DEVELOPMENT AND EVALUATION OF HERBAL NANOGEL FOR RHEUMATOID ARTHRITIS

Sandhya Bagde[1]*, Mohini Sihare[2]

Abstract:
In order to determine whether Vitex negundo leaf extracts have anti-arthritic efficacy in rats, the study's goal is to create and test a topical herbal gel comprising the extracts. The physical appearance, net content, viscosity, extrudability, pH, spreadability, in vitro diffusion profile, and primary skin irritation tests of six herbal gel formulations made with 1.5% of the gelling agents carbopol 934 (F1-F6) were assessed. According to ICH recommendations, a stability study was completed for the topical herbal gel formulation, and Freund's Complete Adjuvant (FCA) induced arthritis technique was used to assess the anti-arthritic activity. Haematological and biochemical markers were also evaluated, along with body weight and paw volume. The gels that were created adhered to the rules and were homogenous and stable. F4 outperformed the other formulations in terms of release characteristics (84.37%). The skin irritation test revealed no erythema or edoema, indicating the gel is safe and nontoxic. In comparison to rats with the disease, topical administration of the herbal gel F4 containing carbopol 934 demonstrated considerable (p 0.001) anti-arthritic action. The anti-arthritic activity of the gel formulation was substantiated by a decrease in paw volume, no agglutination of C-reactive protein and rheumatic factor, a decrease in TNF level, and a return to normal haematological and biochemical parameters.

Keywords: Vitex negundo, Rheumatoid Arthritis, Herbal, Silver Nitrate Nanoparticles, Nanogel.

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Introduction

The use of nanomedicines to increase the bioavailability of several chemical and herbal bioactive components has shown encouraging results [1]. As a type of nanomedicine, nanogels have outstanding stability, drug loading capacity, biologic consistency, great penetrating ability, and the potential to react to external stimuli. Nanogels have gained popularity in several industries, including gene delivery, delivery of chemotherapy drugs, diagnostics, organ targeting, and herbal remedies [2].

About 0.5–1% of people worldwide suffer with arthritis, an autoimmune disease. Commonly recommended medications for rheumatoid arthritis include steroidal and non-steroidal anti-inflammatory, disease-modifying anti-rheumatic, and immunosuppressive medications. These medications are known to have a variety of side effects, including gastrointestinal problems, immunodeficiency, and humoral changes. More people are becoming aware of the Siddha and Ayurvedic systems of medicine as an alternative method for treating arthritis. Vitex negundo is the herb that is most frequently used to treat arthritis in traditional medicine. In addition to being planted as a hedge plant, Vitex negundo Linn. (Verbenaceae), also known as Nirgundi in Hindi, grows gregariously in wastelands and contains several flavonoids, including casticin, orientin, isoorientin, luteolin, lutecin-7-O-glucoside, corymbosin, glycosidic iridoids, alkaloids, and terpenoids [3]. Strong anti-arthritic, anti-inflammatory, anti-pyretic, anti-convulsant, hepatoprotective, and bronchial relaxant properties of VN have been demonstrated. They can be used as tonics, vermifuges, lactagogues, emmenagogues, antibacterial, antipyretic, and anti-histaminic agents, among other things.

A topical herbal gel was created using VN and assessed for anti-arthritic activity in order to explore the scientific evidence for the use of these plants in the treatment of arthritis since there is no information on preclinical research on the topical gel's anti-arthritic activity. VN was created as a gel because it may be applied directly to the afflicted area, is simple to administer, has a localised action, causes no pain or irritation when applied, has no first-pass impact, and does not degrade in the gastrointestinal tract. Although VN is used medicinally in all its parts, traditional medicine mostly treats arthritis using its leaves [4]; as a result, a topical gel formulation was created employing the leaf extracts of both plants.

Material and Method

Materials

Vitex negundo's ripe, fresh leaves were gathered and verified. Sigma-Aldrich was used to acquire Freund's complete adjuvant (FCA), diclofenac sodium, triethanolamine, propylene glycol, and disodium edetate. We bought carbopol 934 and carbopol 940 from Mumbai's Loba Chemie Pvt. Ltd.

Preparation of extracts

After carefully removing any remaining materials and earthy residue from the Vitex negundo leaves, they were cleaned and allowed to dry in the shade. Using a 7-day cold maceration method, methanol was extracted from coarsely powdered Vitex negundo leaves. The extracts were then kept at 4-8 o C for later use after being filtered and concentrated under decreased pressure in an IKA Rotary evaporator (Model No RN 10 digital V, ILMAC Germany).

Animals

For the anti-arthritic assessment, Wistar strains rats (12-week-old healthy, weighing 150-200 g of either sex in the animal house of Pharmacy college, Nagpur, India) were chosen. Albino rabbits with an average weight of 2.2 kg were utilised for the primary skin irritation test, while albino female mice weighing 20–30 g were employed for the acute toxicity research. They were kept in climate-controlled housing with 10- to 14-hour light and dark cycles, and temperatures of 23.2 oC and 50.5% relative humidity. Individual polypropylene cages with sterile rice husk bedding and unlimited access to food and water were employed to house the animals. The Committee for the Purpose of Control and Supervision on Experiments on Animals (CPSCEA) and Institutional Animal Ethical Committee authorised the experiment designs and methods.

Preliminary phytochemical investigations

According to the conventional approach outlined by Harborne, the principal secondary metabolites including alkaloids, flavonoids, saponins, phenols, terpenoids, protein and amino acids, carbohydrates, and glycosides were evaluated. Standard operating procedures were used.

Phytochemical analysis of plant extracts

In each instance, the appropriate solvent was used to prepare a stock concentration of 1% (W/V). The presence of active phytochemicals was examined in the mixture of all three herbs extracts together with positive and negative controls. The presence

of the active chemical components in all three herbs extracts, such as alkaloids, flavonoids, tannins, carbohydrates, phenolic compounds, terpenoids, glycosides, steroids, fixed oils and fats, was determined using preliminary phytochemical screening. Typically, adding the proper chemical reagent(s) to the herbs extract in test tubes was how phytochemical component presence was determined.

Test of Alkaloids
Mayer's test
Basic nitrogenous chemicals known as alkaloids have distinct physiological and pharmacological action. Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer's reagent, which causes alkaloid solution to create whitish yellowish precipitate when a few drops of are added. The alcoholic herbs extract was dried by evaporation, and the residue was heated with 2% hydrochloric acid in a bath of boiling water. The mixture was filtered and treated with a few drops of Mayer's reagent after cooling. After that, the samples were checked for turbidity or yellow precipitation.

Test of Flavonoids
Lead Acetate Test
Extracts were treated with few drops of 10% lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

Test of Glycosides
Aqueous Sodium hydroxide
Extracts were treated with 1 ml water and 1 ml sodium hydroxide. Formation of yellow colour indicates the presence of glycosides.

Test of Steroids
Salkowski Test
A few drops of strong sulphuric acid and chloroform were added to the extracts for treatment. Steroids are present when chloroform layer green fluorescence acid layer becomes a blue red to cherry colour.

Test of Terpenoids
In order to create a monolayer of reddish-brown pigmentation of the interface, 5 ml of each extract were combined with 2 ml of chloroform and 3 ml of concentrated sulphuric acid. This resulted in a beneficial outcome for the terpenoids.

Test of Tannins
To the extract 0.1% ferric chloride solution was added, formation of a dark blue or greenish black color showed the presence of tannins.

Test of Phenols
Ferric Chloride Solution
Extracts were treated with 3-4 drops of 5 % ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Detection of carbohydrate
Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

Benedict's test
Filtrates were treated with Benedict's reagent and heated gently in water bath 10 minutes. Brick red precipitate indicates the presence of reducing sugars.

Synthesis of herbal extract loaded silver Nanoparticles [5-11]
In the one-step green synthesis, 95 ml of a 1 mM aqueous silver nitrate solution were mixed with 5 ml of leaf extract and left at room temperature in the dark for 24 hours. When pure silver ions are reduced, silver nanoparticles are created, and this process was observed by measuring the reaction medium's absorbance in the wavelength range of 300–700 nm using UV spectrophotometry. Centrifugation was used to clean the produced silver nanoparticles (AgNPs) for 15 minutes at 1000 rpm. The supernatant was transferred to a clean, dry beaker for additional particle settlement, and subsequent centrifugation using a cooling microfuge was carried out to have the AgNPs dried, purified, and described.

Characterization of silver nanoparticles
UV-Vis Spectroscopy Analysis
Using quartz cells in a UV-vis spectrophotometer (Lark, model: LI-UV-7000), UV-visible spectrum studies of green produced Ag nanoparticles were taken between the wavelength range of 200-700 nm. As a blank, double-distilled water was employed. Around 440 nm is predicted to be the location of Ag nanoparticles' distinctive peak.

Fourier transform infrared (FTIR) spectral analysis
By using the KBr pellet technique and registering amplitude waves with a range of 400 to 4000 cm1, the infrared spectra for the plant extract and manufactured AgNPs were obtained for the identification of functional groups in a (Perkin Elmer Spectrum 2, Germany) spectrophotometer IR affinity-1.

Particle Size and Zeta Potential Analysis

The Malvern Zeta sizer Instrument Ltd, based on the DLS approach, was used to measure the zeta potential and particle size distribution. Both the zeta potential measurement and the examination of size distribution were done using a solution of produced nanoparticles that was about 50 l in volume and distributed in 2 mL of distilled water.

Drug entrapment efficiency (DEE)

The supernatant, after centrifugation of silver nanoparticles (15,000 rpm for 40 min), was collected, filtered through 0.22 µm membrane filter and amount of drug present was measured at specific wavelength by UV–Visible spectrophotometer. The amount of drug in supernatant was calculated using the equation y = 0.0164x + 0.0076, (R2 = 0.996) where y represents absorbance and x represents concentration (mcg/ml). Amount of drug present in the supernatant was subtracted from the total amount of drug added and accordingly DEE was calculated.

$$\textbf{Encapsulation efficiency (\%)} = \frac{\textbf{Total drug(mg)} - \textbf{Free drug (mg)} \times 100}{\textbf{Total drug (mg)}}$$

Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was used to examine the size and shape of the silver (Jeol, Japan). A voltage of 80 kV was being used to accelerate the microscope. First, distilled water was used to dilute the silver samples (1:10) before a 20-L aliquot was put on a grid that had been covered in carbon. The excess solution was then blotted using filter paper after being kept on the grid for 1 minute. Prior to imaging, the grids were left in the grid box for two hours to dry.

In-*vitro* drug release study

The *in vitro* dissolution study of silver nanoparticles formulation was performed in dissolution test apparatus, USP standard type II. Study was carried out in pH 7.4 phosphate buffer solution by taking formulation quantity in a muslin cloth and placed in 900 ml dissolution media rotated at 50 rpm and maintained at 37±0.5˚C. Aliquots of 5 ml dissolution medium was removed at 15, 30, 45, 60, 75 and 90 min, respectively. Meanwhile, an equal volume of the same medium was replenished. Dissolution samples were filtered through 0.22 µm filters and analysed spectrophotometrically at specific wavelength.

Preparation of gel base

To prevent agglomeration, carbopol 934 was slowly dissolved while being stirred in 60 mL of demineralized water for 1 hour. Triethanolamine and disodium edetate were then individually diluted in 10 mL of demineralized water and agitated for 10 min. 12 mL of demineralized water were combined with 4.83 mL of propylene glycol while being stirred for ten minutes. The pH of the carbopol solution was raised to 7.4 by agitating the mixture for 10 minutes after the addition of triethanolamine solution and disodium edetate. After swirling for 10 minutes, propylene glycol solution was added to create a clear, uniform gel basis.

Preparation of nanogel formulation

According to the drug formulation manual, 6 topical nanogel formulations were created using VNME (methanol leaf extract of Vitex negundo) silver nanoparticles. Formulations F1 to F6 were created using the gel base of carbopol 934 (1.5%). In Table, formulation component details are listed. Due to its improved quality attributes, the F4 formulation made with carbopol 934 was tested for its ability to treat arthritis.

Table: Nano Gel formulations with carbopol 934

Nanogel code	Vitex negundo extract loaded silver nanoparticles (g)	Carbopol 934 (g)	Triethanolamine (g)	Disodium EDTA (g)	Propylene glycol (g)	D.M. water (100 g)
F1	0.5	1.5	1.5	0.005	5	Q. S
F2	1	1.5	1.5	0.005	5	Q. S
F3	1.5	1.5	1.5	0.005	5	Q. S
F4	2	1.5	1.5	0.005	5	Q. S
F5	2.5	1.5	1.5	0.005	5	Q. S
F6	3	1.5	1.5	0.005	5	Q. S

Quality Control of Topical Herbal Gel Formulation

Estimation of active constituents in gel formulation (net content)

Each formulation (1 g) was placed in a 50 mL volumetric flask, filled to the specified level with methanol, and thoroughly shaken to dissolve the active ingredients. A Whatman filter was used to filter the solution, and 0.1 mL of the filtrate was pipetted out and diluted to a volume of 10 mL with methanol. Utilizing a standard curve constructed at a certain wavelength (max of the active ingredients in the extracts), the number of active constituents was determined spectrophotometrically.

Extrudability

About 20 g of nanogel were placed inside a closed collapsible tube, which was then tightly clamped to prevent any rollback. The nanogel was extruded once the cap was removed. Weighing was done after the extruded nanogel was collected. It was determined what proportion of the nanogel was extruded.

pH measurement

Using a digital pH metre, the pH of the nanogel was measured by fully submerging the glass electrode in the nanogel system to cover the electrode. The measurement was done three times, with the average of the values being recorded.

Appearance and Homogeneity

Physical appearance and homogeneity of the prepared nanogels were evaluated by visual perception.

Viscosity

Viscosity of nanogel was determined using Brookfield viscometer (S-62, model LVDV-E) at 25 oC with a spindle speed of the viscometer rotated at 12 rpm.

Spreadability

Glass slides with uniform dimensions were selected from two sets. Over one of the slides, the herbal nanogel formulation was applied. The nanogel was sandwiched between the two slides in an area that took up 7.5 cm along the slides when the other slide was positioned on top of the nanogel. The upper slides were covered with 100 g of nanogel, which was then evenly pushed between the two slides to form a thin layer. The excess nanogel that was sticking to the slides was scraped off once the weight was removed. The two slides were secured in place such that there was no least movement and that only the upper slides could be released by the weight being linked to it. Carefully connected to the upper slide was a 20 g weight. Under the impact of the weight, it took the upper slide 7.5 cm to move in the amount of time necessary to separate from the lower slide. Three times the experiment was run, and each time the mean time was used to calculate the results. In order to determine spreadability, the following formula was used

$$S = m \times l/t$$

where, S= spreadability, m-weight tied to upper slides (20 g), l- length of the glass slide (7.5 cm), t- time taken in sec.

In-vitro permeation in rat skin

Franz diffusion cells were used to conduct in vitro diffusion tests on all formulations. The diffusion cell equipment was made locally as an open-ended cylindrical tube with a diffusion area of 3.8 cm2 and a 3.7994 cm2 area. As receptor medium, phosphate buffer (pH 7.4) was employed. As a dialysis membrane, rat abdomen skin was employed. The stratum corneum side of the skin was in close contact with the release surface of the formulation in the donor cell due to the skin's attachment to the diffusion cell (donor cell). A donor compartment received 100 mL of isotonic phosphate buffer solution, pH 7.4, before being installed on the diffusion cell. One gramme of nanogel-equivalent formulation, which was weighed, was applied to the rat's skin and slightly submerged in 100 mL of receptor media while being swirled constantly. The system as a whole was kept at 37°C. At predetermined intervals up to 8 hours, a 5 mL aliquot was taken, and its concentration was measured spectrophotometrically at a predetermined wavelength. The diffusion medium was withdrawn in equal amounts and then replaced with brand-new diffusion medium. Each time (in h) interval's cumulative percent release was determined.

Release kinetics

Data were acquired and fitted to various mathematical models to determine the release pattern of the active ingredient from the herbal nanogel. First order kinetics is the dependent kinetics, where drug release may occur after swelling and erosion or only diffusion, while zero order kinetics is a concentration independent kinetics. Higuchi's model was used to validate the data and establish the outcome.

Stability studies of topical herbal nanogel formulation

The major goal of the stability testing is to offer proof of how the drug product's quality changes over time under the impact of temperature and humidity. According to ICH requirements, a stability chamber was used for a 6-month period during the stability investigation for the topical herbal nanogel formulation. In a humidity chamber (Floor standing model, 3 units in one with individual humidity and temperature controller, 300 X 300 X 300 mm, 15- 60°C, Technico, India) at 25°C 2°C/60% RH 5% RH, 32°C 2°C/60% RH 5% RH, and 40°C 2°C/75% RH 5% RH, the chosen topical herbal nanogel formulation including At the first, first, second, third, and sixth months, samples were taken out and tested for sterility and changes in colour, odour, homogeneity, pH, viscosity, net content, and microbial load.

Anti-arthritic activity

FCA-induced arthritis model in rats was used to examine the effectiveness of the topical herbal nanogel formulation. Four groups of six rats each were created from the total population of the rats. Nanogel base was topically administered to Group 1 as a standard control. By injecting a 0.1 mL (0.1% w/v) suspension of deceased Mycobacterium tuberculosis bacteria homogenised in liquid paraffin into the left hind foot in the sub plantar region of rats, arthritis was caused in groups 2 to 4. Group 2 was used as a control for arthritis. For 21 days, arthritis was allowed to develop in Groups 2 to 4 that had received FCA. Rat paw volume and body weight from the control and treatment groups were assessed using a digital Vernier calliper on days 4, 8, 14, and 21 of the experiment. Diclofenac sodium gel (Voveran gel, purchased from a neighbourhood pharmacy) and the herbal nanogel formulation F4 were applied topically for 22 to 42 days to the left knee joint region of Group 3 (used as the reference standard) and Groups 4 respectively after the development of arthritis was confirmed. On the 25th, 29th, 35th, and 42nd days of the treatment period, the rat paw volume of the control and treatment groups as well as the animal body weight were assessed using a digital Vernier calliper. The animals' results of the pain test were recorded at the conclusion of the 42 nd day.

Result and Discussion

Phytochemical analysis of herbal extract

Vitex negundo herbal extract was examined phytochemically using the following solvents: methanol, ethanol, petroleum ether and chloroform. All the components of herb, including carbohydrates, alkaloids, flavonoids, tannin, terpenoids, glycosides, phenolic compounds, and steroids, are found in the petroleum ether extract. The herbal ethanol extract, however, has every component except for alkaloids and carbohydrates. There are steroids, alkaloids, flavonoids, tannin, terpenoids, glycosides, phenolic chemicals, and tannins. Carbohydrates, alkaloids, flavonoids, terpenoids, glycosides, and steroids can all be found in the chloroform extract of herbs. Absent are tannin and phenolic chemicals. Steroids, flavonoids, tannin, and sugars are found in the methanol extract of herbs. Flavonoids, terpenoids, and steroids are the main ingredients found in Vitex negundo herb preparations. The results were displayed in the table below.

Table: 1 Preliminary Phytochemical screening of various extracts of herbs

Constituents	Ethanol	Methanol	Petroleum ether	Chloroform
Alkaloids	-	+	+	+
Flavonoids	+	+	+	+
Tannins	+	+	+	-
Carbohydrate	-	-	+	+
Terpenoids	+	+	+	+
Glycosides	+	-	+	+
Steroids	+	+	+	+
Phenols	+	-	+	-

Where,
+ Present
- Absent

Green Synthesis of AgNPs

AgNPs were synthesised using plant extract in a green manner. When plant extract was added to the silver nitrate solution, the solution's colour changed from pale yellow to dark brown, indicating that silver ions had been reduced and silver nanoparticles had formed.

Characterization of Silver Nanoparticles

UV-Visible Spectral Analysis

Vitex negundo exhibits a distinctive peak in the wavelength region between 400 and 450 nm in its UV absorption spectra. The Mie scattering phenomenon may be responsible for the peak specificity in this area. Figure 1 displays the silver nanoparticle's UV spectrum.

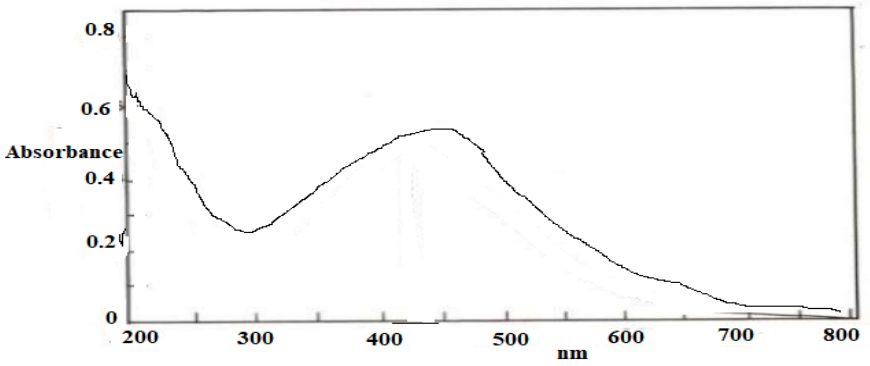


Fig. 1: UV-Visible spectra of Silver Nitrate Nanoparticles

After the reaction was finished, the average particle size in the aqueous reaction mixture was measured using a zeta sizer set to dynamic light scattering mode. The silver ions were converted into nanoparticles, as evidenced by the observation that the average particle size was 85.45 nm. The silver nanoparticle's PDI (polydispersity index), which measures homogeneity and globule distribution, was discovered to be 0.267. Figure 2 displays the particle size and polydispersity index.

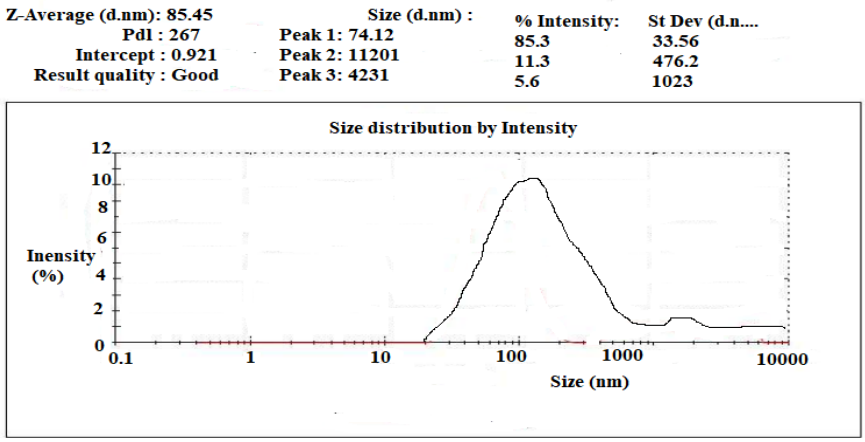
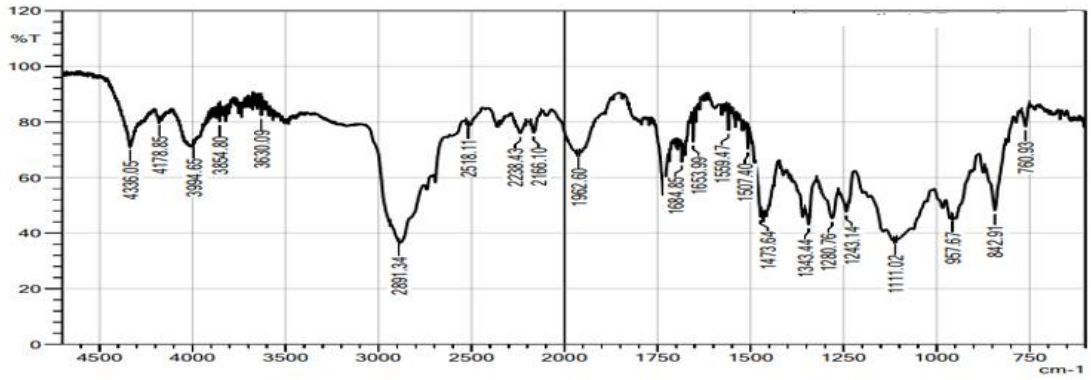


Fig. 2: Particle size and poly dispersity index of silver nitrate nanoparticles

FT-IR Spectroscopy Characterization

Ag nanoparticles' FTIR spectra (Fig. 3b) indicated the O-H stretching vibrations of phenols and alcohols in the band at 3483.50 cm^{-1}. C-H stretch alkanes linked to a prominent band. The C-H stretch alkanes, carboxylated group, asymmetric stretching vibrations of methylene groups, and alkenes group, respectively, were represented by the peaks at 2962.71 cm^{-1}, 1599.02 cm^{-1}, 1314.51 cm^{-1}, and 657.74 cm^{-1}. This said that the Vitex negundo leaf extract's phytochemicals were present around the produced Ag nanoparticles. The peak at 1314.51 cm^{-1} in the FTIR spectrum of Vitex negundo leaf extract (Fig. 3a) indicated C-O stretch of alcohols. While peaks at 1559.47 cm^{-1} and 1343.44 cm^{-1} suggested the N-H stretching of an aromatic secondary amine and the C-N stretching of aromatic amine groups, respectively, the band at 1684.85 cm^{-1} indicated a C=O stretch of acyl chlorides. The flavonoids, phenols, alkaloids, saponins, and carbohydrates found in the aqueous extract of Vitex negundo leaf correspond to these functional groups. Their presence had previously been discovered in the leaf extract's phytochemical tests.

a)



b)

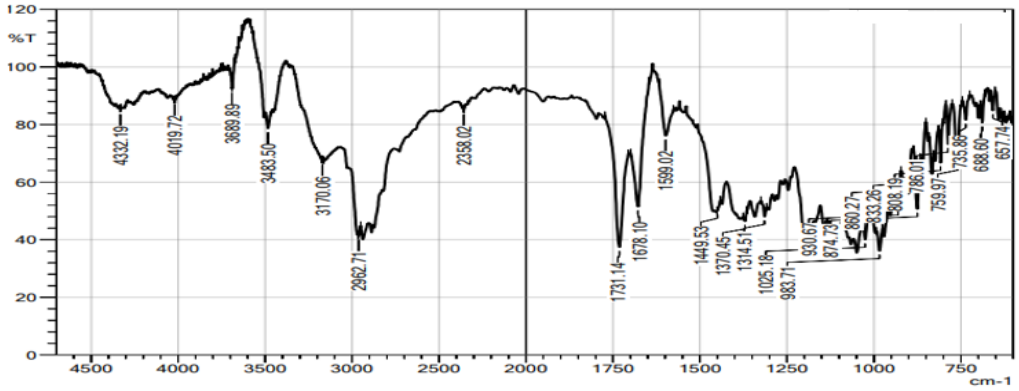


Fig. 3: a) FTIR spectrum of Vitex negundo leaf extract. **b** FTIR spectrum of Ag nanoparticles.

Entrapment efficiency (%)

The effectiveness of silver nanoparticle entrapment. After that, the effectiveness of entrapment did not much rise. Entrapment effectiveness was discovered to be 85.32%. The use of polymers determined the efficiency percentage that was best.

Transmission electron microscopy

Figure 4 depicts the S1's dimensions and shape. Utilizing transmission electron microscopy, they were investigated (TEM). Different fields were used to measure the particle sizes and shape. The electro-silver micrograph's particles were spherical and had well defined particle sizes. According to figure 4, the size has been reported as the mean diameter. It is obvious that the silver nanoparticles are spherical in shape and have tightly regulated particle sizes. Additionally, and as would be predicted, preparation circumstances have a significant impact on particle size. The results from the PCS data are consistent with the observed particles' average particle size, which ranged from 5 to 50 nm.

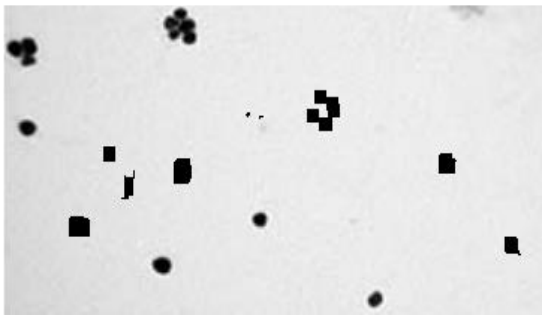


Figure 4. TEM images of spherical Silver, the scale bars were 50 nm and magnifications 50 kx.

In-vitro dissolution study

Silver nanoparticle in-vitro release experiments revealed that for the first 90 minutes, there was absolutely no drug release in simulated stomach juice (pH 1.2 acidic). Both the colonic medium and the simulated intestinal fluid (pH 7.4

phosphate buffer) contained drug release (pH 6.8 phosphate buffer). The regulated efficacy of in-vitro release profiles in intestinal or colonic medium was shown to be quite high. During the dissolving investigation, it was discovered that the drug release is influenced by the pH of the media as well as the kind of polymer matrix. Increased polymer concentration resulted in considerably greater drug release rates across all formulations. For a 90-minute timeframe, the silver nanoparticles demonstrated a greater drug release. At the conclusion of 90 minutes, it was discovered that the percentage cumulative drug release of silver nanoparticles in pH 7.4 phosphate buffer was 84.37 %. Silver nanoparticles have demonstrated favourable medication release rates based on in-vitro drug release study results.

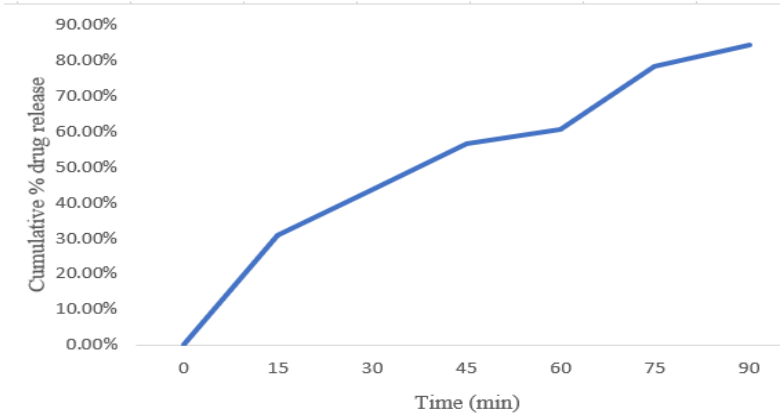


Fig.5: In- vitro dissolution profiles of silver nanoparticles at Phosphate Buffer Solution pH 7.4

Quality control test for formulated silver nanoparticle encapsulated herbal nanogel

The physical appearance, pH, viscosity, spreadability, net content, extrudability, and in vitro diffusion profile of 12 carbopol nanogel formulations F1 to F6 were assessed. The specifics of the study's results, which fell within acceptable ICH criteria, are listed in Table 2.

Table 2: Evaluation parameters for topical herbal nanogel formulation made with 1.5% Carbopol 934

Code	Conc. (%)	pH	Viscosity (poise)	Spreadability (g cm/sec)	Net content (% w/w)	Extrudability	Physical appearance
F1	0.5	7.64	0.3865	31.34	98.56	Good	Greenish, smooth and translucent
F2	1.0	7.64	0.3845	44.09	102	Excellent	Dark green, smooth, homogenous, translucent
F3	1.5	7.87	0.3934	55.23	105	Good	Dark green, smooth, homogenous, translucent
F4	**2.0**	**7.65**	**0.3965**	**64.09**	**99.56**	**Excellent**	**Dark green, smooth, homogenous, translucent**
F5	2.5	7.91	0.3978	72.25	104	Excellent	Dark green, smooth, homogenous, translucent
F6	3.0	7.48	0.3993	75.56	105	Excellent	Dark green, smooth, homogenous, translucent

It was discovered that the prepared nanogels were uniform, attractive, and consistent. According to a study on skin irritation, all the formulations' pH values were within the narrow range of neutral pH (7.48–7.91). As a result, there was no skin irritation. In order to reach and maintain the medication concentration within the therapeutically appropriate range, polymers were added to the developed topical formulations to give a rapid release of the drug. No fluctuation in viscosity was noticed because the polymer concentration was set at 1.5% in all of the nanogel formulations. Furthermore, a topical nanogel formulation made with carbopol polymers was said to have an optimal viscosity value between 0.38 and 0.39 poise. Spreadability values showed that the nanogel formulations are simple to disseminate. Except for F1 and F3, which had 80% of the contents that could be extruded, all of the nanogel formulations from F1 to F6 had outstanding extrudability scores (>90% extrudability, >80% extrudability, >70% extrudability, fair).

***In vitro* diffusion profile and release kinetics**

Figure 6 shows the F1 to F6 formulations' in vitro diffusion profile. For the in vitro release experiments of the nanogel formulations, phosphate buffer saline pH 7.4 was employed because the pH of the membrane in use ranged from 5 to 7.8. All six formulations containing carbopol 934 elicited nearly 100% release from the formulation within 5 hours according to their in vitro release profiles. The generated topical herbal silver nanoparticles loaded nanogel formulations' in vitro release characteristics were extremely encouraging and consistent with commercial diclofenac gel. When compared to the other formulations, F4 had better release characteristics (97.5%) than F1, F2, F3, F5, and F6 (Figure 7 to 9) Our kinetic release investigation led us to the conclusion that the F4 formulation adhered to zero order kinetics. For in vivo experiments, a nanogel formulation containing 2% VNME silver nanoparticles was chosen since zero order kinetics is desired for prolonged release. Commercial diclofenac sodium gel formulation released roughly 90% of its substance in 3 hours, whereas F4 formulation, which contains 2% of each VNME, extended the release of active components up to 5 hours (almost 100%), making it ideal for continuous release and improving patient compliance. As a result, the release data obtained using various mathematical models revealed zero order release kinetics for the nanogel formulation containing 2% VNME (Table 3). The nanogel formulation F4, which contains 2% of VNME, was chosen for in vivo tests because zero order kinetics comes after controlled release.

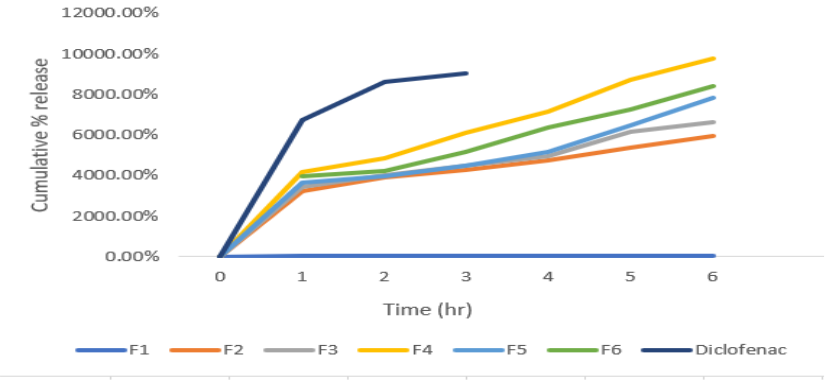


Fig. 6- *In vitro* diffusion profile of topical herbal nanogels (F1-F6) and diclofenac sodium gel.

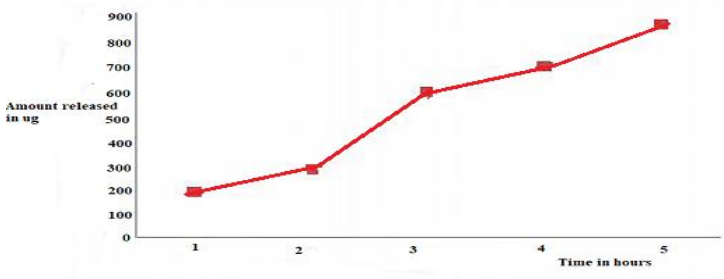


Fig. 7: Zero order plot for F4 topical herbal nanogel formulation.

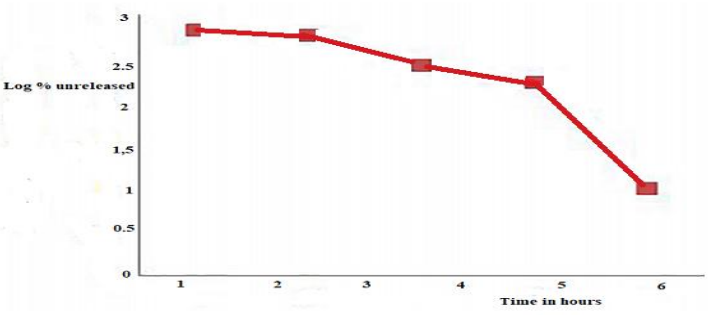


Fig. 8: First order plot for F4 topical herbal nanogel formulation.

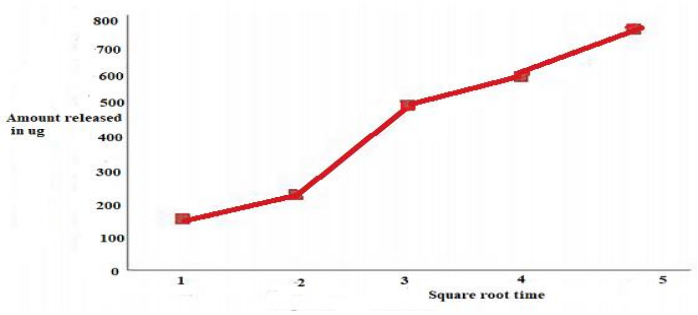


Fig. 9: Higuchi diffusion plot for F4 topical herbal nanogel formulation.

Table 3 - *In vitro* release kinetic study of topical herbal nanogel formulated with Carbopol 934

Formulation code	Zero order R^2	First order R^2	Higuchi diffusion model R^2	Best fitted model
F1	0.972	0.921	>1	Zero order
F2	0.959	0.943	0.938	Zero order
F3	0.921	0.935	>1	First order
F4	0.992	0.915	>1	Zero order
F5	0.989	0.909	0.911	Higuchi
F6	0.910	0.894	0.919	Higuchi

Skin irritation test

No erythema or edoema was seen for any of the formulations, even after 10 days of investigation, indicating that the generated herbal nanogel formulation was found to be safe when the skin irritating effect of the herbal nanogel was tested (Table 4).

Table 4: Primary skin irritation test for herbal nanogel formulation 940 and 934

	Rabbit Numbers					Combined index
	Rabbit Number			Rabbit		
	1	2	3	Control	Average	
1 h						
Erythema score	0	0	0	0	0.00	0.00
Edoema score	0	0	0	0	0.00	
24 h						
Erythema score	0	0	0	0	0.00	0.00
Edoema score	0	0	0	0	0.00	
48 h						
Erythema score	0	0	0	0	0.00	0.00
Edoema score	0	0	0	0	0.00	
72 h						
Erythema score	0	0	0	0	0.00	0.00
Edoema score	0	0	0	0	0.00	
7 days						
Erythema score	0	0	0	0	0.00	0.00
Edoema score	0	0	0	0	0	0.00
10 days						
Erythema score	0	0	0	0	0.00	0.00
Edoema score	0	0	0	0.00		

Stability testing

Stability studies were carried out in accordance with ICH criteria for F4 formulation (made using carbopol 934) since it displayed higher quality features in order to ensure the quality, safety, and efficacy throughout the shelf life. After stability testing for 0,1,2,3 and 6 months, no changes in the topical herbal gel formulation's colour, odour, homogeneity, pH, viscosity, or net content were found. The study's findings made it abundantly evident that the topical nanogel F4 formulation is stable (Table 5).

Table 5: Stability studies of topical herbal nanogel formulation

Sr.no.	Parameters	Topical herbal nanogel formulation (F4) containing 2% w/v of VNME														
		Storage condition														
		25 °C ± 2 °C/60% RH ± 5% RH					32 °C ± 2 °C/60% RH ± 5% RH					40 °C ± 2 °C/75% RH ± 5% RH				
		Months					Months					Months				
		0	1	2	3	6	0	1	2	3	6	0	1	2	3	6
1	Colour	No change in Colour					No change in Colour					No change in Colour				
2	Odour	No change in odour					No change in odour					No change in odour				
3	Homogeneity	Smooth					Smooth					Smooth				
4	pH	6.33	6.32	6.29	6.27	6.24	6.33	6.30	6.31	6.28	6.26	6.32	6.30	6.27	6.25	6.24
5	Viscosity (poise)	0.375	0.375	0.370	0.365	0.360	0.375	0.373	0.370	0.366	0.362	0.375	0.370	0.368	0.360	0.354
6	Net content (%)	98	98	97	96	96	98	97	97	96	97	98	96	95	94	94
7	Microbial load (Bacteria and fungi)	No microbial growth was observed at 24,48 and 72 h					No microbial growth was observed at 24,48 and 72 h					No microbial growth was observed at 24,48 and 72 h				
8	Sterility test	No microbial growth was observed at 24,48 and 72 h					No microbial growth was observed at 24,48 and 72 h					No microbial growth was observed at 24,48 and 72 h				

Body weight

Following the rat arthritis induction, the average change in body weight was seen across all groups (Table 6). When compared to the normal group of rats, the diclofenac sodium gel and topical herbal nanogel formulation F4 treated groups showed gains in body weight while the arthritic control group showed weight loss.

Table 6: Effect of diclofenac sodium, F4 herbal nanogel formulation on body weight changes in FCA Induced arthritic rats

Groups	Initial body weight (g)	Body weight after 21 days of FCA induction	Body wt after treatment 25 th day	Body wt after treatment 29 th day	Body wt after treatment 35 th day	Body wt after treatment 42 th day	Weight gain (g)
Normal control	147.4 ± 0.85	173.3 ± 1.71	174.3 ± 1.90	178.51 ± 1.55	185.52 ± 1.13	193.72 ± 1.26	21.55 ± 1.90
Arthritic control	146.4 ± 0.85	143.81 ± 0.80	142.21 ± 0.95	139.40 ± 1.06	137.00 ± 1.14	133.70 ± 1.27	-12.00 ± 1.35
Diclofenac sodium topical gel (1% w/w)	146.00 ± 1.16	143.21 ± 1.26	144.00 ± 1.42	145.21 ± 1.36	147.51 ± 1.84	150.80 ± 1.67	7.68 ± 0.68
Topical herbal nanogel formulation (2% w/w)	146.6 ±1.06	142.71 ± 1.39	143.21 ±1.50	144.00 ±1.35	145.80 ± 1.57	148.52 ± 1.72	6.81 ± 0.48

Acute oral toxicity study

A detailed study on acute and sub-chronic toxicity of these plants was already reported by us revealed that the VN extracts were nontoxic up to the dose of 2000 mg/kg.

Paw volume

On days 25, 29, 35, and 42 following topical administration of diclofenac sodium gel and the herbal nanogel formulation (F4) for 22 to 42 days, changes in rat paw volume were noted (Table 7 and Figure 10). The increase in paw volume in the arthritis control groups indicated the onset of arthritis. On the 21st day following FCA induction, a significant (p0.01) reduction in rat paw volume was seen in groups treated with topical herbal nanogel formulation F4 and diclofenac sodium gel. Visual arthritic grading methods were used to determine the severity of arthritis. The results of the arthritic test, which were summarised in Table 8, showed that the groups treated with diclofenac sodium gel and topical herbal nanogel formulation F4 experienced much less discomfort from FCA-induced arthritis. When compared to the arthritic control group of rats, significant changes were seen in the flexion pain test score, mobility score, and stance score for all the treated group of rats. This change in arthritic test results confirms the topical herbal nanogel formulation F4's anti-arthritic properties. The formulation F4 was chosen for the anti-arthritic study out of formulations F1 to F6 because its quality control assessment results were positive and its in vitro release characteristics were favourable and consistent with those of commercially available diclofenac sodium gel. The most popular model with clinical and pathological alterations comparable to those found

in human rheumatoid arthritis is FCA-induced arthritis. The development of polyarthritis in the rat following FCA treatment is unusual and is associated with an immune-mediated inflammatory response. The anti-arthritic action was substantiated by the selective reduction of arthritic score (Table 8) and the considerable weight reduction of the thymus and spleen in all treated groups when compared to arthritic rats.

Table 7: Evaluation of anti-arthritic activity of herbal nanogel formulation F4 in FCA induced arthritic rats

Groups	Rat paw volume (mm)					
	Before treatment		After treatment			
	Initial	After 21 days	25th day	29th day	35th day	42nd day
Normal control	4.95 ± 0.15	5.18 ± 0.25	5.10 ± 0.21	5.40±0.12	5.65 ±0.14	5.78±0.80
Arthritic control	4.91 ± 0.13	11.63 ± 0.16	10.68 ± 0.28	10.71 ±0.18	10.78±0.25	10.80±0.12
Diclofenac sodium topical gel (1 % w/w)	5.12 ± 0.15	10.42±0.17	10.49±0.25	9.84± 0.19	8.95±0.25	8.21± 0.07
Topical herbal nanogel formulation F4 (2% w/w)	4.98 ± 0.14	10.41 ±0.16	10.35 ± 0.16	9.78 ± 0.15	9.17 ±0.18	8.75 ± 0.20

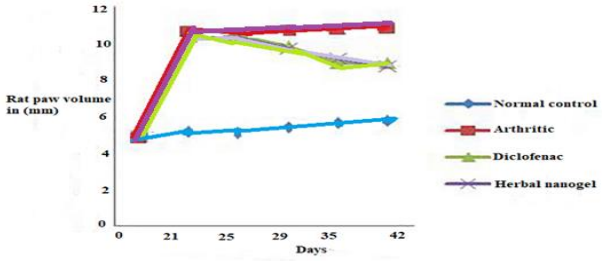


Fig. 10: Herbal gel formulation F4 in FCA induced arthritic rats.

Table 8: Alterations in various pain test scores in FCA Induced arthritis in rats

Groups	Pain test		Mobility score	Stance score
	Extension	Flexion		
Arthritic control	9.8 ± 0.35	8.35 ± 0.35	1.35 ± 0.23	1.53 ± 0.24
Diclofenac sodium topical gel (1 % w/w)	5.18 ± 0.19	4.68 ± 0.23	2.70 ± 0.24	2.35 ± 0.22
Topical herbal nanogel formulation (2 % w/w)	4.68 ± 0.24	3.69 ± 0.35	3.18 ±0.24	2.85 ± 0.18

Conclusion

The proposed topical herbal gel formulation's anti-arthritic properties could be attributed to the luteolin and apigenin found in Vitex negundo leaf extracts extracted in petroleum ether. A promising topical herbal gel for the treatment of arthritis was discovered to be the produced formulation F4, which contained 2% of each VNPEE and 1.5% of carbopol 934. The utility of this formulation for patients with joint inflammatory diseases may be strengthened by additional clinical investigations.

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