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EXPLORING THE ANTI-INFLAMMATORY ACTIVITY OF FORMULATED *AZADIRACHTA INDICA* (NEEM) LOADED MICROSPONGES GEL

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Keywords:

Azadirachta indica, Microsponges, Anti-Inflammatory, Drug Delivery, *In-vitro* Release, Therapeutic Formulation

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ABSTRACT: This research investigates the anti-inflammatory potential of *Azadirachta indica*-loaded microsponges gel, presenting a novel therapeutic approach. The microsponges gel, formulated with *Azadirachta indica*, aim to enhance drug delivery efficiency for improved anti-inflammatory effects. The microsponges gel is formulated using Carbopol 934P (1% w/v) to optimized encapsulated microsponge. Prepared formulation was evaluated for pH, spreadability, viscosity, and *ex-vivo* drug deposition study. Characterization studies, *in-vitro* release assessments, and anti-inflammatory assays contribute to a comprehensive evaluation of the formulated microsponge gel. Preliminary results demonstrate successful formulation of *Azadirachta indica*-loaded microsponge gel. Characterization studies confirm the structure and composition. *In-vitro* release studies reveal sustained drug release, and initial anti-inflammatory assays indicate promising activity. This study underscores the potential of *Azadirachta indica*-loaded microsponge gel as a promising anti-inflammatory therapeutic strategy. The formulation's sustained release profile and demonstrated efficacy in anti-inflammatory assays pave the way for further investigation and potential clinical applications.

INTRODUCTION: Topical drug delivery systems are gaining popularity, with successful administration of various drugs for both local and systemic effects. Gels, as compared to ointments, offer superior potential as a vehicle for topical drug delivery due to their non-sticky nature and low-energy formulation requirements [1]. Skin serves as an accessible and non-invasive route for drug delivery, boasting a large surface area with exposure to circulatory and lymphatic networks.

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Gels, composed of natural or synthetic polymers forming a three-dimensional matrix in a dispersion medium or hydrophilic liquid, are particularly promising. Upon application, the liquid evaporates, leaving the drug entrapped in a thin film of the gel-forming matrix that adheres to the skin [2].

Inflammation, or phlogosis, represents a physiological response of living tissues to injuries, resulting in the local accumulation of plasmatic fluid and blood cells. While it serves as a defense mechanism, the intricate events and mediators involved in the inflammatory reaction can contribute to, perpetuate, or worsen various diseases. Therefore, the use of anti-inflammatory agents becomes crucial in the therapeutic management of such pathologies. Medicinal plants have been extensively employed in the folk

medicine of many countries to address diverse inflammatory conditions, particularly skin inflammations. However, the actual efficacy and relevant active principles of many of these plants remain unknown. Consequently, there is a need for experimental studies aimed at demonstrating the pharmacological properties of these plants and identifying their pertinent active principles [3].

Current anti-inflammatory drugs, both steroidal and non-steroidal, often present a range of side effects. Consequently, research is focusing on identifying anti-inflammatory agents from natural sources. Neem (*Azadirachta indica*) is a plant with seeds and leaves traditionally used in medicine. Neem acts as a potent antioxidant, primarily attributed to the presence of flavonoids and polyphenols [4, 5]. Its anti-inflammatory activity is linked to flavonoids, which serve as antioxidants and potential inhibitors of cyclooxygenase, lipoxygenase, and nitric oxide synthase [6-8].

Despite Neem's historical use for treating inflammation, there is no existing report on the development of gel formulations using Neem extract. Therefore, this study aims to formulate and investigate an effective anti-inflammatory gel derived from the ethanolic extract of Neem [9, 10].

MATERIALS AND METHODS:

Materials: *Azadirachta indica* extract was obtained as a gift sample from Amsar Pvt. Ltd., Indore, Madhya Pradesh, India. The reagents including propylene glycol were obtained from High Purity Laboratory Chemicals, Mumbai. Carbopol 934P, triethanolamine, and n-methyl-2-pyrrolidone were procured from SD Fine-Chem. Limited, Mumbai.

Methods:

Preparation of Gel Containing AZI Microsponges: Carbopol 934P (1% w/v) was first soaked in water for two hours before being homogenously mixed with a magnetic stirrer at a speed of 600 rpm. Then, carbopol gel was evenly infused with AZI microsponges. To balance the pH, triethanolamine (2% v/v) was added. Propylene glycol and N-methyl-2-pyrrolidone were added as permeation promoters to this aqueous dispersion.

Evaluation of Gel Containing AZI Microsponges: The gel was further assessed for the following factors after being visually examined for consistency, colour, and homogeneity.

pH Determination: After placing the electrode tip into the prepared gel for two minutes, the pH of the gel was determined using a pH metre (standardized using buffer, pH 7 before to use). The formulation's pH was measured three times, and the mean value was computed.

Spreadability: A weighted sample was placed between two glass slides to test the gel's spreadability. A weight of 500 g was then placed on the slides for roughly 5 minutes, after which no further spreading was anticipated. The initial and ultimate spread circle diameters were measured in cm and used as benchmarks for spreadability.

Viscosity: In rheology, viscosity in centipoise, which represents a fluid's resistance to flow, is measured. By employing spindle No. 7 at various rpms at room temperature, the Brookfield viscometer was used to test the viscosity of the gel.

***Ex-vivo* Drug Deposition Studies:** Using a Franz diffusion cell, a drug deposition investigation was conducted on the excised rat abdomen skin. The skin's dermal side was maintained facing the receptor solution, while the epidermal side was left exposed to the environment. A 30 mL phosphate buffer compartment in a receptor compartment that was heated to 37±0.5 °C and agitated at 600 rpm was used. Before applying the sample, the skin was soaked with the diffusion medium for 1 hour. On the donor compartment, a 100 mg gel sample (corresponding to 30 mg curcumin) was administered. At various times, the samples were taken out. The diffusion cell was taken apart at the conclusion of the run (24 hours) in order to determine how much medication had been deposited in the skin. Skin was gently peeled, and medication was found on the skin surface was cleaned with distilled water and analyzed for drug content.

Quantification of AZI from the Skin Samples: A modified method was used to remove the drug from the skin. Shortly after being divided into minute pieces, the skin was homogenised in a tissue homogenizer with 10 mL of phosphate buffer pH 7.4. To fully extract the medication, the homogenised sample was treated to ultrasonication

for 10 min. Centrifuging this solution extract for 10 minutes at 5000 rpm. By measuring absorbance at a wavelength of 428 nm, the supernatant was collected and subjected to UV spectroscopic analysis.

Data and Statistical Analysis: The slope of the linear plot of the total quantity penetrated per unit area (µg/cm2) as a function of time (h) was used to compute the steady-state flux (J, µg/cm2/h). The slope's steady state x-intercept was used to calculate the lag time (tL, h). The flow and donor drug concentration were used to compute the permeability coefficient (KP, cm2/s).

Determination of *In-vitro* Anti-inflammatory Activity of AZI Microsponges Gel:

Preparation of the Extracts: One gram of freeze-dried sample was mixed with 20 mL of methanol (70% *v*/*v*) and vortexed at high speed for about five minutes, and then centrifuged (Hettich, EBA 20) for 10 min at 4500 rpm, and the supernatants were collected. Then, the extracts were filtered through a filter paper (WhatmanNo.42), and then the residue that remained was re-extracted with 70% methanol with the same procedure, and the supernatants obtained were combined with those from the first extraction. The solvent in the combined mixture was evaporated in a rotary evaporator (HAHNVAPOR, Model HS-2005 V, HAHNSHIN Scientific, Seoul, South Korea) at 40 °C. The prepared AZI microsponges gel dried at 40 °C for 12 h in an oven, and then dried extracts were stored at −18 °C in air-tight screw-capped glass vials, until used for the anti-inflammatory bioassays, within one week. The extracts collected were dissolved in methanol to obtain a concentration of 3 mg/mL for each assay.

Membrane Lysis Assay:

Preparation of Erythrocyte Suspension: Whole human blood was collected from a healthy human subject. The blood was centrifuged at 3000 rpm for 5 min in heparinized centrifuge tubes, and washed three times with equal volume of normal saline (0.9% NaCl). After the centrifugation, the blood volume was measured and reconstituted as a 10% (*v*/*v*) suspension with isotonic buffer solution (10 mM sodium phosphate buffer pH 7.4). Composition of the buffer solution (g/L) used was NaH_2PO_4 (0.2), Na_2HPO_4 (1.15), and NaCl (9.0).

Heat-Induced Hemolysis: Briefly, 0.05 mL of blood cell suspension and 0.05 mL of hydromethanolic extracts of flowers were mixed with 2.95 mL phosphate buffer (pH 7.4). The mixture was incubated at 54 °C for 20 min in a shaking water bath. After the incubation, the mixture was centrifuged (2500 rpm for 3 min), and the absorbance of the supernatant was measured at 540 nm using a UV/VIS spectrometer (Shimadzo 1800, Mumbai, India). Phosphate buffer solution was used as a control for the experiment.

The level of haemolysis was calculated using the following equation:

% Inhibition of Haemolysis = 100 X (1-A2/A1)

Where A1 = absorption of the control, and A2 = absorption of test sample.

Effect of Protein Denaturation: The reaction mixture (5 mL) consisted of 0.2 mL of 1% bovine albumin, 4.78 mL of phosphate buffered saline (PBS, pH 6.4), and 0.02 mL of extract, and the mixture was mixed, and was incubated in a water bath (37 °C) for 15 min, and then the reaction mixture was heated at 70 °C for 5 min. After cooling, the turbidity was measured at 660 nm using a UV/VIS spectrometer (Shimadzo 1800, Mumbai, India). Phosphate buffer solution was used as the control. The percentage inhibition of protein denaturation was calculated by using the following formula:

% Inhibition of Haemolysis = 100 X (1-A2/A1)

Where A1 = absorption of the control, and A2 = absorption of test sample.

Proteinase Inhibitory Activity: Briefly, the reaction solution (2 mL) consisted of 0.06 mg trypsin, 1 mL of 20 mM Tris-HCl buffer (pH 7.4), and 1 mL test sample (0.02 mL extract 0.980 mL methanol). The solution was incubated (37 °C for 5 min), and then 1 mL of 0.8% (*w*/*v*) casein was added, and the mixture was further incubated for an additional 20 min. At the end of incubation, 2 mL of 70% perchloric acid was added to terminate the reaction. The mixture was centrifuged, and the absorbance of the supernatant was measured at 210 nm against buffer as the blank. Phosphate buffer solution was used as the control. The percentage

inhibition of protein denaturation was calculated by using the following formula:

% Inhibition of Haemolysis = 100 X (1-A2/A1)

Where A1 = absorption of the control, and A2 = absorption of test sample.

Lipoxygenase Inhibition Assay: Briefly, a mixture of a solution of sodium borate buffer (1 mL, 0.1 M, pH 8.8) and lipoxygenase (10 µL, final concentration 8000 U/mL) was incubated with 10 mL flower extract in a 1 mL cuvette at room temperature (30 ± 2 °C) for 5 min. The reaction was initiated by the addition of 10 µL linoleic acid substrate (10 mmol). The absorbance of the reaction solution was measured at 234 nm using a UV/VIS spectrometer (Shimadzo 1800, Mumbai, India). Phosphate buffer solution was used as the control, and the percentage inhibition of lipoxygenase was calculated using the following equation [3, 11]:

% Inhibition = 100 X (absorbance of the control- absorbance of the sample) /absorbance of the control

RESULT AND DISCUSSION:

Evaluation of Microsponge Gel:

Visual Examination: The homogeneity of the preparation was demonstrated by the formation of the non-opaque, pale yellow gel with a pH of 6.69± 0.314.

Spreadability and Viscosity: Table 1 displays the spreadability result. The viscosity of the microsponge and carbopol gels were measured using a Brookfield viscometer. There is no discernible difference between the two gels' viscosities, and both gels' viscosities decrease as rpm increases.

TABLE 1: EVALUATION OF SPREADABILITY, HARDNESS AND ADHESIVENESS OF GELS

Formulation	Spreadability (diameter in cm)	Hardness (g)	Adhesiveness (g.s.)
Carbopol gel	2.45	3.87±0.35	17.45
Formulation gel	2.23	3.20±0.24	16.34

***Ex-vivo* Drug Deposition Studies:** The *ex-vivo* drug release profile of AZI is shown in **Fig. 1**. This data reveals that therapeutic drug concentrations were sustained for a long time with 80.23% of the drug released in 24 hours and that microsponge-loaded gel increased the drug residence duration in skin. By homogenising the skin with 10 mL of phosphate buffer pH 7.4, the amount of drug (AZI) that was still present in the skin at the conclusion of the trial was discovered to be 207.61 5.03 g/cm2.

The supernatant from this solution extract was centrifuged, and it was then subjected to UV-Vis spectroscopic analysis at a wavelength of 445 nm. Based on the slope of a linear plot of the cumulative quantity permeated each hour, the permeation flux and permeability coefficient are determined to be 10.876 g cm-2 h-1 and 0.3625X106 cm-2 h-1, respectively unit area (µg/cm2) as a function of time (h). This *ex-vivo* drug release profile data were found to be fitted best into the zero-order model (R2 = 0.974) with anomalous transport mechanism of drug release unit area (µg/cm2) as a function of time (h). This *ex-vivo* drug release profile data were found to be fitted best into the zero-order model (R2 = 0.974) with anomalous transport mechanism of drug release.

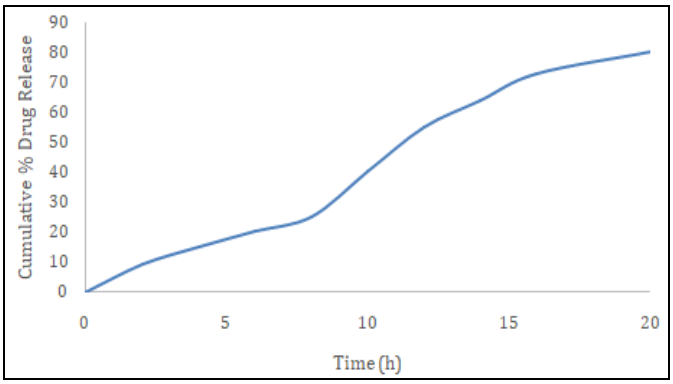

FIG. 1: *EX-VIVO* DRUG RELEASE PROFILE OF AZI

In-vitro Anti-inflammatory Activity:

Heat Induced Haemolysis: The percent inhibition of heat-induced haemolysis of red blood cells at different concentrations of flower extract of AZI microsponges gel (DW), in the range of 25–100 µg/mL, is shown in **Fig. 2.** Methanolic extracts of gel were able to inhibit haemolysis in a concentration-dependent manner. Inhibition % of haemolysis from these leaf extracts were within the range from 9.23% to 24.2%, at the concentrations of 25–100 µg/mL.

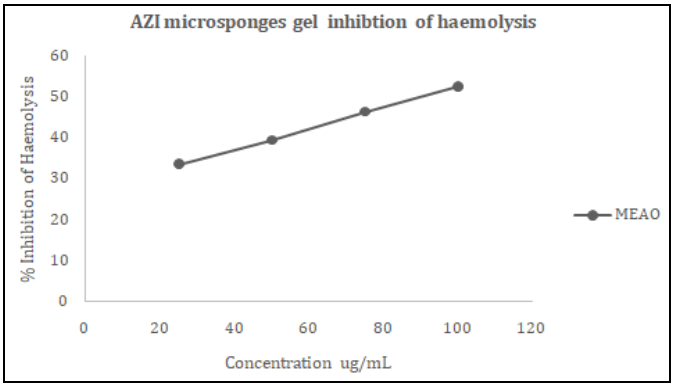

FIG. 2: AZI MICROSPONGES GEL INHIBITION OF HAEMOLYSIS (DATA ARE REPRESENTED AS THE MEANS± STANDARD DEVIATIONS OF THREE REPLICATE DETERMINATION)

Protein Denaturation: Methanolic extracts of gel were able to inhibit protein denaturation in a concentration-dependent manner, and the inhibitory effect of gel at different concentrations (25–100 µg/mL) on protein denaturation is shown in **Fig. 3.**

Inhibition % of protein denaturation of these leafy vegetables was within the range from 43.12% to 72.12% at the concentration range of 25–100 µg/mL.

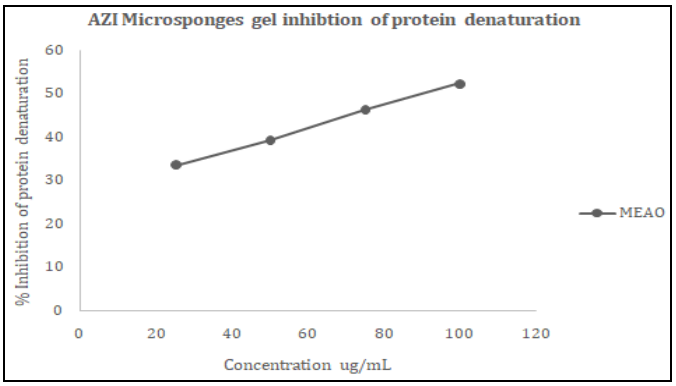

FIG. 3: *AZI* MICROSPONGES GEL INHIBITION OF PROTEIN DENATURATION (DATA ARE REPRESENTED AS THE MEANS± STANDARD DEVIATIONS OF THREE REPLICATE DETERMINATION)

Proteinase Inhibitory Activities: Proteinase inhibitory activity of gel is shown in **Fig. 4**, and the inhibition levels were within the range of 22.23–36.56%.

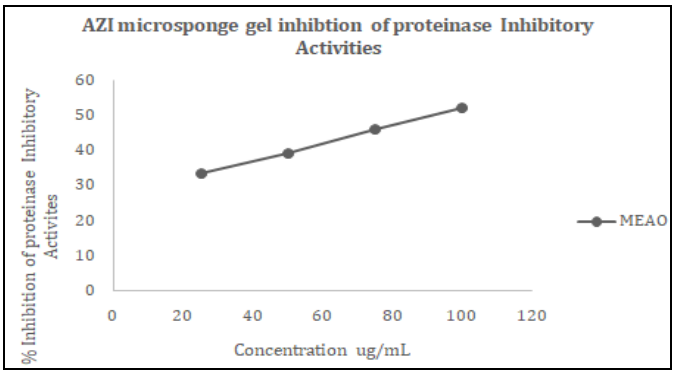

FIG. 4: AZI MICROSPONGES INHIBITION OF PROTEINASE ACTIVITY (DATA ARE REPRESENTED AS THE MEANS± STANDARD DEVIATIONS OF THREE REPLICATE DETERMINATION)

Lipoxygenase Inhibition Activity: Results for lipoxygenase inhibitory activity of different leafy vegetables are shown graphically in **Fig. 5.** Inhibition levels were within the range of 33.45–52.34% within the concentrations of 25–100 µg/mL.

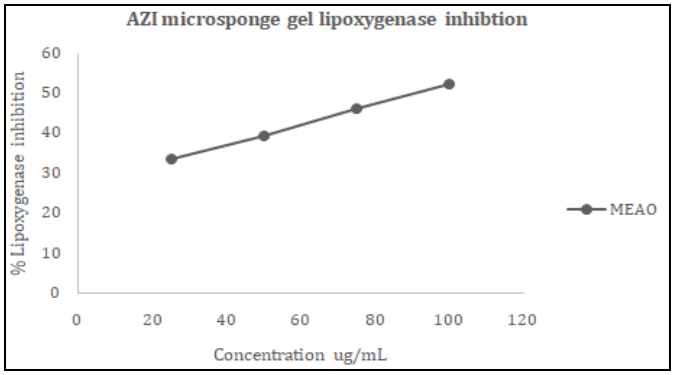

FIG. 5: AZI MICROSPONGES GEL LIPOXYGENASE INHIBITION (DATA ARE REPRESENTED AS THE MEANS± STANDARD DEVIATIONS OF THREE REPLICATE DETERMINATION)

The flowers of AZI microsponges gel showed an improved ability to inhibit lipoxygenase activity (about 50.0%) at 100 µg/mL concentration.

CONCLUSION: The present study presents a cost-effective and efficient method for the production of microsponges loaded with AZI using the quasi-emulsion solvent diffusion technique. Utilizing propylene glycol and N-methyl-2-pyrrolidone, drug-loaded microsponges were successfully synthesized, they were used as permeation promotors to this aqueous dispersion. Accordingly, the gel incorporating 1% Carbopol 934P emerged as the optimized formulation based on superior results compared to other formulations. Consequently, the gel containing AZI-loaded microsponges holds promise as a suitable formulation for treating inflammation. Further, the *ex-vivo* drug release profile analysis employing a Franz diffusion cell revealed that the AZI microsponges loaded in the carbopol gel demonstrated 80.23% of drug release in 24 h. The zero-order model (R2 = 0.974) with an anomalous drug transport mechanism was determined to best suit the drug release profile data. Therefore, it was determined that the AZI microsponge gel made in this study had promise as a novel drug delivery method that offered sustained drug release and, therefore, would be more beneficial than standard formulation treatment in the administration of drugs topically and orally.

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CONFLICTS OF INTEREST: Authors have no conflict of interest.

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