Research Article

Polyherbal formulation as an alternative therapy for inflammatory conditions: *In vitro* insights

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Abstract

Background: Inflammation is a key factor in many acute and chronic conditions, necessitating safe and effective long-term treatments. Polyherbal formulations, combining multiple herbal extracts, offer potential synergistic benefits with reduced side effects. This study evaluates the antioxidant and anti- inflammatory properties of a polyherbal extract containing *Zingiber officinale, Boswellia serrata, Ocimum sanctum,* and *Cinnamomum camphora* through in-vitro assays. **Objective:** To assess the anti-inflammatory and antioxidant properties of a polyherbal formulation by evaluating oxidative stress markers, inflammatory markers, and cell responses. **Materials and methods**: transdermal patch was formulated using solvent casting with herbal extracts, PVP, and HPMC. Antioxidant activity was tested using the DPPH assay and Anti-inflammatory effects were evaluated using MTT cytotoxicity, ELISA for TNF-α inhibition, COX-II inhibition, and protein denaturation assays. **Results:** The formulation exhibited strong antioxidant and anti-inflammatory activity. *Boswellia serrata* demonstrated the 55.56% free radical scavenging among the extracts, outperforming standard ascorbic acid in its effectiveness. The MTT assay showed an IC50 of 44.39±0.21 µl/ml. ELISA demonstrated a reduction in TNF-α upto 42.41 pg/ml. The COX-II inhibition assay yielded an IC50 of 0.2493 ± 0.11 µl/ml, outperforming celecoxib. Protein denaturation inhibition was comparable to diclofenac. The patch had good mechanical strength, with thickness (0.46–0.62 mm) and folding endurance (>100 folds). **Conclusion:** The polyherbal transdermal patch effectively reduces inflammation and oxidative stress, inhibiting TNF-α and COX-II activity and suppressing protein denaturation. This study suggests it could be a viable natural alternative for inflammation management and study paves the way for future clinical investigations to validate these findings and optimize formulation design and dosage for enhanced clinical efficacy.

***Keywords*:** Herbal synergism, Antioxidant activity, Inflammation management, Cytokine suppression

Introduction

Transdermal medication Discrete, self-contained dosage forms that enable drugs to reach the bloodstream at a controlled rate when applied to intact skin are referred to as deliveries. Transdermal patches—also referred to as skin patches—are medicated adhesive patches applied to the skin that allow a prescribed dosage of medication to pass through the bloodstream through the skin. For the medication released by TDDS to have a therapeutic impact, it needs to navigate through the stratum corneum, epidermis, and dermis before entering the bloodstream and reaching the intended target area (Ace Jr, 2001). It can be applied at high doses and stays on the skin for a long period, which gives it an edge over other dermal drug delivery techniques. The transdermal patch is made up of four main types and three components: the adhesive, backing layer, and patch matrix:

1) Drug in a matrix type: Adhesive and a backing layer are applied to the matrix, which contains the active pharmaceutical ingredients directly incorporated into the film patch material.
2) Adhesive-type drug: The medication is embedded in a self-sticking polymer and encased in a backing layer.
3) Drug in reservoir type: This type incorporates a porous polymeric membrane to control the release rate while

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dispersing the drug in a matrix.

4) Multilamellar type: A drug-loaded matrix with multiple layers and a membrane between the layers is present (Suwandecha and Changklang, 2023).

The steps listed below are involved in medication transdermal permeation:

I. Absorption through the corneum stratum.

II. Drug penetration via the dynamic epidermis.

III. The drug's absorption into the dermal papillary layer through the capillary network.

The medication needs specific physicochemical properties in order to systematically penetrate the stratum corneum and reach the target area (Ace Jr, 2001).

The objective of this study was to develop and evaluate a transdermal drug delivery system using polyherbals such as *Zingiber officinale*, *Boswellia serrata*, *Ocimum sanctum*, and *Cinnamomum camphora*. The use of different polymers, such as PVP and HPMC, in the solvent casting process to increase the absorption of drugs and decrease adverse effects. One of the most often utilized components in traditional medicine and culinary is the rhizome of the *Zingiber officinale*, or ginger. The primary bioactive elements of *Zingiber officinale*, such as flavonoids, phenolic compounds (gingerols, gingerdiol, shogaol, and paradol), and terpene compounds (zingiberene, β-bisabolene, α-farnesene, and α-curcumene), have been linked to the plant's biological and therapeutic qualities. Numerous pharmacological properties have been associated with ginger and its compounds, including anti- inflammatory, antioxidant, anticancer, anti-lipidemic, anti-diabetic, antipyretic, and analgesic effects. Furthermore, studies have demonstrated that ginger extract can improve the quality of life for arthritis sufferers by reducing pain, stiffness, and physical function. This may be achieved by blocking the expression of many inflammatory cytokines and reducing the production of prostaglandins (Maleki et al., 2023). Boswellia serrata contains Acetyl-11-keto-β-boswellic acid and boswellic acid are main phytoconstituents which exerts anti-inflammatory activity. The *Boswellia serrata* extract's ability to prevent arthritis was assessed using an anti-arthritic animal model (Majeed et al., 2021; Banji et al., 2022). The *Ocimum sanctum* plant, is also referred to as the basil or tulsi plant. The leaves and the stem of the *Ocimum sanctum* plant may contain different types of phytochemicals which includes flavonoids, terpenoids, saponins, and tannins. The ability of the phenolic-rich fraction extracted from the basil leaves that inhibit the activity of two inflammatory enzymes (Lipoxygenase (LOX) and COX)(Satyendra et al., 2021). There are various chemical components of camphor (*Cinnamomum camphora*), and each has a unique composition of essential oil. Five chemical constituents can be distinguished in *Cinnamomum camphora* based on the primary constituents of its leaf oil: Camphor, 1,8-cineole, linalool, iso-borneol, and borneol forms. It inhibits the synthesis of IL- 6, TNF- α, and interleukin (IL)-1β. It Inhibits heat-induced erythrocyte hemolysis and hypotonic solution-induced erythrocyte hemolysis, and treats allergic dermatitis, such as atopic dermatitis (Lee et al., 2022).

Materials and methods

Materials

All the herbal extracts [*Boswellia serrata*, *Zingiber officinale*, *Ocimum sanctum*] and an essential oil [*Cinnamomum camphora*] procured from amines biotech Pvt. Ltd and Avi naturals Pvt. Ltd, Vadodara respectively. Excipients such as PVP, HPMC, Propylene glycol and Polyethylene glycol procured from sulab Pvt. Ltd, Vadodara. These botanicals were investigated as part of pre-formulation research, which included standardizing the oils and evaluating their morphology in compliance with WHO recommendations.

Formulation of Transdermal Patch

The polyherbal patch was developed using the solvent casting procedure, which ensured even dispersion of the

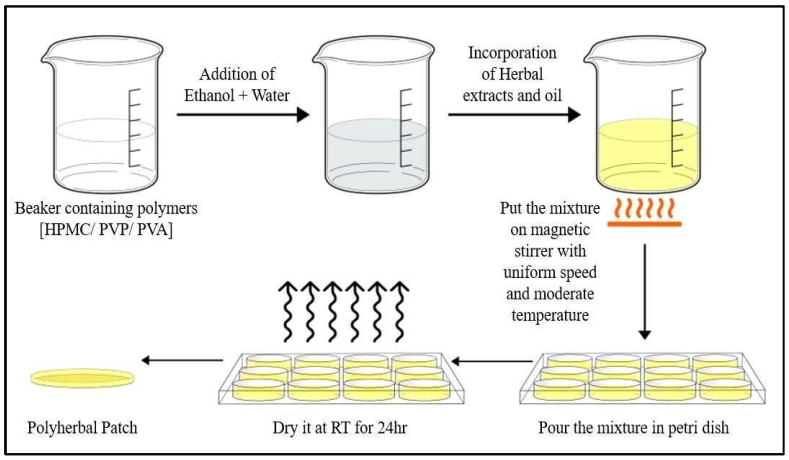


Figure 1: Preparation of Polyherbal patch by solvent casting method (Saleem and Idris, 2016).

active herbal ingredients. This method works well for transdermal drug delivery because it provides better adhesion qualities and controlled drug release. Extracts of *Zingiber officinale, Boswellia serrata, Ocimum sanctum*, and *Cinnamomum camphora* were carefully combined with excipients such ethanol, PVP, HPMC, and polyethylene glycol as part of the formulation. The resulting mixture was cast into moulds and dried to form a flexible and stable transdermal patch (Figure 1).

Evaluation of Patch:

1. Organoleptic Characteristics: Physical examinations were conducted to assess the created patch's smoothness, colour, clarity, flexibility, and appearance.

2. Thickness: Vernier calliper was used to measure the patch's thickness. At multiple sites, the thickness uniformity was measured, and an average was determined.

3. Folding Endurance: The patch was taken and folded in the same direction several times until it broke. It was noted how often the patch could be folded without breaking.

4. Moisture Content: After being weighed, the prepared patch was placed in the desiccator with fused calcium chloride and kept for a day. Following that, it was removed and weighed again. The following formula was used to determine the moisture content percentage:

% of moisture content = [Initial weight − Final weight]/ Final weight × 100

5. Moisture uptake: the weighed films that were maintained at 84% RH absorption for 24 hours at room temperature in a desiccator with a saturated potassium chloride solution. The films were reweighed 24 hours later, and the percentage moisture uptake was calculated using the following formula:

% of moisture uptake = [Final weight – initial weight]/ initial weight × 100

6. Tensile strength: A textile analyst assessed it. It is made up of two grips for load cells. While the upper one could be moved, the lower one was fixed. Between these cell grips, a 2 by 2 cm film strip was fastened, and force was exerted until the film broke (Hajera et al., 2014).

Patch Composition

The composition of several formulations (F1–F4) is shown in the table 1, which also shows a steady rise in the concentration of active herbal extracts while maintaining the excipients and polymer matrix constant. The modifications help to ensure that the patch formulation is optimized for optimal efficacy.

Determination of DPPH free radical scavenging assay:

Using the DPPH assay method, the extracts' capacity to scavenge radicals was assessed. To prepare a 0.3 mM DPPH solution, 4.3 mg of DPPH (2, 2-Diphenyl-1-picrylhydrazyl) was dissolved in 6.6 ml of methanol. The test tubes were covered with aluminum foil to protect solution from light. After adding 150µl of DPPH to 3 ml of methanol, the absorbance at 516 nm was observed right away for the control measurement. Test samples in the following concentrations: 25 µl, 50 µl, 100 µl, 150 µl, 200 µl, and 250 µl. Each sample was diluted with methanol up to 3 ml, and then 150 µl of DPPH was added. After 15 minutes of darkness, the samples were examined for optical density at 516 nm with methanol serving as a blank (Morgan, 1998).

In vitro Study

In vitro study was performed by Akaar biotechnologies Pvt. Ltd.

1. MTT Assay

The MTT Assay was used to assess the sample's cytotoxicity on the RAW 264.7 cell line. The cells (10000 cells/well) were cultivated in a 96-well plate for 24 hours in DMEM media (Dulbecco's Modified Eagle media-AT149-1L) supplemented with 1% antibiotic solution and 10% FBS (Fetal Bovine Serum, HIMEDIA-RM 10432) at 37°C

Table 1: Composition of Formulation

Sr. No.	Ingredients	F1 [%]	F2 [%]	F3 [%]	F4 [%]
1	*Zingiber officinale*	1.2	2.5	5.0	10
2	*Boswellia serrata*	5.0	10	15	20
3	*Ocimum sanctum*	2.5	5.0	10	20
4	*Cinnamomum camphora*	0.1	0.5	1	1.2
5	HPMC	50			
6	PVP	20			
7	Polyethylene glycol	3			
8	Propylene glycol	0.5			
9	Ethanol	4			
10	Water	16			

with 5% CO_2. Cells were treated with varying doses the following day (concentration as per mentioned in excel sheet). The cells without treatment were considered as Control. The cell culture was cultured for 24 hours before being supplemented with MTT Solution (concentration as specified in the excel sheet) and incubated for an additional 2 hours. After removing the culture supernatant at the end of the experiment, the cell layer matrix was dissolved in 100 µl of Dimethyl Sulfoxide (DMSO–SRL-Cat no. 067685) and measured at 540 and 660 nm using an Elisa plate reader (iMark, Biorad, USA). The Graph Pad Prism -6 software was used to determine the IC50. Utilizing an AmScope digital camera (10 MP Aptima CMOS), images were taken under an inverted microscope (Olympus ek2).

2. Protein Expression Analysis with ELISA-TNF-*α:*

Reagents: Wash buffer (20X), Assay Diluent (1X), Standard (TNF – α), Biotin Conjugated Detection Antibody, Concentrated Streptavidin: HRP Conjugate.

Methodology: The GENLISATM Human TNF α ELISA kit instructions (Cat No.: KB1145) were followed to conduct the experiment. After adding 100 µl of sample for the cell culture treated at 22.2 µl/ml (IC50 dose/2) and cells untreated (control), the plate was sealed and allowed to sit at room temperature for two hours. Control cells were those that received no treatment. After four rounds of washing with a wash buffer (1X), the plate was firmly tapped upside down on absorbent paper to blot the buffer. After adding 100µl of diluted detection antibody (Biotin Conjugated Detection Antibody) solution to each well, the plate was sealed and allowed to sit at room temperature for an hour. Once again, the plate was cleaned using wash buffer (1X), and each well received 100µl of diluted Streptavidin-HRP solution. The plate was then sealed and allowed to incubate for an hour. 100µl of TMB substrate (3,3',5,5'-Tetramethylbenzidine) solution was added to the plate after it had been cleaned with wash buffer (1X), and it was then left in the dark for half an hour. Each well received 100 µl of stop solution to halt the reaction, and the absorbance at 450 nm was measured 30 minutes later (Kang et al., 2007).

3. Enzyme Inhibition Assay - Cox-II:

Assay for Enzyme Inhibition Sample dilutions were made in buffer (Tris Cl buffer, 100mM, pH 8.0) as specified in the excel sheet. In a designated well of a 96-well plate, reaction buffer (Enzyme in Tris/heme/phenol; 100mM/1µM/1µM buffer-Bovine Hemin Chloride – SRL-78372, Phenol – Fischer Scientific-35953) was added. Five microliters of substrate (arachidonic acid, 10 mM-SRL-20975) and five microliters of TMPD solution (17 mM-HiMedia GRM445-5G) were added to start the reaction. The plate was then allowed to sit at room temperature for ten minutes, and the absorbance was measured at 595 nm using a microplate reader (iMark, BioRad). As a positive control, the inhibitor Colexcib (final concentration of 25 µM; TCI-C2816) was employed (Telagari and Hullatti, 2015).

4. Protein Denaturation Inhibition Assay

45µl of egg albumin (5% in PBS) was combined with 5µl of the sample at several concentrations, and the mixture was incubated for 30 minutes at 37°C. Following incubation, each tube received 150µl of sodium phosphate buffer (pH 6.3), and samples were incubated for an additional 30 minutes at 70°C. Using spectrophotometry, turbidity was measured at 660 nm. 5 µl of PBS was used in place of extracts for the control test, and egg albumin was absent from the product control test (Bácskay et al., 2023).

Enzyme calculations:

The following formula was used to determine the percentage inhibition of protein denaturation:

$$\%\ Inhibition = (A\ Test - A\ Control / A\ Control) \times 100$$

Results

DPPH Antioxidant Assay

The findings show that the polyherbal extracts had a strong ability to scavenge free radicals, with *Boswellia serrata* showing the strongest effectiveness of all the extracts examined. The formulation appears to have considerable antioxidant capability, as evidenced by the activity being comparable to that of standard ascorbic acid (Figure 2).

Evaluation parameters of prepared patch:

1. Organoleptic evaluation of patch.

The prepared patch as shown in Fig.3 exhibited a jellified consistency with a pale color and slightly opaque clarity. The patch was flexible and smooth upon handling and did not cause any skin irritation, indicating its potential suitability for transdermal application.

2. Thickness of patch:

The thickness of the formulated transdermal patches varied between 0.46 mm and 0.62 mm across different batches. Specifically, Formulation F1 measured 0.54 mm, F2 measured 0.62 mm, F3 recorded the lowest thickness at 0.46 mm, and F4 also measured 0.54 mm. Among these, Formulation F2 exhibited the highest thickness, while F3 showed the lowest. These differences may be attributed to variations in polymer concentration, and the overall uniformity observed suggests consistency in the casting process, which is essential for reliable drug delivery.

3. Folding endurance:

In terms of mechanical strength, the folding endurance of the transdermal patches varied among the formulations.

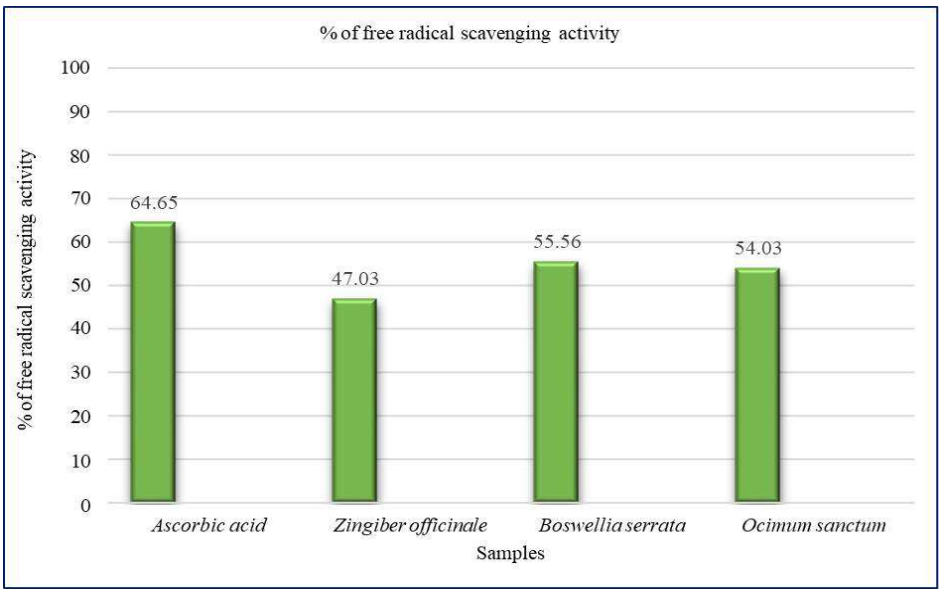


Figure 2: Comparative antioxidant activity of extracts with standard Ascorbic acid.

Formulations F1, F3, and F4 withstood more than 100 consecutive folds without breaking, indicating excellent flexibility and mechanical integrity. Formulation F2 exhibited a slightly lower folding endurance of 88 folds; however, this value is considered satisfactory for transdermal applications, indicating that all formulations exhibit sufficient mechanical resilience for effective application and prolonged wear.

4. Moisture content:

The moisture content of the transdermal patches ranged from 4.02% to 5.25%, with Formulation F3 exhibiting the lowest value at 4.02% and Formulation F4 the highest at 5.25%. Formulations F1 and F2 recorded moisture contents of 4.23% and 4.51%, respectively. These values indicate that all formulations retained minimal moisture, supporting their physical stability and enhancing their potential shelf-life.

5. Moisture uptake:

Moisture uptake values among the formulated patches ranged from 2.33% to 4.17%, reflecting their ability to withstand humid conditions without compromising physical integrity. The lowest uptake was observed in Formulation F3 (2.33%), whereas Formulation F4 recorded the highest (4.17%). Formulations F1 and F2 showed intermediate values of 3.16% and 3.32%, respectively. These results indicate that the patches possess adequate moisture resistance, contributing to their overall durability and suitability for transdermal application.

Tensile strength:

Tensile strength evaluation revealed that the patches maintained sufficient elasticity and mechanical strength. The results suggest that the polymer concentration plays a crucial

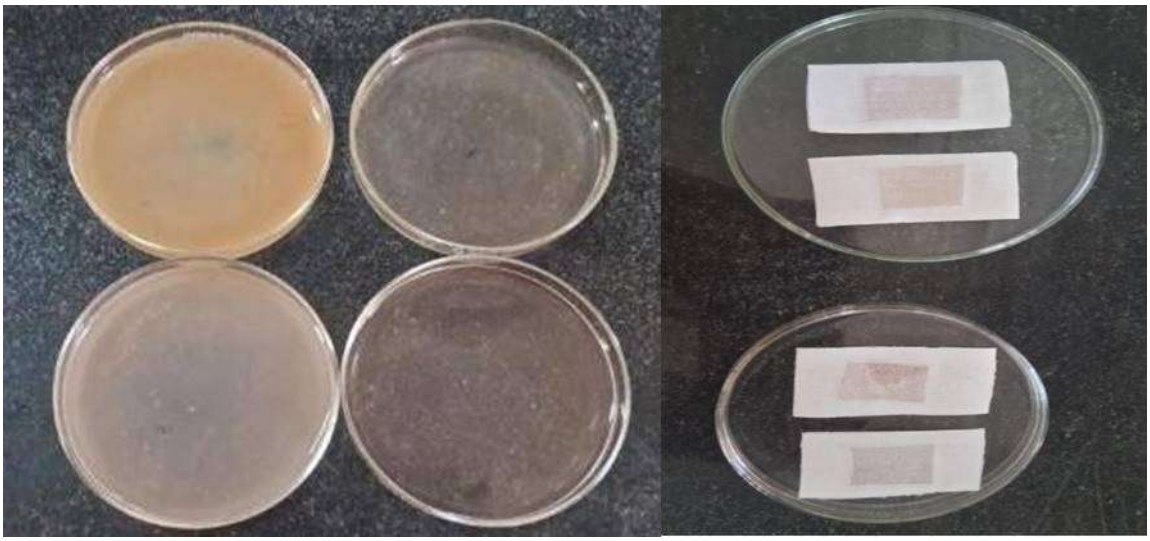


Figure 3: polyherbal patch

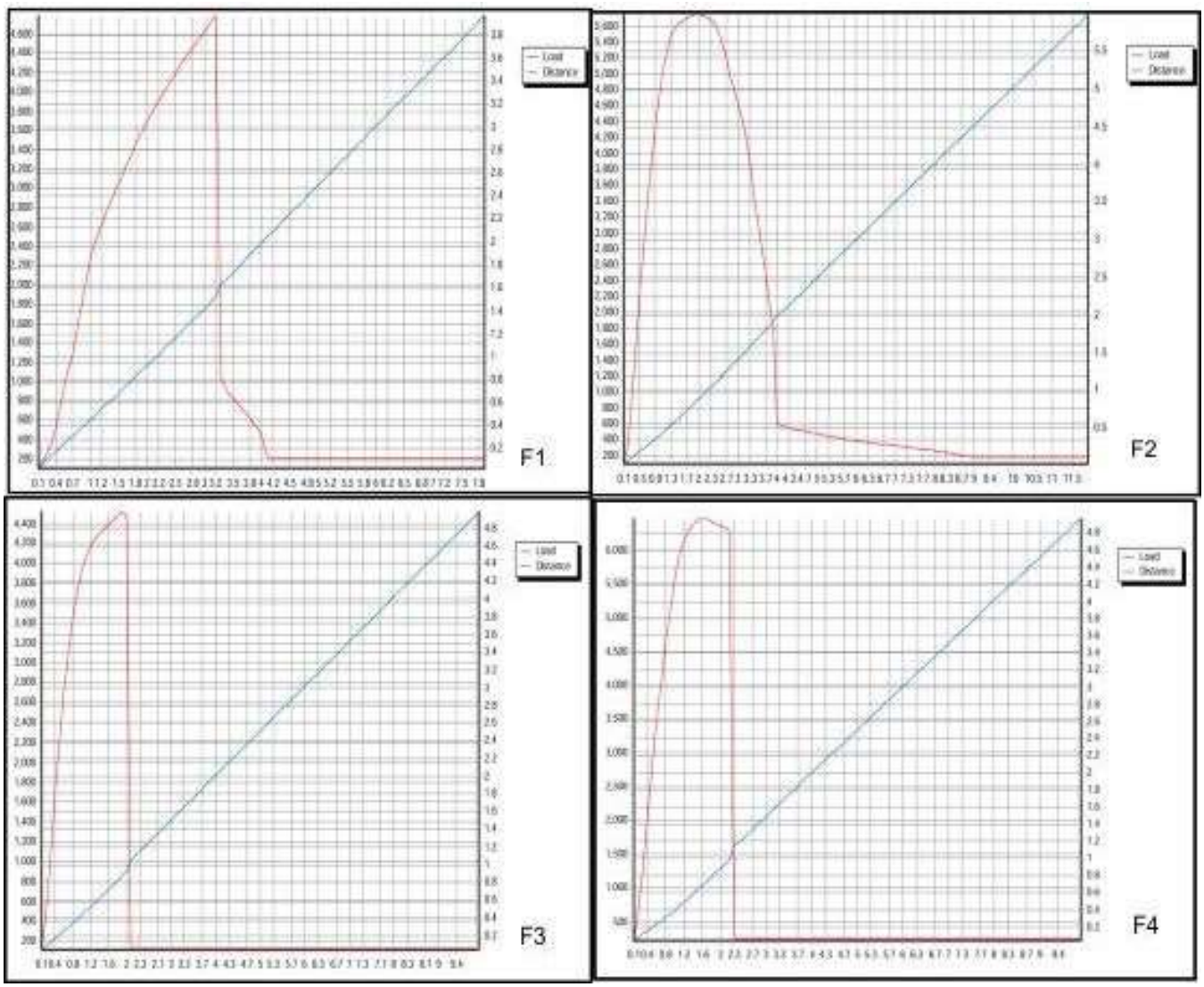


Figure 4: Tensile strength of (a) F1 (b) F2 (c) F3 (d) F4

role in determining patch durability, with F2 and F4 exhibiting higher tensile strength (Figure 4).

In vitro study

MTT Assay

Cell viability percentage (MTT Assay - RAW 264.7 cells)

The MTT assay results indicate that the IC50 value of formulation F3 was 44.39 ± 0.21 µl/ml. This suggests that the polyherbal extract exhibits a dose-dependent cytotoxic effect on RAW 264.7 cells, supporting its potential anti-inflammatory properties (Figure 5).

Protein Expression Analysis with ELISA-TNF-*α*

The ELISA results show a significant reduction in TNF-α levels in treated samples compared to the control. The mean TNF-α concentration decreased from 60.93 pg/ml in the control to 42.41 pg/ml in the treated sample, indicating effective cytokine suppression by the polyherbal patch (Figure 6).

Enzyme Inhibition Assay

The COX-II inhibition results indicate that formulation F3 exhibited an IC50 value of 0.2493 ± 0.11 µl/ml, significantly lower than Celecoxib (6.587 ± 0.025 µg/ml), demonstrating potent inhibitory effects on the enzyme responsible for inflammation (Figure 7).

Protein Denaturation Inhibition Assay

The results indicate that formulation F3 exhibited an IC50 value of 4.786 ± 0.01 µl/ml, showing comparable inhibition of protein denaturation when compared to standard Diclofenac (IC50: 8.241 ± 0.013 µg/ml) (Figure 8). This suggests that the polyherbal patch effectively inhibits protein denaturation and performs competitively against the standard, highlighting its potential in managing inflammation.

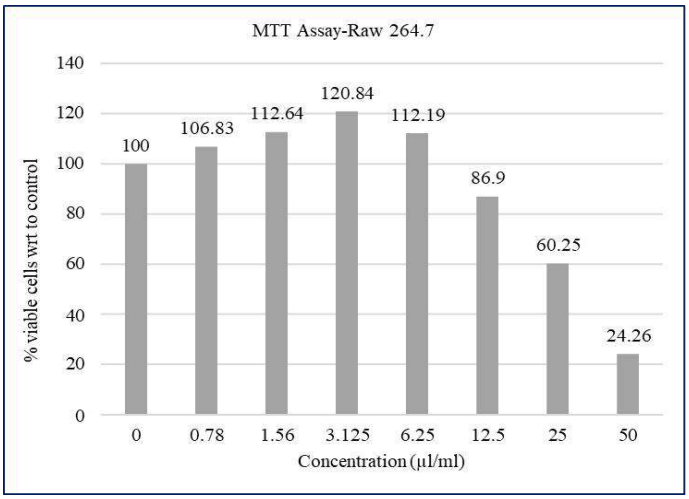


Figure 5. Cell viability percentage (MTT Assay - RAW 264.7 cells)

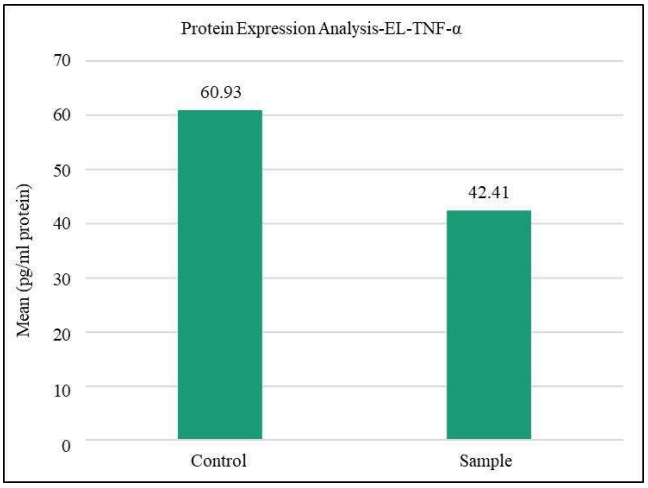
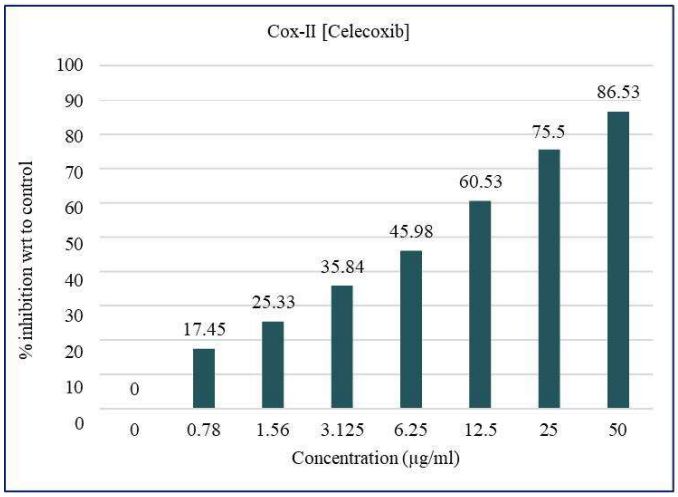


Figure 6. Protein Expression Analysis with ELISA-TNF-*α*



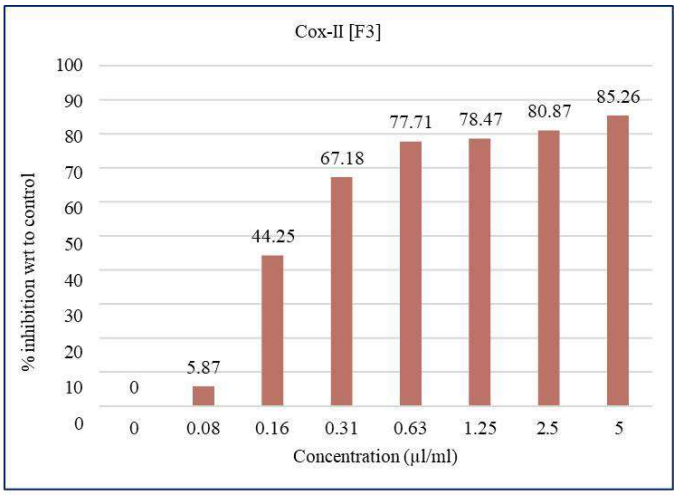
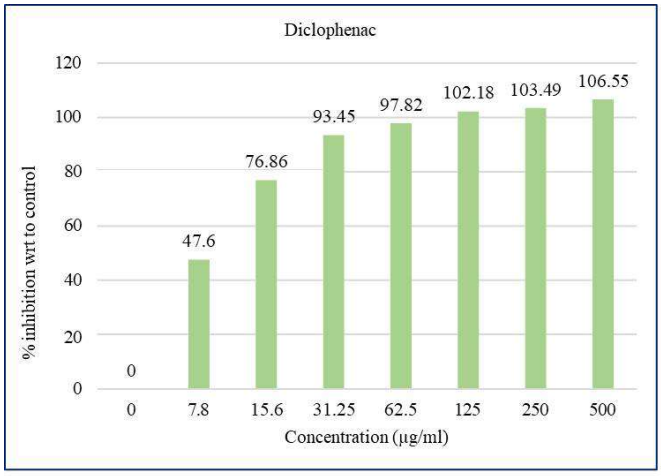


Figure 7. Enzyme Inhibition Assay of Celecoxib and F3



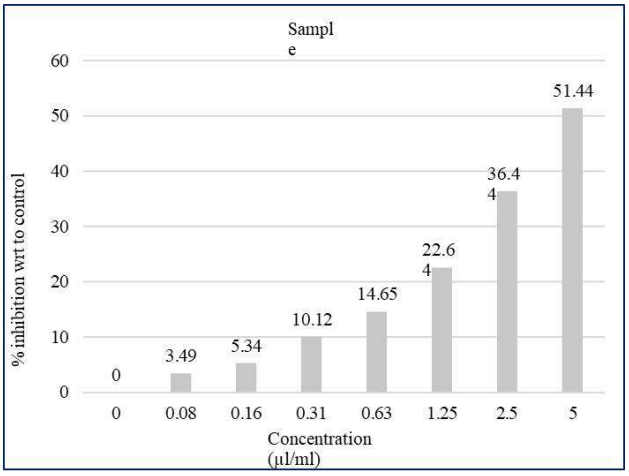


Figure 8. Protein Denaturation Inhibition Assay of Diclophenac and sample

Conclusion

This study demonstrated the successful development and evaluation of a polyherbal transdermal patch composed of *Zingiber officinale*, *Boswellia serrata*, *Ocimum sanctum*, and *Cinnamomum camphora*. The formulation showed promising antioxidant and anti-inflammatory activity, with *Boswellia serrata* exhibiting the highest free radical scavenging potential at 55.56%, in comparison to the standard ascorbic acid. In vitro studies confirmed the patch's biological effectiveness, as shown by an IC50 value of 44.39 ± 0.21

µl/ml in the MTT assay, significant TNF-α inhibition (42.41 pg/ml), and potent COX-II inhibition (IC50: 0.2493 ± 0.11 µl/ml), outperforming celecoxib. Additionally, protein denaturation inhibition was comparable to that of diclofenac, further validating its anti-inflammatory capacity.

Physicochemical evaluations, including optimal thickness (0.46–0.62 mm), high folding endurance (>100 folds), and controlled moisture parameters, confirmed the patch's stability and suitability for transdermal application. The synergistic effect of the polyherbal formulation, driven by the presence of flavonoids, phenols, and essential oils, reinforces its therapeutic potential. Overall, the study supports the use of this polyherbal transdermal system as a promising, plant-based alternative for managing inflammation and oxidative stress. This study suggests it could be a viable natural alternative for inflammation management and study paves the way for future clinical investigations to validate these findings and optimize formulation design and dosage for enhanced clinical efficacy.

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