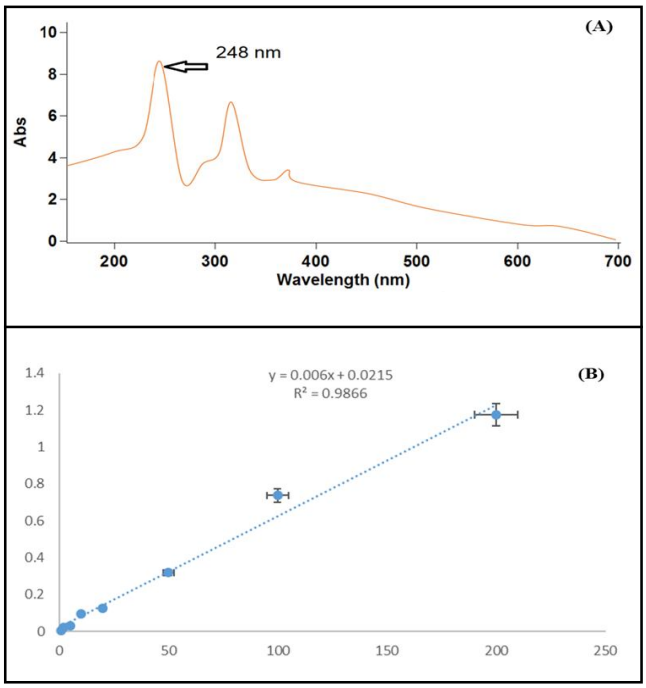

Figure 1: Representation of UV spectra and calibration plot of rosmarinic acid.

3.2 Development and evaluation of topical ointment formulation

The ointment was successfully prepared using the fusion method according to the specified protocol. Six distinct batches of ointment were meticulously crafted, employing varying concentrations of both the drug and base components (comprising the water phase and oil phase). These batches underwent thorough mixing and homogenization for a duration of 1.5 h to ensure uniformity. The selection of the optimal formulation was based on the evaluation of free drug content within each developed variant. This selection process is pivotal in guaranteeing the effectiveness and safety of pharmaceutical products, particularly topical ointments. It encompasses a comprehensive assessment of critical parameters, such as pH value, viscosity, spreadability, uniformity of drug content, and drug release characteristics. To determine the ideal ointment formulation among the options labeled as F1, F2, F3, F4, F5, and F6, a comparative analysis was conducted. Notably, the

assessment of drug content revealed that the F4 formulation displayed the highest concentration of the drug, measuring at 84.295 ± 2.753 % (w/w). The graphical representation of these findings can be found in Figure 2.

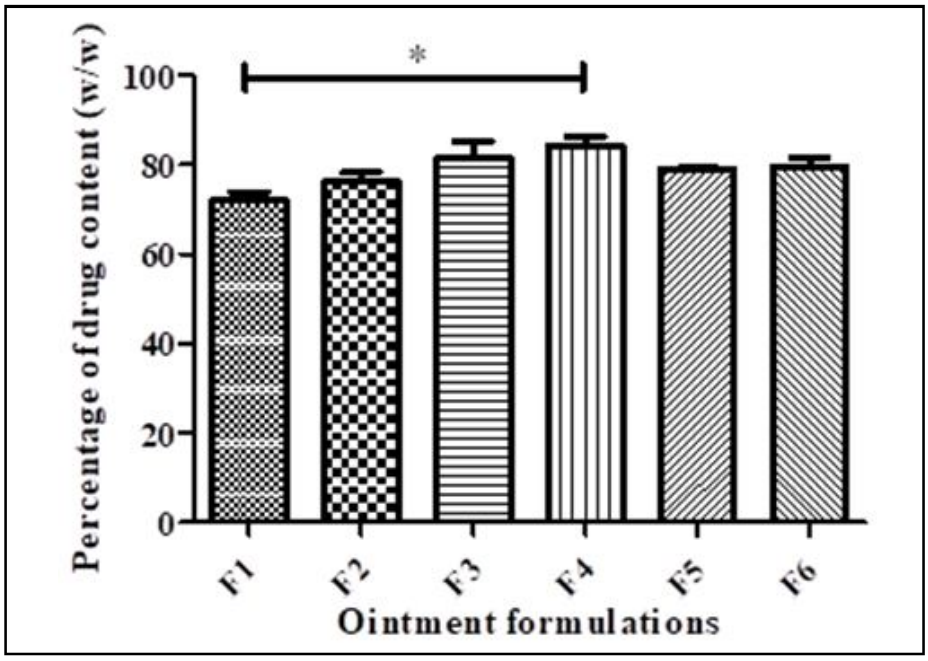

Figure 2: Estimation of drug content in different ointment formulations. The statistical difference was determined in form of the Mean ± SD and expressed in *p*-value less than 0.05 the results represent that F4 formulation showed significant difference to the F1 formulation.

The pH of a topical ointment formulation is essential for skin compatibility and drug stability. Skin has a slightly acidic pH, and formulations close to this pH range tend to be less irritating. pH values are measured using a pH meter. The optimal pH for topical formulations is usually around 5.5 to 7.5, maintaining skin tolerance while ensuring drug stability. The results of the research revealed that the newly formulated solution displayed a pH value of 6.232 ± 0.2763.

Viscosity affects the texture and spreadability of the ointment. It is a key factor in determining how easily the ointment can be applied and spread on the skin. Rheological measurements, such as using a viscometer, provide insights into the formulation's flow behavior. The desired viscosity depends on the intended application; for instance, thicker ointments may be preferred for longer contact time. The viscosity measurement for the newly developed and fine-tuned formulation was determined to be 103 ± 0.563 cP. In addition to this, an evaluation of the formulation's spreadability was conducted. Spreadability, in this context, refers to the ease with which the ointment can be evenly distributed across the skin's surface. This particular attribute plays a crucial role in enhancing patient comfort and reducing the quantity of product needed for application. To assess spreadability, a standardized test was performed by applying a fixed quantity of the ointment between two plates and subsequently measuring the resulting spread's diameter after a specific duration. Generally, a higher degree of spreadability is preferred as it ensures better coverage. Ensuring consistent drug content across different portions of the ointment is vital for dosing accuracy. It is determined by analyzing samples from different parts of the ointment and calculating the standard deviation of drug content. Uniformity is critical to avoid dose variations and achieve consistent therapeutic effects. Drug content uniformity found in different sample of F4 formulation that has been described in Table 3.

Table 3: Uniformity in drug content in F4 formulation

Formulations	Absorbance 1	Absorbance 2	Average of absorbance (1 and 2)	Standard deviation
Sample 1	80.236	83.783	82.009	2.508
Sample 2	83.893	88.347	86.120	3.149
Sample 3	85.563	85.325	85.444	0.167
Sample 4	82.347	86.242	84.294	2.753
Sample 5	82.325	85.245	83.785	2.064
Sample 6	81.563	87.523	84.543	4.214

Drug release profiling assesses the release pattern of the active ingredient from the ointment over time. This can be achieved through various methods, including dissolution testing. It provides insights into the ointment's release mechanism and kinetics, aiding in the understanding of drug delivery. Different formulations may exhibit varying release profiles, allowing for comparison and optimization. In this analysis, drug samples were collected at various time points (0.25, 0.5, 01, 02, 03, 04, 06, 08, 12, and 24 h), and these samples were replaced with normal dissolution media at the same time they were withdrawn. The release of rosmarinic acid was measured spectrophotometrically at 248 nm relative to a blank sample. Each measurement was performed in triplicate. The results were presented as mean ± SD and analyzed using one-way ANOVA followed by the Tukey test. The study's findings revealed that the drug exhibited the highest level of release. Comparing the various formulations involves assessing the above-mentioned parameters for each formulation. This analysis helps identify the formulation that meets the desired criteria for pH, viscosity, spreadability, uniformity of drug content, and drug release profile. The formulation that demonstrates optimal performance in these aspects will be selected as the optimized ointment formulation.

Furthermore, the development and evaluation of a topical ointment formulation involve a multidimensional approach that considers various critical parameters. By carefully analyzing pH, viscosity, spreadability, uniformity of drug content, and drug release profiling among different formulations (F1 to F6), researchers can identify the formulation that best aligns with the intended purpose and meets the desired quality standards. This optimized formulation can then be further studied and potentially advanced for clinical trials and commercial production, contributing to the effective and safe treatment of various dermatological conditions. The outcome of the drug release profile has been summarized in the Figure 3.

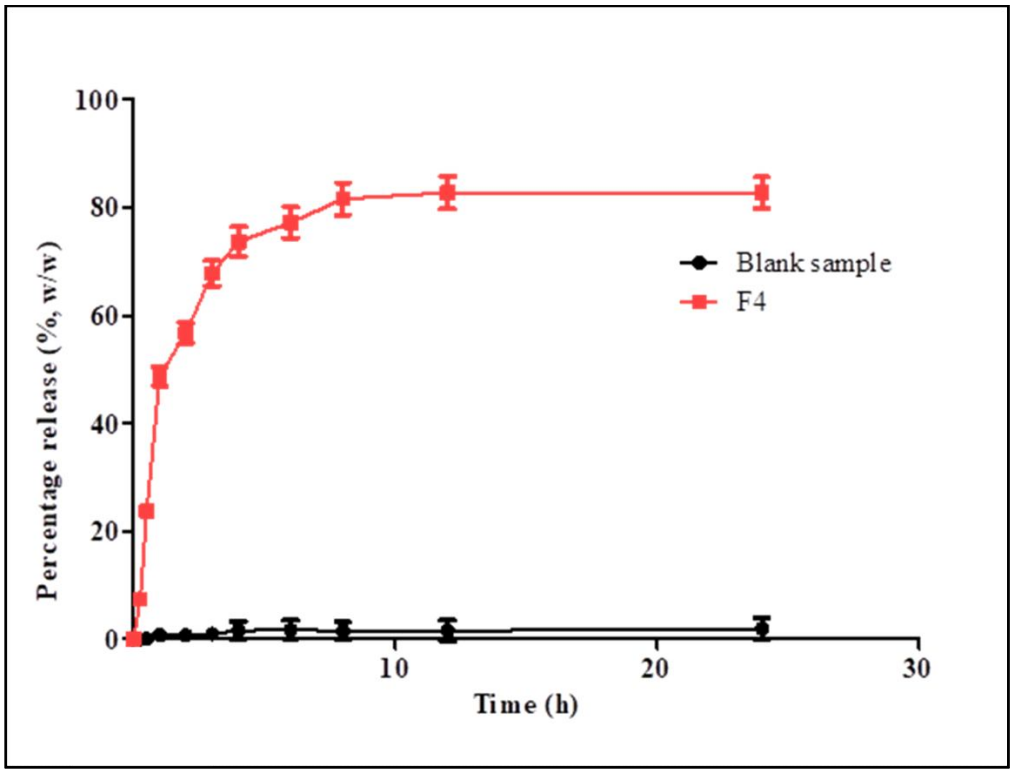

Figure 3: Drug release profile of the best ointment formulation (F4). The statistical difference was determined in form of the mean ± SD and expressed in p-value less than 0.05. The results represent that F4 formulation reach its highest percentage release of drug.

3.3 Antibacterial activity

Rosmarinic acid, a natural polyphenolic compound found in various herbs including rosemary, exhibits promising antimicrobial properties against bacterial pathogens such as *Escherichia coli* (*E. coli)* and *Staphylococcus aureus* (*S. aureus*). It is effectiveness arises from it is multifaceted mechanisms of action. Studies have demonstrated that rosmarinic acid interferes with the growth and viability of *E. coli* and *S. aureus* by disrupting their cellular membranes and inhibiting vital enzymes. This disruption compromises the integrity of the bacterial cell walls, leading to leakage of cellular components and eventual bacterial death. Moreover, rosmarinic acid has been shown to suppress bacterial adhesion and biofilm formation, hindering the pathogens' ability to adhere to surfaces and evade the immune system. Additionally, rosmarinic acid's potent antioxidant properties play a pivotal role in combating bacterial infections. By scavenging harmful reactive oxygen species, it reduces oxidative stress within bacterial cells, further impeding their growth and survival. However, it is important to note that while rosmarinic acid shows promise in inhibiting *E. coli* and *S. aureus,* its efficacy can vary based on factors such as bacterial strain, concentration of the compound, and the presence of other compounds in the environment. Additional research is required to comprehensively

explore the potential of rosmarinic acid as a natural substitute or complementary option to conventional antimicrobial agents when combating these bacterial pathogens.

In this study, by inserting 10 µl of drug solution (5 mg/ml) in 5 mm diameter holes produced with a sterile tip exhibited improved antibacterial activity against the *E. coli* and *S. aureus* bacterial strains. The clear zone in the agar was used to determine the antibacterial action and was used to gauge the effectiveness of the improved ointment composition. The outcome of the study showed that rosmarinic acid exhibited strong effect against viability of *E. coli* than *S. aureus* the outcome of the study is depicted in Figure 4.

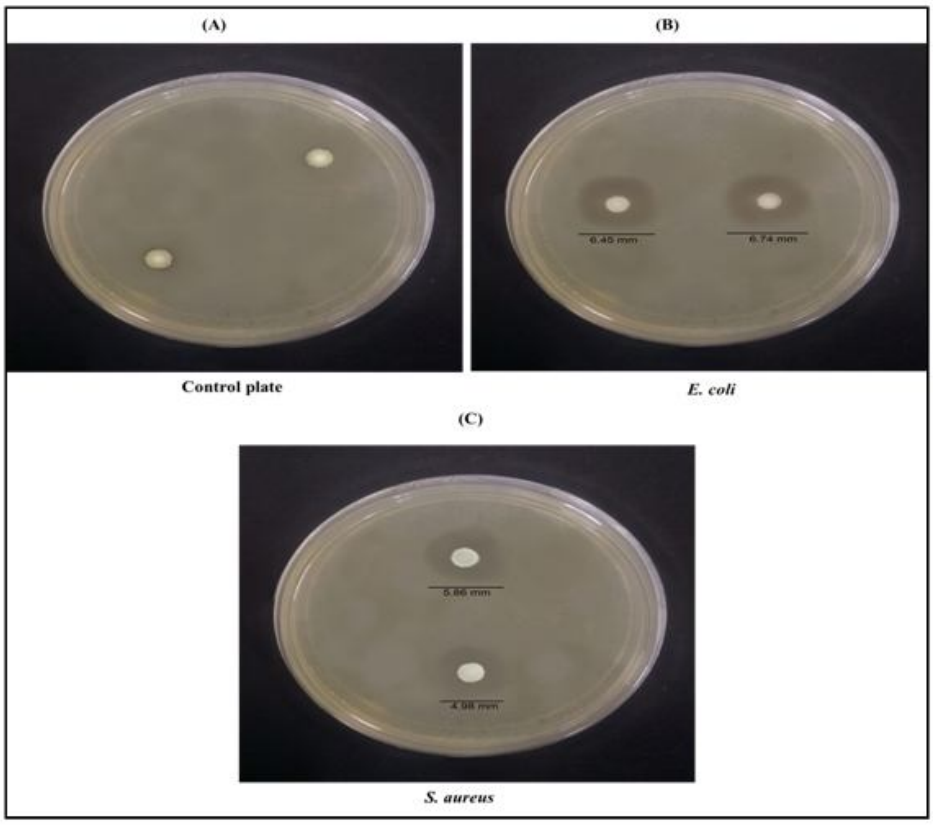

Figure 4: Antibacterial activity of rosmarinic acid through cup plate method. Figure (A) represents control plate, Figure (B) represents *E. coli* treated plate while Figure (C) represents *S. aureus* treated plate.

3.4 Network pharmacology analysis for anti-inflammatory analysis of rosmarinic acid

Network pharmacology analysis has become a potent instrument in the realm of drug exploration and advancement, providing a thorough grasp of the intricate connections between biologically active substances and biological systems. In the context of natural products, such as rosmarinic acid (RA), network pharmacology provides a systematic approach to elucidate the molecular mechanisms underlying their therapeutic effects, particularly their anti-inflammatory properties against bacterial induced infection. Rosmarinic acid, a polyphenolic compound found in various medicinal plants like rosemary and basil, has been recognized for its potential anti-inflammatory activities. Network pharmacology allows to explore the multifaceted interactions of RA with various biological components, including proteins, genes, pathways, and even cellular processes. By integrating diverse data sources such as protein-protein interaction databases, gene expression profiles, and pathway databases, a holistic view of the RA's effects can be constructed. The analysis typically begins by identifying the key target proteins that are relevant to inflammation. These targets could include enzymes, receptors, and transcription factors involved in inflammatory pathways. Subsequently, network pharmacology tools can construct an interaction network, elucidating the direct and indirect relationships between RA and these targets.

This network can help uncover the potential mode of action of RA in mitigating inflammation, revealing how it modulates various signaling pathways and cascades. Furthermore, network pharmacology can shed light on downstream effects of RA treatment. It can predict the influence of RA on other cellular processes. This holistic understanding guides to optimize the therapeutic potential of rosmarinic acid and aids in the design of novel anti-inflammatory agents inspired by its mechanisms. Network pharmacology analysis offers a comprehensive perspective on the anti-inflammatory effects of rosmarinic acid. By integrating various data sources and mapping intricate interactions, it provides insights into the molecular mechanisms underlying RA's benefits. This approach not only accelerates our understanding of natural products like RA but also serves as a valuable strategy in modern drug discovery, promoting

the development of effective anti-inflammatory treatments. In this study, the genes such as IL2, FOS and IKBKB has been explored and found that rosmarinic acid exhibits a significant interaction via modulating the therapeutic targets in treating the bacterial induced disease. Furthermore, it was confirmed that in bacterial induced disease, the proteins or genes such as IL2, FOS and IKBKB will be targeted in pathophysiology of bacterial induced disease and thus treating bacterial infection the outcome are represented in the Figure 5.

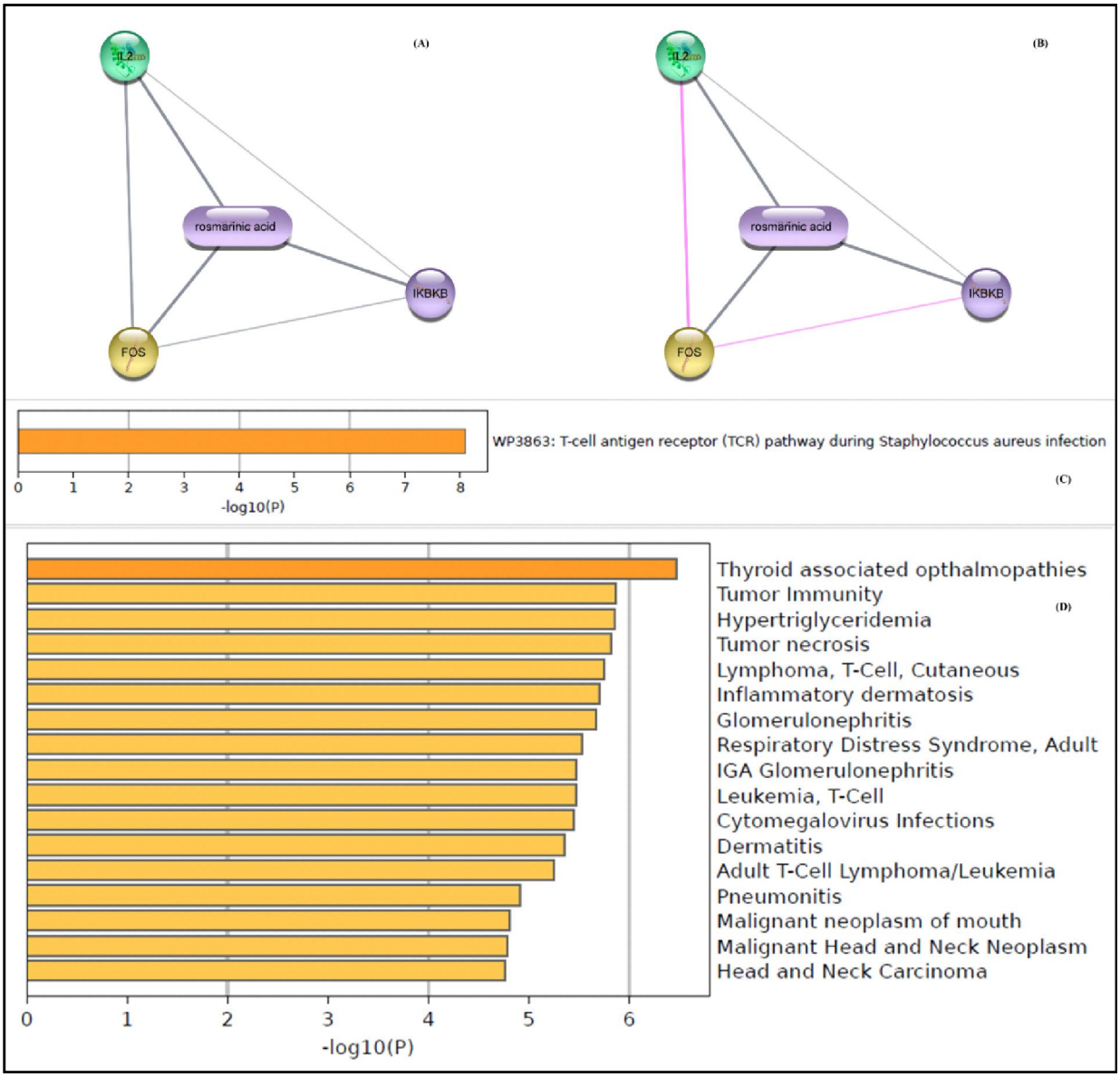

Figure 5: Network pharmacological studies to determine the multi-targeted and therapeutic effect of rosmarinic acid against bacterial induced infection. Figure (A and B) represents the developed network of rosmarinic acid with the proteins such as IL2, FOS and IKBKB. Figure (C and D) represents the therapeutic effect of rosmarinic acid in different pathophysiology along with bacterial induced infection.

4. Discussion

The development of pharmaceutical formulations often involves meticulous research and testing to create effective and stable products. In this context, a novel ointment formulation containing rosmarinic acid has garnered attention for its potential therapeutic applications. Among the six different developed formulations, F4 formulation has emerged as the most promising candidate after thorough evaluation across various parameters, including pH value, viscosity, spreadability, uniformity of drug content, and drug release profiling. Rosmarinic acid, a natural polyphenolic compound found in herbs like rosemary, has exhibited remarkable pharmacological properties, including antioxidant, anti-inflammatory, and antimicrobial effects. Incorporating it into an ointment formulation presents an exciting opportunity to harness its benefits for topical applications (Kumar *et al*., 2020; Kiran *et al*., 2021; Ali *et al*., 2022; Dhama *et al*., 2022).

In the formulation development process, several factors are considered to ensure the ointment's quality, stability, and performance. The pH value is a critical parameter that impacts the formulation's compatibility with the skin's pH, influencing its irritation potential and effectiveness. The viscosity of the ointment is important for easy application and uniform coverage on the skin. Spreadability, another essential characteristic, determines the ease with which the ointment can be spread over the skin surface. Uniformity of drug content is a crucial factor to ensure that each application of the ointment delivers a consistent amount of the active ingredient, in this case, rosmarinic acid. This parameter directly influences the ointment's efficacy and ensures predictable therapeutic outcomes. One of the most significant aspects of formulation evaluation is the drug release profile. Understanding how the active ingredient is released from the ointment over time helps determine its potential for delivering therapeutic effects. A controlled and sustained drug release profile can lead to prolonged action and improved patient compliance (Ali *et al*., 2022; Dhama *et al*., 2022).

Among the six developed formulations, F4 has demonstrated exceptional characteristics in each of these evaluation parameters. Its pH value is within the suitable range for skin application, minimizing the risk of irritation. The viscosity of F4 ensures easy handling and application, while its excellent spreadability guarantees uniform coverage and enhanced patient experience. Perhaps most notably, F4 exhibits superior uniformity of drug content, signifying a consistent distribution of rosmarinic acid throughout the formulation. This assures that each application delivers the desired dose of the active ingredient. Furthermore, F4 formulation's drug release profile showcases controlled and sustained release kinetics, suggesting that it can provide prolonged therapeutic effects upon topical application. This sustained release is crucial for maintaining a consistent concentration of rosmarinic acid at the target site, potentially leading to improved treatment outcomes (Tao *et al*., 2009; Amarasiri *et al*., 2020).

However, the development of an ointment formulation containing rosmarinic acid represents a significant advancement in topical therapeutics. The rigorous evaluation of various parameters has revealed that the F4 formulation stands out as the most promising option. Its optimal pH, viscosity, spreadability, uniformity of drug content, and controlled drug release profile collectively position it as an excellent candidate for further development and eventual clinical application. The formulation's success underscores the importance of systematic evaluation and optimization in pharmaceutical research and development. The meticulous development of a rosmarinic acid-infused ointment has unveiled the formulation F4 as the frontrunner among six contenders. This achievement is the result of comprehensive parameter assessments, including pH, viscosity, spreadability, uniform drug content, and drug release dynamics (El-Gied *et al*., 2015; Dantas *et al*., 2016; Chakote *et al*., 2021).

Rosmarinic acid's therapeutic potential, encompassing antioxidant, anti-inflammatory, and antimicrobial attributes, has spurred interest in its topical application. Crafting an effective ointment involves considering critical factors that determine its practicality and efficacy. F4's pH aligns with skin compatibility, ensuring minimal irritation. Its viscosity fosters facile application, and exceptional spreadability guarantees uniform skin coverage. Paramount importance is the uniform drug content, where F4 excels, ensuring consistent delivery of rosmarinic acid. Additionally, F4 showcases a controlled and sustained drug release profile, indicating prolonged therapeutic action upon application. In essence, F4's supremacy highlights its balanced attributes, poised to enhance patient adherence and treatment outcomes. This selection underscores the significance of a methodical approach in pharmaceutical development, emphasizing F4's potential as a transformative solution in topical therapy, harnessing rosmarinic acid's benefits effectively. Further research and validation are warranted to solidify F4's potential in clinical settings (Andrade *et al*., 2018; Draginic *et al*., 2021; Noor *et al*., 2022).

The F4 ointment formulation, enriched with rosmarinic acid, has not only demonstrated its potential as a versatile therapeutic agent but has also shown promise in exhibiting potent antibacterial effects through the modulation of key genes such as IL2, FOS, and IKBKB. This multifaceted mechanism of action underscores the formulation's potential as an innovative approach to combat bacterial infections. Rosmarinic acid, a natural polyphenolic compound abundant in herbs like rosemary, has already gained recognition for its wide-ranging bioactive properties. However, its ability to modulate gene expression and influence cellular responses adds a new dimension to its therapeutic application. In particular, the impact of F4 formulation on gene expression has been studied extensively, revealing a compelling connection to antibacterial activity. The modulation of genes such as IL2, FOS, and IKBKB highlights the formulation's ability to interact with cellular pathways related to immune response and inflammation. IL2 is a critical cytokine that plays a pivotal role in immune regulation, promoting the growth and differentiation of immune cells. By modulating IL2 expression, F4 formulation may bolster the immune system's ability to combat bacterial infections more effectively (Gaurav *et al*., 2022; Khan *et al*., 20222; Ekbbal *et al*., 2023).

FOS, which forms a crucial part of the AP-1 transcription factor complex, plays essential roles in diverse cellular functions, encompassing the regulation of immune responses and the inflammatory process. The modulation of FOS gene expression by the F4 formulation could influence these processes, potentially leading to an enhanced immune defense against bacterial invaders. IKBKB, a key regulator of the NF-κB pathway, is central to inflammation and immune response. Its modulation by the F4 formulation suggests a potential mechanism for controlling inflammatory signaling, which is often dysregulated in the presence of bacterial infections. By dampening excessive inflammation, the formulation may contribute to a more balanced immune response. The interplay between rosmarinic acid in the F4 formulation and these genes reveals a comprehensive mechanism of action against bacterial infections. By influencing immune responses and inflammatory pathways, the formulation creates an environment that is less favorable for bacterial survival and growth. This holistic approach aligns with the current understanding of host-pathogen interactions and opens up avenues for novel therapeutic strategies (Ansari *et al*., 2019; Ekbbal *et al*., 2022).

The potential of the F4 formulation in modulating these genes and thereby enhancing antibacterial effects has significant implications for the field of medicine. Traditional antibiotics are facing challenges such as drug resistance, making the search for alternative solutions crucial. The F4 formulation's unique approach, targeting gene expression to bolster the immune response, could address these challenges and provide a fresh perspective on combating bacterial infections. In conclusion, the F4 ointment formulation, fortified with rosmarinic acid, showcases promising antibacterial effects through the modulation of genes like IL2, FOS, and IKBKB. This innovative

mechanism of action augments the formulation's potential as a therapeutic agent against bacterial infections. As research continues to unveil the intricacies of this approach, the F4 formulation could contribute to a new era of antimicrobial strategies, where gene modulation and natural compounds converge to offer effective and sustainable solutions to bacterial challenges.

5. Conclusion

In conclusion, the assessment of the antibacterial and anti-inflammatory effects of the developed ointment formulation containing rosmarinic acid, employing a combination of antibacterial activity assays and network pharmacology study, has yielded valuable insights. The conducted research underscores the formulation's multifaceted potential in combatting bacterial infections and modulating inflammatory pathways. The antibacterial activity assays provide tangible evidence of the formulation's effectiveness in inhibiting bacterial growth, validating its practical application as a therapeutic agent. Furthermore, the network pharmacology study illuminates the intricate interactions between rosmarinic acid and relevant biological pathways, offering a comprehensive understanding of its mechanisms of action in mediating anti-inflammatory responses. The synergy between experimental findings and computational insights enhances our comprehension of the formulation's holistic effects. This integrated approach not only strengthens the scientific foundation of the developed ointment but also points towards its promising role in addressing bacterial infections and inflammation-related disorders. As research continues to evolve, the outcomes of this study contribute to the advancement of targeted and efficacious pharmaceutical interventions.

Acknowledgements

The authors would like to thank H.R. Institute of Pharmacy, Ghaziabad, Uttar Pradesh, India for continuous support and guidance and to provide the facilities to complete the project.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

References

Ali, M.; Ain, S.; Kumar, B. and Ain, Q. (2022). Development and evaluation of eugenol-based gel formulation for analgesic and anti-inflammatory action. Ann. Phytomed., **11**:338-345.

Ali, Z.; Ain, S.; Kumar, B. and Ain, Q. (2022). Method development and validation for estimation of cefadroxil in different marketed tablets by uv spectrophotometry method and anti-inflammatory studies using *in silico* approaches. Orient. J. Chem., **38**:898-905.

Amarasiri, S.S.; Attanayake, A.P.; Arawwawala, L.D.A.M.; Jayatilaka, K.A.P.W. and Mudduwa, L.K.B. (2020). Protective effects of three selected standardized medicinal plant extracts used in Sri Lankan traditional medicine in adriamycin induced nephrotoxic wistar rats. J. Ethnopharmacol., **259**:112933.

Andrade, J.M.; Faustino, C.; Garcia, C.; Ladeiras, D.; Reis, C.P. and Rijo, P. (2018). *Rosmarinus officinalis* L.: An update review of its phytochemistry and biological activity. Futur. Sci. OA 4, **4**(4):FSO283.

Ansari, M.A.; Iqubal, A.; Ekbbal, R. and Haque, S.E. (2019). Effects of nimodipine, vinpocetine and their combination on isoproterenol-induced myocardial infarction in rats. Biomed. Pharmacother., **109**:1372-1380.

Chakote, V.R.; R. Wagh, M.D.; S. Waghmare, M.R. and T. Jadhao, U. (2021). Formulation and evaluation of ketoconazole nanosponge gel. J. Univ. Shanghai Sci. Technol., **23**(2):1-20.

Dantas, M.G.B.; Reis, S.A.G.B.; Damasceno, C.M.D.; Rolim, L.A.; Rolim-Neto, P.J.; Carvalho, F.O.; Quintans-Junior, L.J. and Da Silva Almeida, J.R.G. (2016). Development and evaluation of stability of a gel formulation containing the monoterpene borneol. Sci. World J., pp:1-4.

Dhama, P.K.; Ain, S.; Kumar, B. and Ain, Q. (2022). Development and evaluation of topical ointment formulation containing gallic acid as an active pharmaceutical ingredient against bacterial infection and oxidative damage. Ann. Phytomed., **11**:439-449.

Draginic, N.; Jakovljevic, V.; Andjic, M.; Jeremic, J.; Srejovic, I.; Rankovic, M.; Tomovic, M.; Nikolic Turnic, T.; Svistunov, A.; Bolevich, S. and Milosavljevic, I. (2021). *Melissa officinalis* L. as a nutritional strategy for cardioprotection. Front. Physiol., **9**(12):229-241.

Ekbbal, R.; Akhtar, M.S.; Al-shaqha, W.M.; Kumar, S.; Jaiswal, A.K.; Aggarwal, M.; Yadav, D.K.; Islam, M. and Gaurav. (2023). Regulation of interleukin 2, proto-oncogene c-fos and poly (ADP-ribose) glycohydrolase genomic expression by rosmarinic acid in attenuation of diabetes and associated complications. Reviews, **37**:3321-3331.

Ekbbal, R.; Iqubal, A.; Ansari, M.; Ahmad, S. and Haque, S. (2022). Evaluation of cardioprotective potential of isolated swerchirin against the isoproterenol-induced cardiotoxicity in wistar albino rats. Pharmacogn. Mag., **18**(77):10-21.

El-Gied, A.A.A.; Abdelkareem, A.M. and Hamedelniel, E.I. (2015). Investigation of cream and ointment on antimicrobial activity of *Mangifera indica* extract. Journal of Advanced Pharmaceutical Technology and Research, **6**(2):53.

Gaurav; Khan, M.U.; Basist, P.; Zahiruddin, S.; Ibrahim, M.; Parveen, R.; Krishnan, A. and Ahmad, S. (2022). Nephroprotective potential of *Boerhaavia diffusa* and *Tinospora cordifolia* herbal combination against diclofenac induced nephrotoxicity. South African J. Bot. **151**:238-247.

Gaurav; Sharma, I.; Khan, M.U.; Zahiruddin, S.; Basist, P. and Ahmad, S. (2023). Multi-mechanistic and therapeutic exploration of nephroprotective effect of traditional ayurvedic polyherbal formulation using *in silico*, *in vitro* and *in vivo* approaches. Biomedicines, **11**(1):168.

Gautam, G. (2022). Network pharmacology-based validation of traditional therapeutic claim of momordica charantiain alleviating diabetic nephropathy. J. CAM Res. Prog., **1**:102.

Khan, M.U.; Basist, P.; Gaurav; Zahiruddin, S.; Penumallu, N.R. and Ahmad, S. (2024). Ameliorative effect of traditional polyherbal formulation on TNF-α, IL-1β and caspase-3 expression in kidneys of wistar rats against sodium fluoride induced oxidative stress. J. Ethnopharmacol., **318**:116900

Khan, M.U.; Gaurav; Zahiruddin, S.; Basist, P.; Krishnan, A.; Parveen, R. and Ahmad, S. (2022). Nephroprotective potential of Sharbat-e-Bazoori Motadil (sugar-free) in HEK-293 cells and Wistar rats against cisplatin induced nephrotoxicity. J. King Saud Univ. - Sci., **34**(3):101839.

Kiran, K.; Kaur, A. and Azmi, W. (2021). Multidrug-resistant pathogens and their plasmid curing by lactic acid bacteria. Ann. Phytomed., **10**(2): 101-112.

Kumar, R.S.; Kishan, R.K.; Venkateshwar, R.K.N.; Duganath, N. and Kumanam, R. (2010). Simultaneous spectrophotometric estimation of curcuminoids and gallic acid in bulk drug and ayurvedic polyherbal tablet dosage form. Int. J. Pharm. Qual. Assur., **2**:56-59.

Kumar, V.; Ain, S.; Kumar, B.; Ain, Q. and Gaurav. (2020). Optimization and evaluation of topical gel containing solid lipid nanoparticles loaded

with luliconazole and its anti-fungal activity. Int. J. Pharm. Res., 12(2):2901-2910.

Mehrotra, N. (2020). Medicinal plants, aromatic herbs and spices as potent immunity defenders: Antiviral (COVID-19) perspectives. Ann. Phytomed., 9(2):30-49.

Noor, S.; Mohammad, T.; Rub, M.A.; Raza, A.; Azum, N.; Yadav, D.K.; Hassan, M.I. and Asiri, A.M. (2022). Biomedical features and therapeutic potential of rosmarinic acid. Arch. Pharm., Res., 45:205-228.

Tao, Y.I.; Li, W.; Liang, W. and Van Breemen, R.B. (2009). Identification and quantification of gingerols and related compounds in ginger dietary supplements using high-performance liquid chromatography-tandem mass spectrometry. J. Agric. Food Chem., 57(21):10014-10021.

Venkatachalam, T.; Chitra, M.; Kalaiselvi, P.; Chitra, A.; Sumathi, K.; Babu, C.M.S.; Senthilkumar, N. and Sattanathan, K. (2021). Phytochemical screening and antidiabetic potentiality of *Pavetta indica* L. (Angiosperms: Rubiaceae) methanol extract on streptozotocin induced diabetic mice. Ann. Phytomed., 10(2):292-297.

Citation **Nikhil Kumar, Brijesh Kumar, Vivek Kumar Sisodia, Arvind Rathour, Ram Kumar Roy and Vijay Kumar (2023). Determination of antibacterial and anti-inflammatory effect of developed ointment formulation containing rosmarinic acid. Ann. Phytomed., 12(2):784-794. http://dx.doi.org/10.54085/ap.2023.12.2.92.**