RESEARCH PAPER

FORMULATION AND EVALUATION OF CURCUMA LONGA AND PIPER NIGRUM LOADED HERBAL NANOHYDROGEL FOR IN-VITRO ANALYSIS OF ARTHRITIS

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Abstract
The present study was undertaken to formulate and evaluate a Curcuma longa–Piper nigrum loaded herbal nanohydrogel for potential topical management of arthritis-associated inflammation. Ethanolic extracts of Curcuma longa rhizomes and Piper nigrum fruits were prepared by Soxhlet extraction and subjected to pharmacognostic, phytochemical, FTIR and antioxidant evaluation. The extracts demonstrated appreciable phytoconstituent content, including phenolics, flavonoids, alkaloids and saponins, with Curcuma longa showing 45.78 mg/100 mg flavonoids and 43.21 mg/100 mg phenolics. DPPH and superoxide radical-scavenging activities increased with extract concentration. Nine nanohydrogel formulations (F1–F9) were developed using chitosan–TPP ionic gelation followed by incorporation into Carbopol 934 hydrogel. The formulations were evaluated for pH, spreadability, viscosity, extrudability, drug/extract content, particle size, PDI, zeta potential, entrapment efficiency, drug loading, percentage yield and in-vitro release. F9 showed a particle size of 271.6 ± 5.48 nm, PDI of 0.214 ± 0.010, zeta potential of +36.8 ± 0.91 mV, entrapment efficiency of 92.48 ± 1.06%, drug loading of 12.86 ± 0.19% and drug content of 96.42 ± 0.86%. F9 released 98.6% of the incorporated actives at 12 h and followed the Korsmeyer–Peppas model ($R^2 = 0.9953$; $n = 0.9676$), consistent with Case II/Super Case II transport according to the thesis interpretation. In-vitro anti-inflammatory assessment showed 74.4 ± 0.69% inhibition in the protein-denaturation assay and 70.2 ± 0.91% inhibition in the egg-albumin denaturation assay. These findings support the feasibility of the developed herbal nanohydrogel as a topical delivery system; further ex-vivo, animal and clinical investigations are required before therapeutic conclusions can be made.

Keywords: Curcuma longa; Piper nigrum; herbal nanohydrogel; chitosan; sodium tripolyphosphate; Carbopol 934; rheumatoid arthritis; antioxidant activity; protein denaturation; topical drug delivery.
How to cite this article: Manish Kumar Sharma
Rajkumari Lodhi* Dr. Satish Nayak. FORMULATION AND EVALUATION OF CURCUMA LONGA AND PIPER NIGRUM LOADED HERBAL NANOHYDROGEL FOR IN-VITRO ANALYSIS OF ARTHRITIS. Int J Drug Deliv Technol. 2026;16(87s):1852-1864. DOI: 10.25258/ijddt.16.87s.199.

1. Introduction

Arthritis is a chronic inflammatory condition characterized by joint pain, swelling, stiffness, reduced mobility, and progressive damage to joint tissues. It includes several disorders, among which osteoarthritis and rheumatoid arthritis are the most common. Osteoarthritis is primarily associated with the degeneration of articular cartilage, whereas rheumatoid arthritis is an autoimmune disorder involving persistent inflammation of the synovial joints. These conditions can significantly affect the quality of life by limiting physical activity and daily functioning. Conventional treatments, including non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and disease-modifying antirheumatic drugs, are commonly used to manage arthritis. However, prolonged use of certain medications may be associated with adverse effects, such as gastrointestinal irritation, cardiovascular complications, renal toxicity, and other drug-related problems. Consequently, the development of alternative and complementary approaches using medicinal plants and advanced drug-delivery systems has attracted considerable research interest.

Medicinal plants have been widely investigated for their potential anti-inflammatory, antioxidant, and analgesic properties. Herbal formulations contain various bioactive constituents that may act on multiple biological pathways involved in inflammation and tissue damage. Among these plants, *Curcuma longa* (turmeric) and *Piper nigrum* (black pepper) are particularly interesting because of their traditional medicinal applications and pharmacologically active constituents. Their combination may provide a useful basis for developing a topical herbal formulation intended to reduce inflammatory responses associated with arthritis.

Curcuma longa, belonging to the family Zingiberaceae, is a widely used medicinal plant commonly known for its yellow rhizome. Its major bioactive constituents include curcuminoids, particularly curcumin, demethoxycurcumin, and bisdemethoxycurcumin. Curcumin has been extensively studied for its anti-inflammatory and antioxidant properties. It may influence inflammatory pathways involving nuclear factor-kappa B (NF-κB), cyclooxygenase-2 (COX-2), pro-

inflammatory cytokines, and other mediators associated with inflammation. By modulating these pathways, curcumin may help reduce inflammatory responses and oxidative stress. Oxidative stress is relevant to arthritis because excessive production of reactive oxygen species can contribute to cellular injury and the progression of joint inflammation. Despite its promising pharmacological potential, curcumin has limitations, including poor aqueous solubility, limited absorption, and relatively low bioavailability. These characteristics can restrict its effectiveness when administered through conventional formulations and highlight the need for improved delivery approaches.

Piper nigrum, commonly known as black pepper, belongs to the family Piperaceae. It is an important culinary spice and medicinal plant containing several bioactive compounds, of which piperine is the principal alkaloid. Piperine has been investigated for its antioxidant, anti-inflammatory, and bioavailability-enhancing properties. It may influence inflammatory signalling pathways and has been studied for its ability to improve the absorption or bioavailability of certain compounds, including curcumin. Combining *Piper nigrum* with *Curcuma longa* may therefore offer a promising strategy for developing a herbal formulation that incorporates complementary phytochemical properties. Nevertheless, the effectiveness and safety of this combination must be established through appropriate formulation studies and experimental evaluation rather than assumed from the properties of the individual constituents.

Nanotechnology-based drug-delivery systems have emerged as promising approaches for improving the delivery of poorly water-soluble phytoconstituents. Nanohydrogels are three-dimensional, hydrophilic polymeric networks capable of absorbing and retaining water while incorporating active ingredients within their structure. Depending on their composition and preparation method, these systems can provide improved dispersion of active compounds, controlled drug release, enhanced formulation stability, and better contact with the application site. A topical nanohydrogel may also offer advantages such as ease of application, a non-greasy feel, improved patient convenience, and the possibility of reducing systemic exposure compared with some orally administered formulations. However, the extent of skin penetration and local therapeutic activity depends on the formulation characteristics and must be established experimentally.

2. plants profile The formulation of *Curcuma longa* and *Piper nigrum* into a herbal nanohydrogel may provide a suitable approach for combining the anti-inflammatory potential of curcumin with the complementary pharmacological properties of piperine. The nanohydrogel base can serve as a carrier for these plant-derived constituents and may help overcome certain limitations associated with their conventional topical application. The formulation can be developed using suitable polymers, gelling agents, stabilizers, and other excipients selected according to their compatibility with the herbal extracts. Important formulation parameters include the concentration of plant extracts, polymer type, pH, viscosity, homogeneity, spreadability, and physical stability. If nanosized particles are incorporated into the hydrogel, their particle size, polydispersity index, and zeta potential may also be evaluated, depending on the formulation design. These characteristics are important for determining the quality, reproducibility, and potential performance of the final product.

The evaluation of the developed herbal nanohydrogel is essential to establish its physicochemical properties and preliminary biological activity. Physical evaluation may include colour, appearance, odour, homogeneity, pH, viscosity, spreadability, extrudability, and stability under appropriate storage conditions. Drug or phytoconstituent content and in-vitro release studies can provide information about the amount of active material present and its release from the formulation. In-vitro anti-inflammatory activity may be investigated using suitable laboratory methods, such as inhibition of protein denaturation or other validated inflammation-related assays. These tests can provide preliminary evidence of the formulation's ability to influence processes associated with inflammation. A suitable standard anti-inflammatory drug, untreated control, vehicle control, and individual herbal formulations may be included, where appropriate, to compare the activity of the combined formulation. The selected assay, experimental conditions, and statistical analysis should be clearly defined to ensure reliable interpretation of the results.

Although both turmeric and black pepper have been studied for their pharmacological properties, the effectiveness of their combination in a nanohydrogel cannot be established solely from the existing evidence on the individual plants. Further research is required to determine the optimum formulation composition, compatibility of the extracts, release characteristics, stability, and in-vitro anti-inflammatory performance. In addition, in-vitro findings alone cannot confirm clinical effectiveness, skin penetration into joint tissues, or the ability to treat arthritis in humans.Therefore, the present study aims to formulate and evaluate a *Curcuma longa* and *Piper nigrum*-loaded herbal nanohydrogel for the in-vitro assessment of its potential anti-inflammatory activity relevant to arthritis. The study will focus on developing a suitable topical formulation, evaluating its physicochemical characteristics, investigating the release of active constituents, and assessing its preliminary anti-inflammatory activity using an appropriate laboratory model. The findings may

contribute to the development of a plant-based topical drug-delivery system and provide a scientific foundation for further studies on its safety, efficacy, and potential application in arthritis management.

3. Materials and Methods

3.1. Collection and Identification of Curcuma Longa and Piper Nigrum

The healthy fresh rhizomes of Curcuma Longa and fruits of Piper Nigrum (About 0.5 to 1 kg) was collected from the local market of Bhopal.

3.2. Organoleptic characters of Curcuma Longa and Piper Nigrum

Organoleptic properties like size, shape, color, odor, taste, fracture and surface, are the critical parameters for the rapid identification and consumer acceptance. Sensory evaluation -visual macroscopy, colour, odour, taste, fracture and surface are the common features helped in identification of the crude drug.

3.3. Extraction of Curcuma Longa and Piper Nigrum by Soxhlation

The very coarse powder form was extracted by Soxhlation method by using ethanol (Analytical grade) as solvent. The very coarse powder form was packed in Soxhlet extractor .A total amount of 150 g very coarse powder was extracted with 300 ml of ethanol solvent. At first the extractor was properly cleaned and dried. In the extractor, the lower end of siphon tube was blocked with a thick bed of surgical cotton. Then the very coarse powder was packed in the extractor up to upper end of siphone tube. Another thick bed of surgical cotton was placed above crude drugs just below the lower end of siphone tube. Then, the extractor was placed over round bottom flask of 500 ml capacity. The solvent was poured int o the extractor unless until one solvent siphoning will be taken place. Care was taken that after first siphoning of solvent; the addition of solvent to extractor must be continued up to that extent that the solvent will remain up to that level marked in s iphoning tube. Now the condenser was connected to cool water inlet and outlet system, was placed over the extractor. The whole set up was fitted to stand and round bottom flask was kept over the heating mantle. The extraction was done at a temperature of 30 to 34 °C, depending on boiling point of solvent. Extraction was continued unless until the solvent in siphoning tube becomes colourless. For each solvent, around four to five cycles were run to obtain thick slurry. The thick slurry was then concentrated under reduced pressure using rotary evaporator to obtain crude extract.

3.4. Pharmacognostic (physical) evaluation of C. Longa and P. Nigrum powder

Physical standards of crude ethanolic extracts of Curcuma Longa and Piper Nigrum are to be determined for drugs, wherever possible. These are rarely constant for crude drugs, but may help in evaluation, especially with reference to moisture content, foreign organic matter, ash (Total ash, acid insoluble ash, water soluble ash and sulphated ash).

3.4.1. Determination of Moisture Content (Loss on Drying)

The percentage of active chemical constituents in crude drugs is mentioned based onair - dried material. Hence, the moisture content of a drug should be determined and should also be controlled. The presence of moisture in crude drugs can lead to its deterioration due to either activation of certain enzymes or growth of microbes. The moisture content of a drug should be minimized to prevent decomposition of crude drugs either due to chemical changes or microb ial contamination. About 1.5 g of crude drug (Very coarse powder form) was weighed into a flat and thin porcelain dish. The drug was dried in an oven at 105 °C up to a constant weight. The drug was cooled and reweighed. The loss in weight is usually record ed as moisture content of crude drug and it was calculated by using the following equation. Moisture loss (%) = [(W1 – W2)/W1] × 100 Where, W 1 is the initial weight of crude drug in g and W 2 is the final weight of crude drug in g after drying.

3.4.2. Determination of Foreign Organic Matter

The parts of the organs other than those named in descriptions of the drug are defined as foreign organic matter. If the maximum limit of foreign organic matter exceeds, deterioration in quality of the drug takes pla ce. Accurately 100 g of sample was weighed, and it was spread on a white tile uniformly without overlapping. Then the sample was inspected visually using a lens (Magnification is 5x). The foreign organic matter was separated manually. After complete separation, the collected foreign organic matter was weighed and the percentage in w/w present in the sample was calculated by using the following formula. Foreign Organic matter (%) = (W1/ W2) × 100 Where, W 1 is the weight of foreign organic matter in g a nd W 2 is the initial weight of crude drug (100 g).

3.4.3. Determination of Volatile Oil Contents

The Pharmaceutical significance of aromatic drugs is due to their odorous principle i.e., volatile oils. Such crude drugs are standardized based on their volatile oil content. The official method for the estimation of volatile oil in a crude drug is hydrodistillation based on distilling the drug with water and distillate is collected in the graduated tube, from which the aqueous portion of the distillate automatically returns to the distillation flask. The volatile oil content of crude drug was determined by using Clevenger apparatus (Borosilicate Clevenger Apparatus, Radial Scientific Equipment Pvt. Ltd., India). About 25 g of very coarse powder drug was taken with 250 ml of water in a distillation flask. Few pieces of porcelain were added to avoid bumping during distillation. The apparatus was set in position. The receiver and return tubes were filled with water. Then the flask was heated through heat

ing mantle. The heating was continued till no more oil was collected. On completion of distillation, the heat was removed and the receiving tube was allowed to cool up to room temperature. The volatile oil and water layers were allowed to separate and the volume of volatile oil was recorded.

3.4.4. Determination of Ash values

The residue remaining after incineration is the ash content of the drug, which simply represents inorganic salts, naturally occurring in drug or adhering to it or deliberately added to it, as a form of adulteration. Ash contains inorganic radicals like phosphates, carbonates and silicate salts of sodium, potassium, magnesium and calcium. The importance of knowing ash value is to assess the quality and purity of a crude drug. i. Determination of total ash value Accurately 5 g of air -dried powdered drug was accurately weighed in tarred silica crucible. It was incinerated gradually by increasing the heat (Temperature not more than 450 °C) until free from carbon, cooled and weighed accordingly. It wa s then kept in a desiccator (Esel Desiccator, Esel International, Mumbai, India). The percentage of total ash with reference to the air -dried powdered drug was calculated using the below mentioned formula. Total ash value (%) = (w/W) × 100 where, w is the weight of obtained ash in g and W is the weight of crude drug in very coarse powder form taken in g. ii. Determination of acid insoluble ash value The ash from the dish used for total ash was washed with 25 ml of dilute hydrochloric acid using 100 ml beaker an d boiled for 5 min. The residue was filtered through an ash - less filter paper. Then the residue was washed twice with hot water. The crucible was ignited, cooled and weighed. The percentage of acid insoluble ash was calculated with reference to the air -dried sample of crude drug, as per the equation mentioned below. Acid insoluble ash value (%) = (w1/w2) × 100 Where, w 1 is the weight of obtained acid insoluble ash in g and w 2 is the weight of crude drug in very coarse powder form taken in g. iii. Determination of water insoluble ash value The ash from the dish used for total ash was washed with 25 ml of water and boiled for 5 min. The insoluble matter was collected on an ash-less filter paper. Then the residue was washed twice with hot water. The crucible was ignited to a constant weight at low temperature, cooled and weighed. The percentage of water insoluble ash was calculated with reference to the air -dried sample of crude drug using the following formula. Water insoluble ash value (%) = (W/w) × 100 Where, W is the weight of obtained water insoluble ash in g and w is the weight of crude drug in very coarse powder form taken in g. iv. Determination of sulphated ash value About 5 g of powdered drug was taken in a silica crucible and ignited thoroughly. The residue was cooled and moistened with 1 ml of dilute sulphuric acid. It was heated gently until white fumes were no longer evolved and again ignited until all black particles had disappeared. The crucible was allowed to cool, and t he weight of the residue was taken. The percentage of sulphated ash with reference to the air - dried drug was calculated as per the formula given below. Sulphated ash value (%) = (P1/P2) × 100 Where, P1 is the weight of obtained sulphated insoluble ash in g and P2 is the weight of rude drug in very coarse powder form taken in g. v. Determination of extractive values The extracts obtained by exhausting crude drugs are indicative of approximate measures of their chemical constituents. Taking into consideration the diversity in chemical nature and properties of contents of drugs, various solvents are used for determination of extractives. The solvent used for extraction is able to dissolve appreciable quantities of substances desired. Extractive value determinati on may help in evaluation, specifically with reference to solubility in different solvents. Evaluation of a drug basically needs its identification and can be done by morphological or microscopical characters. Many a times, the drug identified by its diagn ostic characters is of substandard quality due to either faulty collection or incorrect storage. So, to prove its acceptability as a drug, extractive value determination can be applied, wherever possible. The determination of extractive value helps for eva luation of crude drugs, give ideas about the nature of chemical constituents present in crude drugs and also useful for the estimation of specific constituents, soluble in that particular solvents used for extraction. About 5 g of powdered drug (Very coarse form) was transferred into a dry 250 ml conical flask. It was macerated with 100 ml of each solvent (Water, Ethanol, n -butanol, Petroleum ether and Chloroform respectively) in a stoppered flask for 24 h. In the first 6 h, the flask was frequently shaken and then allowed to stand for 18 h. Then the mixture was filtered into 50 ml of cylinder. When sufficient filtrate was collected, 25 ml of filtrate was transferred to a weighed, thin porcelain dish. The filtrate was evaporated to dryness by placing the dish on a boiling water bath. The drying was completed in a Hot air oven at 100 °C. The extract was cooled in a desiccator and weighed. The percentage (w/w) of extractive with reference to the air -dried drug was calculated.

3.5. FTIR Analysis of Extract

The FTIR analysis of the extract was carried out to identify the major functional groups present. A small quantity of the dried extract was mixed with KBr and compressed into a thin pellet. The pellet was placed in an FTIR spectrophotometer, and th e spectrum was recorded over the range of approximately 4000 –400 cm^{-1} . The characteristic absorption peaks obtained were compared with standard reference values and interpreted to identify the functional groups present in the extract.

3.6. Preliminary phytochemical screening of extracts

Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio -resource of drugs of traditional systems of medicine, modern medicines, nutraceu ticals, food supplements, folk medicines, pharmaceutical intermediates, and chemical entities for synthetic drugs. Many plants are claimed to possess the antibiotic properties in the traditional system and are also used extensively by the tribal people wor ldwide. The plants may be considered as a biosynthetic laboratory, not only for the chemical compounds such as carbohydrates, proteins and lipids that are utilized as food by man, but also for a multitude of compounds like glycosides, alkaloids, volatile o ils, tannins, etc., that exert a pharmacological effect. The compounds that are responsible for therapeutic effect are usually the secondary metabolites.

3.7. Quantitative Estimation of phytoconstituents in extract

i) Determination of total phenolics Phenolics are characterized by one aromatic ring bearing one or more hydroxyl groups. Antioxidant action of phenolic compounds is due to their high tendency to chelate metals. Phenolics possess hydroxyl and carboxyl groups and are able to bind particularly iron and copper. Phenols react with phosphomolybdic acid in Folin-Cioocalteau reagent in alkaline medium to produce a blue -coloured complex which can be estimated spectrophotometrically. Folin-ciocalteau colorimetic assay The amount of total phenolic in extracts was determined with the Folin Ciocalteu reagent. Gallic acid was used as a standard and the total phenolic were expressed as mg/g gallic acid equivalent (GAE). Concentration of 0.0 1, 0.02, 0.03, 0.04 and 0.05 mg/ml of gallic acid were prepared in methanol. Concentration of 0.1and 1mg/ml of plant extract were also prepared in methanol and 0.5ml of each sample were introduced in to test and mixed with 2.5ml of a 10 fold dilute folin Ciocalteu reagent and 2ml of 7.5% sodium carbonate. The tubes were covered with parafilm and allowed to stand for 30 minutes at room temperature before the absorbance was at read at 760 nm spectrometrically. All determination was performed in triplicate. The folin -Ciocalteu reagent is sensitive to reducing compounds including polyphenols.They produce a blue colour upon reaction.This blue colour was measured spectrophotometrically. Line of regression from Gallic acid was used for estimation of unknown phenol content. $y = 0.005x + 2.569$ and $R2 = 0.991$ Thus the goodness of fit was found to be good for selected standard curve. By putting the absorbance of test sample (y = absorbance) in line of regression of above mentioned gallic acid. ii) Determination of Total Flavonoid Content : Flavonoids are polyphenolic compounds ubiquitous in nature and are categorized according to their chemical structure as flavonols, flavones, flavanoes, isoflavones, catechins, anthocyanidins and chalcones. Favonoids react with AlCl3 to produce a coloured product, which can be measured spectrophotometrically. Total flavonoids were measured by a colorimetric assay. An aliquot of diluted sample or standard solution of rutin was added to a 75 µl of $NaNO_2$ solution, and mixed for 6 min, before adding 0.15 mL AlCl 3 (100 g/L). After 5 min, 0.5 mL of NaOH was added. The final volume was adjusted to 2.5 ml with distilled water and thoroughly mixed. Absorbance of the mixture was determined at 510 nm against the same mixture, without the sample, as a blank. Total flavonoid content was expressed as mg Rutin/g dry weight (mg rutin/g), through the calibration curve of Rutin. All samples were analysed in three replications. Line of regression from rutin was used for estimation of unknown flavonoid content. $y = 0.001x - 0.020$ and $R2 = 0.994$ Thus the goodness of fit was found to be good for selected standard curve. By putting the absorbance of test sample (y = absorbance) in line of regression of above mentioned rutin. iii) Spectrophotometric Quantification of Total Saponin Content: Standard curve 50, 62.5, 75, 87.5, 100, 112.5 and 125.5 µg/ml of the standard saponin solutions was made with aqueous methanol (80%). Diosgenin was taken as standard prepared by dissolving 10 mg(diosgenin) in 16 ml methanol and 4 ml of distilled w ater. Vanillin reagent (8%, 0.25 ml) and sulphuric acid (72% v/v, 2.5 ml) was combined. After proper mixing the tubes were placed in water bath at 60°C for 10 minutes and cooled in ice bath for 3-4 mins. The absorbance was done at 544 nm against the reagent blank. 0.1gm of sample was prepared by dissolving in aqueous methanol (80%). v) Spectrophotometric Quantification of Total Alkaloid Content: For the standard curve of atropine, total 5 different concentrations were used. 100 µg/ml solution was initially prepared from atropine (1 mg in 10 ml of distilled water). From this stock solution, exactly 0.5, 1, 1.5, 2 and 2.5 ml of atropine solutions was transferred to five different separating funnel. To each and individual funnels, 5 ml of phosphate buffer (pH4.7) and 5ml of Bromocresol green (BCG) solution was added and mixed vigorously. The formed complex mixture is extracted with chloroform. Chloroform fraction was collected and final volume was adjusted in 10 ml volumetric flask with chloroform. Absorption at a wavelength of 470 nm of each flask was measured and calibration graph was drawn. Plant extract (1mg/ml) was admixed in HCl(2N) and filtered. The pH of the plant extract was fixed to neutral with 0.1 N NaOH. Solution(1ml) was transferred to a separating funnel and around bromocresol green(5ml) and phosphate buffer(5ml) was combined and mixed properly. The mixture was further extracted with chloroform (5ml)

and transferred to 10 ml of volumetric flask and final volume was adjusted. The estimation was done by absorbance at 470 nm.

3.8. Determination of in vitro antioxidant activity

i) DPPH (2, 2 diphenyl 2 picryl hydrazyl hydrates) radical scavenging activity Principle: DPPH (di - phenyl picryl hydrazyl) is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Due to its odd electron, the methanolic solution of DPPH shows a strong absorption band at 517 nm. When the solution of DPPH is mixed with a suitable reducing agent, the electrons become paired off. This gives rise to a reduced form of DPPH as shown in fig 5.1. The solution loses color stiochometrically with the number of electrons taken up. Such reactivity has been widely used to test the ability of compounds/ plant extracts to act as free radical scavenger. Reduction of DPPH radicals can be observed by the decrease in absorbance at 517 nm. Procedure: 0.1mM solution of DPPH in methanol was prepared. 1.5ml of DPPH solution was added to 1.5ml of extract solution at different concentrations. The mixtures were shaken vigorously and incubated in the dark for 30 min. Thirty minutes later, the absorbance was measured at 517nm. Ascorbic acid was used as standard. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Assay was done in triplicates. The capability to scavenge the DPPH radical was calculated using the following equation: % inhibition= (A control – A test/std / A control) × 100

ii) Superoxide radical scavenging activity: The scavenging activity of superoxide anions by extract was assessed using the established protocol. This approach hinges on the ability to inhibit the formation of formazan from nitroblue tetrazolium (NBT) in the presence of superoxide ions. All solutions were formulated using a 100 mM phosphate buffer at a pH of 7.4. Specifically, a mixture was prepared comprising 1 ml of 156 mM NBT, 1 ml of 468 mM reduced Nicotinamide adenine dinucleotide (NADH), and 3 ml of extract at concentrations ranging from 20 to 100 µg/ml. The reaction initiation was triggered by the addition of 100 µl of 60 µM Phenazine methosulphate (PMS), followed by incubation at 25°C for a duration of 5 minutes. The absorbance was subsequently measured at 560 nm utilizing a UV -visible spectrophotometer, and percentage inhibition of superoxide radicals was calculated.

3.9. In vitro anti-arthritic activity

Basic consideration Rheumatoid arthritis (RA) is a systemic autoimmune oxidative stress chronic inflammatory disease primarily involves in release of inflammatory mediators (matrix metalloproteinases, prostaglandin E, Tumor Necrosis Factor - α, and interleukin -1β) in synovial (small peripheral, proximal) joints, which leads to the destruction of the joint with cartilage and bone erosion. RA characterized with swelling, redness, pain, and tightness in joints . The basic drugs that are choice for lifetime treatment of RA are non - steroidal anti - inflammatory drugs (NSAIDs), Dise ase-modifying anti -rheumaticdrugs (DMARDs), immunosuppressants, steroids, and cytotoxic drugs. These drugs are associated with many persistent adverse effects. Developments of cost effective and safer alternative anti-arthritic agents for lifelong is necessitate overcoming from the adverse effects of the allopathic drug therapies. Novel topical gel form of herbal drugs are promising alternatives for the development of effective and safe drug against arthritis is due to their ease of availability, target sit e specific, and cost -effectiveness in comparison to allopathic. The phytoconstituents present as active moiety in the ethanolic extract suppresses the oxidative chain reactions which results in reduction in production of oxidative free radicals and neutral izes theses free radicals, thus release of inflammatory mediators at the local areas of synovial get decreased. This results in anti - inflammatory that is anti-arthritic activity of plant. .Preparation of saline phosphate buffer pH 6.4 The saline phosphate buffer of pH 6.4 was prepared by using 1.79 g of disodium hydrogen phosphate, 1.36 g of potassium dihydrogen phosphate and 7.02 g of sodium chloride. The three chemicals were weighed and dissolved in double distilled water. Finally, the volume of the solution was adjusted to 1000 ml. The pH of the final solution was determined by using digital pH meter. The pH of the solution was adjusted to 6.4 by using dilute solution of HCl and NaOH.

3.9.1. In vitro anti-arthritic activity by Protein denaturation inhibition method

The inflammatory and arthritic diseases are mainly caused by the denaturation of tissue protein. Though the root cause of RA is unknown, still it is believed that in vitro denaturation of certain tissue proteins leads to pro duction of autoantigen (Protein denaturation and macroglobulin formation), thus initiating the immune response and producing biochemical changes in connective tissue, which ultimately leads to rheumatoid arthritis . Some natural (Herbal drug that is Curcum a Longa and piper nigrum) component containing novel gel can inhibit the protein denaturation, thus could solve arthritic diseases. Test drugs The normal saline water was used as normal control . The marketed conventional tablet of Celecoxib are used as standard (Positive control) and Test groups of nanohydrogel . Experimental method The in vitro anti-arthritic activity of was performed by protein denaturation method as per the standard procedure mentioned in the literature . The Bovine serum albumin (BSA) solution was prepared in the strength of 5 % w/v by using saline phosphate buffer solution as solvent. The pH of the BSA solution was adjusted to 6.4 by using HCl. About 5 ml of solution

consisting of 4.5 ml of Bovine serum albumin (BSA – 5 % w/v) and 0.5 ml of double distilled water was used as Normal control solution. About 5 ml of solution containing 4.5 ml of Bovine serum albumin (BSA – 5 % w/v) and 0.5 ml of Celecoxib solution using the Celecoxib marketed tablet (Equivalent to Celecoxib strength of 100 µg/ml) was treated as primary standard (Positive) control solution. About 5 ml of solution of formulations consisting of 4.5 ml of Bovine serum albumin (BSA – 5 % w/v) and 0.5 ml of formulation (Equivalent to Extract strength of 100 µg/ml) separately were used as test control solutions. All the above mentioned solutions were incubated separately by using the laboratory grade incubator at 37 °C for 15 min. After incubation, the sample solution mixture was heated separately at 60 °C for 5 min. This heating activity will induce the protein denaturation process. Now the samples were cooled to room temperature. The absorbance of all solutions was determined by using the UV -Visible spectrophotometer (Shimadzu 1900i, Japan) at λmax of 660 nm. From the observed absorbance, the percentage inhibition of protein (BSA) was calculated.

3.9.2. In vitro anti-arthritic activity by egg albumin denaturation inhibition method

The in vitro anti-arthritic activity of formulations was performed by egg albumin denaturation method as per the standard procedure mentioned in the literature. The egg albumin solution was prepared in the strength of 5 % w/v by using saline phosphate buffer solution as solvent. The pH of the egg albumin solution was adjusted to 6.4 by using HCl. About 5 ml of solution consisting of 4.5 ml of egg albumin (5 % w/v) and 0.5 ml of double distilled water was used as Normal control solution. About 5 ml of solution containing 4.5 ml of egg albumin (5 % w/v) and 0.5 ml of Celecoxib solution using the Celecoxib marketed tablet (Equivalent to Celecoxib strength of 100 µg/ml) was treated as primary standard (Positive) control solution. About 5 ml of solution of formulations consisting of 4.5 ml of Bovine serum albumin (BSA – 5 % w/v) and 0.5 ml of formulation (Equivalent to strength of 100 µg/ml) separately were used as test control solutions. All the above solutions were incubated separately by using the laboratory grade incubator at 37 °C for 15 min. After incubation, the sample solution mixture was heated separately at 60 °C for 5 min. This heating activity will induce the protein denaturation process. Now the samples were cooled to room temperature. The absorbance of all five processed sample solutions was determined by using the UV - Visible spectrophotometer (Shimadzu 1900i, Japan) at λmax of 660 nm. From the observed absorbance, the percentage inhibition of protein (BSA) was calculated

3.10. Formulation of nanohydrogel

The nanohydrogel was prepared by ionic gelation of chitosan with sodium tripolyphosphate (TPP), followed by incorporation of the resulting nanoparticles into a Carbopol 934 hydrogel base. Chitosan was dissolved in a suitable dilute acidic medium with continuous stirring, and Curcuma longa (curcumin) and Piper nigrum (piperine) extracts were separately dispersed/dissolved using Tween 80 and propylene glycol before incorporation into the chitosan solution. An aqueous TPP solution was added dropwise under continuous magnetic stirring to form chitosan–TPP nanoparticles by ionic cross-linking. Separately, Carbopol 934 was dispersed in distilled water and allowed to hydrate, followed by incorporation of glycerin and the prepared chitosan–TPP nanoparticle dispersion with gentle stirring. Methyl paraben and propyl paraben were then added, and the formulation was made up to 100 g with distilled water. The pH was adjusted to approximately 5.5, and the prepared formulations were designated F1–F9.

3.11. Characterization of the nanohydrogel

3.11.1. Appearance and homogeneity

The prepared nanohydrogels are visually examined for color, appearance, texture, phase separation, grittiness, and homogeneity. A small quantity is spread between the fingers or on a glass slide to assess smoothness and the absence of coarse particles. An acceptable formulation shou ld be uniform, smooth, and free from visible aggregates or phase separation.

3.11.2. pH

The pH of each nanohydrogel is measured by dispersing a defined quantity of formulation in distilled water and allowing it to equilibrate. The pH is measured using a previously calibrated digital pH meter. Since the formulation is intended for topical application, the pH should preferably be within a skin-compatible range to minimize irritation.

3.11.3. Spreadability

Spreadability is determined by placing a known quant ity of gel between two glass slides and applying a predetermined load. The movement of the upper slide over a specified distance is recorded. Spreadability is calculated from the applied weight, distance travelled, and time required. A formulation with satisfactory spreadability can be applied uniformly over the affected joint area.

3.11.4. Determination of Extrudability of Nanohydrogel

The extrudability of the prepared nanohydrogel was determined to evaluate the ease with which the formulation could be expelled from a collapsible tube. A known quantity of nanohydrogel was filled into a clean, collapsible aluminium or laminated tube and the tube was properly sealed. The tube was placed between two glass slides or on a suitable support, and a definite weight, commonly about 500 g to 1 kg, was applied over the tube for a fixed period. The amount or length of nanohydrogel extruded through the tube opening was then measured. Extrudability was assessed on the basis of

the quantity of gel extruded under the applied pressure and may be expressed as g/cm² using the equation: Extrudability = Applied weight / Area of the tube opening. The test was generally performed in triplicate, and the mean value was calculated. A formulation showing smooth, uniform, and easy extrusi on with minimum applied force was considered to possess good extrudability.

3.11.5. Viscosity

Viscosity is measured using a suitable Brookfield viscometer with an appropriate spindle and rotational speed. The formulation is placed in the sample container and allowed to equilibrate before measurement. Viscosity influences spreadability, residence time, drug release, and ease of topical application. Excessively high viscosity may reduce spreadability, whereas very low viscosity may reduce retention at the application site.

3.11.6. Drug content

Drug content is determined by accurately weighing a specified quantity of nanohydrogel and extracting the incorporated curcumin and piperine using an appropriate solvent. After suitable dilution and filtration, the drug concentration is determined using a validated analytical method. Drug content is expressed as the percentage of the labeled or theoretical drug amount present in the formulation.

3.11.7. Zeta-potential analysis

The hydrodynamic size of particles is calculated using this method by beaming a monochromatic light into the solution and observing the resulting Doppler shift. Once the laser light hits the moving particles, it changes the wavelength of the incoming light and scatters the light at an angle. This angle of scattered light is inversely propositional to the size of the particle. The size distribution of nanoparticles is monitored using Zetasizer (Nano ZS, Malvern instrument, United Kingdom). The Zeta-potential of the nanoparticles is measu red to find the surface charge on it.

3.11.8. Particle size determination

Dynamic Light Scattering (DLS) principle was used to measure the particle size distribution of synthesized nanoparticles using a Malvern Zetasizer Nano ZS (Malvern Panalytical, UK). It was then diluted with deionized water to achieve the desired concentrat ion of nanoparticles within the sample (to avoid multiple scattering), before MBS measurements were taken. The diluted sample was pipetted into a disposable polystyrene cuvette and measured at 25 °C where a laser beam illuminated the sample, scattering lig ht changes due to Brownian motion of nanoparticles were observed. The measured diffusion coefficients were automatically converted into hydrodynamic particle size and polydispersity index (PDI) by the instrument software. Quantitative measurements were made in triplicate, and data are reported as mean ± standard deviation.

3.11.9. Determination of Entrapment Efficiency

The entrapment efficiency of the prepared herbal nanohydrogel was determined by separating the unentrapped drug or phytoconstituents from the nanohydrogel formulation. A known quantity of the nanohydrogel was accurately weighed and dispersed in a suitable solvent or distilled water. The dispersion was subjected to centrifugation at approximately 10,000 –15,000 rpm for 20–30 minutes to separate the entrapped nanoparticles/nanocarrier from the free drug or extract present in the aqueous phase. The supernatant containing the unentrapped drug was carefully collected and suitably diluted. The amount of free drug was determined using a suitable analytical method, such as UV –Visible spectrophotometry, at the predetermined wavelength. The total amount of drug present in an equivalent quantity of formulation was also determined after complete disruption or extraction of the nanohydrogel. Entrapment efficiency was calculated.

3.11.10. In-vitro drug release

In-vitro release is evaluated using a suitable membrane diffusion or Franz diffusion -cell method. A known quantity of nanohydrogel is placed in the donor compartment over a suitable membrane, while the receptor compartment contains an appropriate release medium maintained at approximately physiological temperature. Samples are withdrawn at predetermined intervals and replaced with an equal volume of fresh medium. Curcumin and piperine released into the receptor medium are quantified, and cumulative percentage drug release is plotted against time. The optimized F9 formulation is expected to provide a controlled and sustained release profile.

3.11.11. In vitro release kinetic model

To determine the mechanism and kinetics of drug release, the release data were fitted to different mathematical models, including Zero -order, First -order, Higuchi, Hixson – Crowell, and Korsmeyer – Peppas models. The model showing the highest coefficient of determination (R^2) was considered the best -fit model and was used to de scribe the predominant drug -release mechanism from the gel.

3.11.12. Stability study

The optimized nanohydrogel can be evaluated under appropriate stability conditions according to the applicable ICH framework. Samples are periodically examined for appearance, pH, viscosity, drug content characteristics. Any substantial change in these parameters is recorded to assess the physical and chemical stability of the formulation.

4. Results and Discussion

4.1 Extract yield and pharmacognostic evaluation

The ethanolic extract yield was 24.7 ± 1.05% for Curcuma longa rhizomes and 32.7 ± 1.108% for Piper nigrum fruits. The crude drugs showed

relatively low foreign organic matter and measurable moisture, ash and volatile-oil values, supporting their pharmacognostic evaluation. Ethanol provided the highest extractive value among the solvents evaluated for both plant materials.

Parameter	Curcuma longa	Piper nigrum
Moisture (%)	4.50 ± 0.08	4.31 ± 0.12
Foreign organic matter (%)	0.28 ± 0.02	0.21 ± 0.08
Volatile oil (%)	1.80 ± 0.17	2.40 ± 0.11
Total ash (%)	3.80 ± 0.22	5.30 ± 0.18
Acid-insoluble ash (%)	0.51 ± 0.11	1.60 ± 0.22
Water-soluble ash (%)	8.55 ± 0.16	10.80 ± 0.20
Sulphated ash (%)	0.78 ± 0.12	0.33 ± 0.24

Table 1. Pharmacognostic evaluation of the two crude drugs.

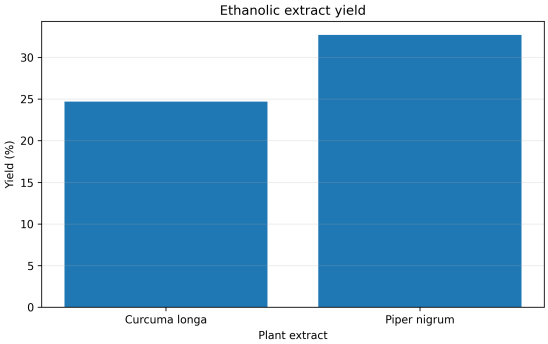


Figure 1. Ethanolic extract yield of Curcuma longa and Piper nigrum.

4.2 Phytochemical profile

Phytochemical	Curcuma longa	Piper nigrum
Alkaloids	++	++
Carbohydrates	+	+
Cardiac glycosides	+	+
Anthraquinone glycosides	–	–
Gums and mucilages	–	–
Proteins and amino acids	–	+
Phenols	+++	++
Triterpenoids	++	+
Saponins	+	+
Flavonoids	+++	+++
Fixed oils/fats	+	–

Table 2. Preliminary phytochemical screening of ethanolic extracts.

Constituent	Curcuma longa (mg/100 mg)	Piper nigrum (mg/100 mg)
Alkaloids	19.23	17.89
Flavonoids	45.78	38.87
Phenolics	43.21	32.95
Saponins	8.90	7.20

Table 3. Quantitative phytoconstituent estimation.

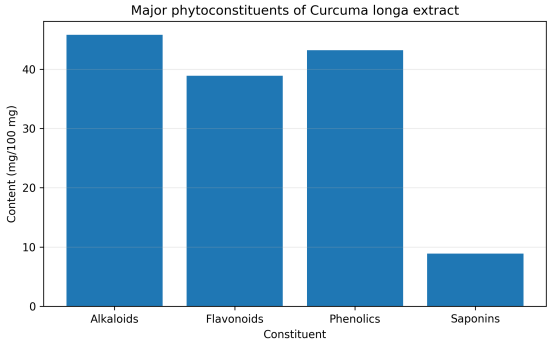


Figure 2. Quantified phytoconstituents in Curcuma longa extract.

4.3 FTIR analysis

FTIR analysis demonstrated characteristic hydroxyl, aliphatic C–H, aromatic C=C, carbonyl, ether and C–O bands in both extracts. Curcuma longa showed prominent bands at 3421.35, 2924.18, 2853.47, 1631.29, 1510.64, 1421.22, 1158.35 and 1026.48 cm^{-1}. Piper nigrum showed bands at 3369.27, 2922.11, 2851.32, 1736.26, 1624.31, 1512.57, 1456.18, 1261.44 and 1028.76 cm^{-1}. The observed functional groups are consistent with the presence of diverse oxygenated and hydrocarbon-containing phytoconstituents reported in the thesis.

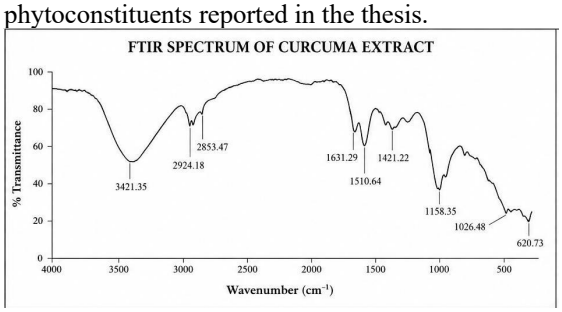


Figure 4. FTIR spectrum of Curcuma longa extract.

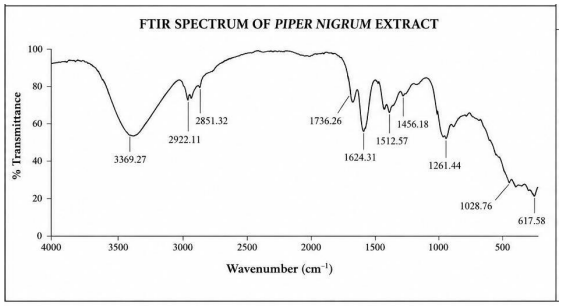


Figure 5. FTIR spectrum of Piper nigrum fruit extract.

Extract	Major FTIR peaks (cm^{-1})	Representative assignment
Curcuma longa	3421.35; 2924.18; 2853.47; 1631.29; 1510.64; 1158.35; 1026.48	O–H; aliphatic C–H; C=O/C=C; aromatic C=C; C–O–C/C–O
Piper nigrum	3369.27; 2922.11; 2851.32; 1736.26; 1624.31; 1512.57; 1261.44; 1028.76	O–H; aliphatic C–H; C=O; C=C; aromatic C=C; C–O

Table 4. Selected FTIR peaks and tentative assignments.

4.4 Antioxidant activity

Concentration (µg/mL)	Ascorbic acid	Curcuma longa	Piper nigrum
20	42.51 ± 3.21	39.24 ± 3.13	37.43 ± 2.12
40	53.27 ± 2.34	46.45 ± 2.51	44.75 ± 1.57
60	66.35 ± 1.96	58.19 ± 2.38	54.18 ± 1.87
80	70.91 ± 3.21	64.38 ± 2.96	62.68 ± 1.54
100	82.43 ± 2.74	81.96 ± 2.43	81.02 ± 3.71

Table 5. DPPH radical-scavenging activity (% inhibition).

Concentration (µg/mL)	Ascorbic acid	Curcuma longa	Piper nigrum
20	29.37 ± 1.61	21.26 ± 2.67	20.16 ± 1.43
40	52.18 ± 1.79	48.26 ± 2.10	45.23 ± 1.67
60	61.06 ± 2.22	60.13 ± 1.79	57.42 ± 1.86
80	69.70 ± 2.32	67.76 ± 1.32	65.53 ± 2.67
100	82.47 ± 1.88	80.76 ± 2.01	79.54 ± 1.78

Table 6. Superoxide radical-scavenging activity (% inhibition).

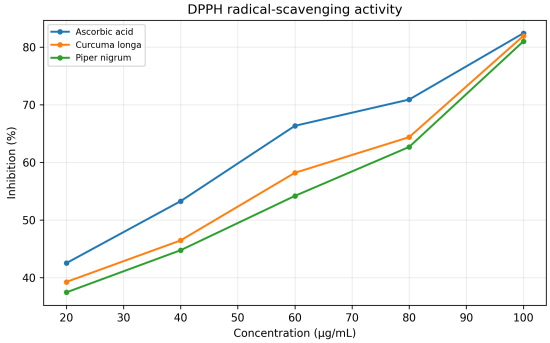


Figure 6. DPPH radical-scavenging activity.

Both extracts demonstrated concentration-dependent antioxidant activity. At 100 µg/mL, Curcuma longa showed 81.96 ± 2.43% DPPH inhibition and 80.76 ± 2.01% superoxide inhibition, while Piper nigrum showed 81.02 ± 3.71% and 79.54 ± 1.78%, respectively. Curcuma longa showed numerically higher activity than Piper nigrum across the tested concentrations in the reported data. The thesis relates this activity to the phenolic, flavonoid, alkaloid and other bioactive constituents identified in the extracts.

4.5 Nanohydrogel characterization

Batch	pH	Spreadability	Viscosity (cP)	Drug content (%)	Extrudability
F1	5.82	5.12	6,120	87.24	5.42
F2	5.86	5.28	6,280	89.16	5.68
F3	5.91	5.46	6,960	90.42	5.94
F4	5.94	5.62	6,740	91.38	6.18
F5	5.97	5.78	7,520	92.26	6.42
F6	6.01	5.94	7,310	93.14	6.67
F7	6.04	6.08	6,120	94.26	6.91
F8	6.07	6.22	7,640	95.18	7.18
F9	5.54	6.38	7,820	96.42	7.46

Table 7. Physical and pharmaceutical properties of F1–F9.

Batch	Particle size (nm)	PDI	Zeta potential (mV)
F1	428.6	0.462	+18.6
F2	401.8	0.421	+21.4
F3	378.4	0.386	+23.7

F4	354.7	0.351	+25.8
F5	332.5	0.318	+28.1
F6	315.8	0.294	+30.2
F7	298.6	0.267	+32.1
F8	284.3	0.238	+34.6
F9	271.6	0.214	+36.8

Table 8. Particle size, PDI and zeta potential of F1–F9.

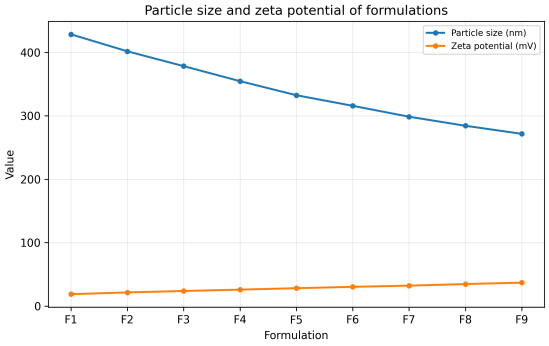


Figure 7. Particle size and zeta potential across F1–F9.

The formulation data showed a progressive reduction in particle size and PDI from F1 to F9, accompanied by an increase in positive zeta potential. F9 exhibited the smallest particle size (271.6 ± 5.48 nm), lowest PDI (0.214 ± 0.010) and highest positive zeta potential (+36.8 ± 0.91 mV), indicating a comparatively uniform and physically stable nanoparticle dispersion as interpreted in the thesis.

Batch	Entrapment efficiency (%)	Drug loading (%)	Percentage yield (%)
F1	68.42	8.26	72.18
F2	71.65	8.94	75.46
F3	75.84	9.62	78.35
F4	79.26	10.18	81.24
F5	82.48	10.76	84.17
F6	85.36	11.32	86.54
F7	88.14	11.86	88.62
F8	90.26	12.34	90.47
F9	92.48	12.86	92.36

Table 9. Entrapment efficiency, drug loading and percentage yield.

F9 demonstrated the highest entrapment efficiency (92.48 ± 1.06%), drug loading (12.86 ± 0.19%) and percentage yield (92.36 ± 0.98%). The combination of these results with the particle-size and physical-property data led to selection of F9 for further evaluation in the thesis.

4.6 In-vitro drug release and kinetic modelling

Time (h)	F1	F2	F3	F4	F5	F6	F7	F8	F9
1	9.8	10.6	11.2	11.8	12.4	13.1	13.7	14.2	14.8
2	17.2	18.4	19.1	20.2	21.1	22.3	23.5	24.4	25.6
4	31.6	33.4	35.2	37.1	39.2	41.5	43.6	45.8	47.6
6	46.8	48.9	51.3	53.7	56.2	58.8	61.4	63.7	66.2
8	60.4	62.8	65.1	67.6	70.1	72.8	75.2	77.4	79.8
10	71.6	73.9	76.3	78.8	81.4	83.7	85.8	87.6	89.5
12	79.8	82.1	84.6	86.7	88.9	90.8	92.4	94.1	98.6

Table 10. In-vitro cumulative drug release (%) from F1–F9.

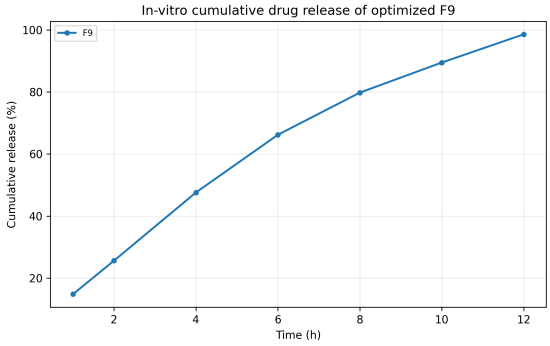


Figure 8. Cumulative drug release profile of optimized F9.

F9 showed the highest cumulative release at each sampling interval and reached 98.6% at 12 h. The thesis reports the Korsmeyer–Peppas model as the best-fitting model for F9, with R^2 = 0.9953 and release exponent n = 0.9676. Based on the interpretation provided in the thesis, n > 0.89 indicated Case II/Super Case II transport, suggesting a major contribution from polymer relaxation, swelling or matrix erosion rather than simple Fickian diffusion.

4.7 In-vitro anti-arthritic/anti-inflammatory activity

Treatment	Protein denaturation inhibition (%)	Egg albumin denaturation inhibition (%)
Celecoxib	76.8 ± 0.88	71.6 ± 0.87
F9 nanohydrogel	74.4 ± 0.69	70.2 ± 0.91

Table 11. In-vitro protein and egg-albumin denaturation inhibition (mean ± SD, n = 3).

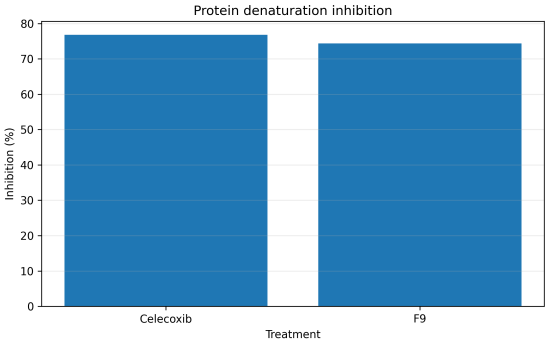


Figure 9. Protein denaturation inhibition by celecoxib and F9.

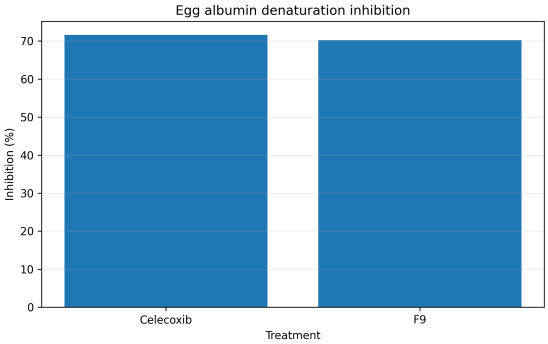


Figure 10. Egg albumin denaturation inhibition by celecoxib and F9.

The F9 nanohydrogel showed 74.4 ± 0.69% inhibition of protein denaturation compared with 76.8 ± 0.88% for celecoxib. In the egg-albumin assay, F9 showed 70.2 ± 0.91% inhibition compared with 71.6 ± 0.87% for celecoxib. Thus, the test formulation produced substantial inhibition in both assays, while the reported values were numerically lower than the standard. The thesis reports statistical significance at $p < 0.01$ using one-way ANOVA followed by Dunnett test.

The observed activity is consistent with the intended anti-inflammatory screening strategy, but these in-vitro assays alone do not establish clinical anti-rheumatoid efficacy. The thesis itself indicates the need for appropriate ex-vivo, animal and clinical studies for further confirmation.

5. Conclusion

The study successfully developed and evaluated a Curcuma longa and Piper nigrum loaded herbal nanohydrogel based on chitosan–TPP nanoparticles incorporated into Carbopol 934 hydrogel. The ethanolic extracts showed measurable pharmacognostic quality parameters, diverse phytochemical constituents and concentration-dependent antioxidant activity. Among the nine formulations, F9 showed the most favorable overall physicochemical profile, including 271.6 ± 5.48 nm particle size, 0.214 ± 0.010 PDI, +36.8 ± 0.91 mV zeta potential, 92.48 ± 1.06% entrapment efficiency, 12.86 ± 0.19% drug loading, 92.36 ± 0.98% percentage yield and 96.42 ± 0.86% drug content. F9 provided 98.6% cumulative release at 12 h and showed strong inhibition of protein and egg-albumin denaturation in the reported in-vitro assays. On the basis of the thesis data, F9 was selected as the optimized formulation and represents a promising research candidate for topical herbal delivery. However, the results should be interpreted as formulation and in-vitro evidence rather than proof of therapeutic efficacy. Further standardized ex-vivo permeation, safety, animal pharmacological and clinical studies are required.

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