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Research Article

Formulation and Comparative Evaluation of Sustained Release Matrix Tablet with a Marketed Diclofenac Gel

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Abstract

In this study, we used natural polymers (Ocimum Sanctum Linn and Acacia powders) in various formulations to create sustained release matrix tablets of Nimesulide. (F1 to F4). The pre-compression characteristics of bulk density, tapped density, angle of repose, compressibility (Carr's index), and Hausner's ratio were assessed for the tablets made using the wet granulation process. Additionally, post-compression parameters such as weight fluctuation, drug content, hardness, thickness, and friability tests were evaluated for the produced tablets. Different formulations demonstrated sustained release of Nimesulide over a ten-hour period, according to in vitro release experiments. Following that, we conducted a permeation investigation of commercially available Diclofenac gel using Franz diffusion cells, and we came to the conclusion that tablet formulations continue to be the best option for producing steady, systemic, and long-lasting therapeutic effects in arthritis. For all-encompassing, long-term arthritis management, tablets continue to be the preferred and more effective primary dosage form. This indicates the potential application of natural polymers in sustained drug delivery systems, improved patient compliance, reduced dose frequency, and repeatable drug release characteristics.

Keywords: Nimesulide, Ocimum Sanctum Linn powder, Acacia powder, sustained release matrix tablets, Diclofenac gel.

1. INTRODUCTION:

The development of novel drug delivery systems has become one of the most active research areas in the field of pharmaceutics.[1] Conventional immediate-release dose forms frequently result in inconsistent therapeutic effects, frequent dosing, and low patient compliance because they are unable to sustain an appropriate plasma drug concentration for prolonged periods of time.[2] Sustained release drug delivery systems (SRDDS) have been developed to enable controlled and predictable drug release, limiting side effects while maintaining a therapeutic drug level for a prolonged length of time.[3]

The most practical and widely used method of administering medications has been oral administration.[4] It offers more versatility in dosage form formulation, patient compliance, and convenience of administration. The drawbacks of traditional dosage forms, such as a short half-life and the possibility of missing a dose, are eliminated by sustained drug delivery.[5] By sustaining a steady plasma concentration over a prolonged period of time, sustained release formulations are intended to distribute medications at a predefined rate. These methods increase patient adherence to treatment, decrease the frequency of doses, and improve bioavailability.[4,6] The fundamental idea behind a sustained release formulation is to either coat the medicine with polymers that control its rate of diffusion or dissolution or embed it within a matrix. Better pharmacokinetic and pharmacodynamic profiles are ensured by controlled drug release, which reduces peak-trough variations in drug concentration that may cause adverse effects or therapeutic failure.[4,7]

For medications like Nimesulide that have limited biological half-lives, sustained release methods are very beneficial. These systems minimize local gastrointestinal irritation, decrease the frequency of doses, and enhance patient convenience and compliance by maintaining constant medication levels.[6,8]

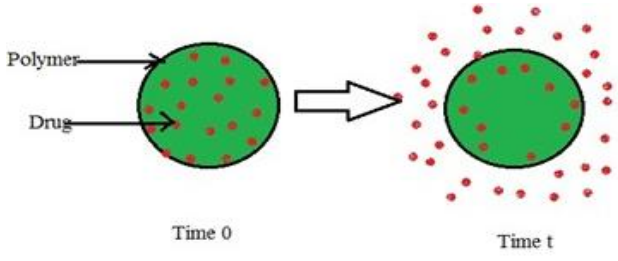

Figure 1: Diffusion Type Matrix

One of the most popular and straightforward dosage forms for continuous release distribution is a matrix tablet. They entail evenly distributing the active pharmaceutical ingredient (API) within a polymeric matrix that regulates the rate of drug release by erosion, diffusion, or a mix of the two.[9] The physicochemical characteristics of the medicine, the kind and amount of polymer, and the production method are some of the factors that influence the design of such systems.[10]

Matrix tablets have a number of benefits.

• Cost-effectiveness and a straightforward production procedure.
• Uniform medication release and improved stability.
• Consistent plasma medication levels result in fewer adverse effects.
Capacity to customize release profiles using a broad range of synthetic and natural polymers.[11]

Nimesulide is a non-steroidal anti-inflammatory medication (NSAID) having analgesic and antipyretic effects that is comparatively COX-2 selective.[12] Acute pain, osteoarthritis symptoms, and primary dysmenorrhea in adults and adolescents over the age of twelve are among its recognized indications.[13] Achieving a steady state blood or tissue level that is both therapeutically effective and non-toxic for a long time is the fundamental objective of therapy.[14] For the treatment of numerous acute and chronic illnesses, sustained release drug delivery systems are intended to increase patient compliance, improve therapeutic efficacy, minimize adverse effects, and lower dosage regimens with less toxicity.[15] Nimesulide is mostly removed via metabolic transformation, with the 4′-hydroxy derivative (M1) being the main metabolite. Urine and feces have been found to contain minor metabolites, mostly in conjugated form. Although their activity is less than that of Nimesulide pharmacological experiments conducted in vivo have demonstrated that the metabolites possess analgesic and anti-inflammatory qualities.[16] A derivative of sulfonamides, Nimesulide has a melting point of about 143°C. The literature claims that it has a weakly acidic pKa of about 6.5, which is explained by the presence of a sulfonamide group.[17] At ambient temperature, it is soluble in methanol and ethanol but nearly insoluble in water (approximately 10 µg/mL).[18]

None of the herbs used have the same repute as holy basil (Ocimum Sanctum) or Tulsi, despite India's enormous variety in ways that no other medicinal system does. Originating in north central India, tulsi is a fragrant shrub belonging to the Lamiaceae (tribe ocimeae) basil family. Today, it grows locally across the eastern global tropics. Tulsi is referred to as "The Incomparable One," "Mother Medicine of Nature," and "The Queen of Herbs".[2,19] In Ayurveda, where it is considered a "elixir of life" with unmatched medical and spiritual qualities

Tulsi, or Ocimum Sanctum Linn, is a powerful adaptable with a unique set of pharmacological qualities that support resilience and well-being. Hundreds of scientific studies, including in vitro, animal, and human trials, have exhaustively examined the therapeutic advantages of Tulsi.[20]

Tulsi mucilage's bioadhesives and film-forming properties allow it to efficiently regulate medication release, resulting in a consistent and extended release pattern.[21] Additionally, it is a natural substance that is easily accessible, biodegradable, and non-toxic, which makes it a desirable substitute for manufactured polymers.[22] These studies show that Ocimum Sanctum Linn (Tulsi) possesses a special combination of antimicrobial (including anti-bacterial, antiviral, antifungal, antiprotozoal, anti-malarial, and anthelmintic properties), antidiarrheal, antioxidant, anti-cataract, anti-inflammatory, Chemopreventive, radio-protective, hepatoprotective, neuro-protective, cardio protective, anti-diabetic, and anti-pyritic. Hydrophilic and hydrophobic polymers are the materials most frequently utilized in matrix system preparation.[23,24]

Hydroxylpropylmethylcellulose (HPMC), hydroxy-propylcellulose (HPC), hydroxyethyl cellulose (HEC), xanthan gum, sodium alginate, poly (ethylene oxide), and cross linked homopolymers and copolymers of acrylic acid are examples of widely accessible hydrophilic polymers. Because the quick creation of a gelatinous layer on the tablet surface depends on tiny particle size, it is typically supplied in micronized forms.[24,25]

Acacia is a natural gum made from the dried exudates of the Acacia Senegal and Acacia seyal trees. It is often referred to as gum acacia or gum arabic. Arabinose, galactose, and rhamnose units make up the majority of this complex mixture of polysaccharides and glycoprotein. Acacia has been widely employed as a binder, stabilizer, emulsifier, and suspending agent in medicinal formulations.[26] Acacia functions as a hydrophilic matrix forming in formulations with prolonged release.[27] It swells and creates a gel layer when hydrated, which regulates the drug's diffusion and dissolving medium penetration. Long-term medication release rate regulation is aided by its superior swelling and film-forming qualities.[28] By cooperatively balancing swelling and erosion mechanisms, the combination of Acacia with other natural polymers, including Tulsi mucilage, can improve matrix strength and accomplish desired release characteristics.[21] A few partially diametrical qualities must be balanced when choosing a binder material.[29] Polyvinylpyrrolidone (PVP) k30 is a useful excipient for both traditional formulations and innovative controlled or targeted delivery systems, acting as a binder, etc., due to its inertness, non-toxicity, and biocompatibility. PVP is utilized in a range of formulations for various applications at varying molecular weights (MWs) and concentrations. Due to its low bulk density, microcrystalline cellulose is the most popular diluent.[30] A unique quality of purified alpha wood cellulose is used to make MCC. The amorphous cellulose parts are removed by strong acid hydrolysis, producing particles that resemble bundle-like, needle-

like microcrystals.[31] In order to successfully manufacture pharmaceutical solid dosage forms, lubrication is crucial, and lubricants are necessary components of a strong formulation to accomplish this.[32] Product performance is improved by lubricants such as magnesium stearate and its powder properties. Lubricants have an impact on tablet characteristics as well as the behavior of the powder mixture.[33] The pharmaceutical industry is well-known for using talc as a glidants to increase powder flowability and enhance tablet manufacturing.[34] Mg3Si4O10 (OH)2 or H2Mg3 (SiO3)435 is the chemical composition of talc, a hydrous magnesium silicate.[35] One of the most important unit activities in the manufacturing of pharmaceutical dosage forms, primarily tablets and capsules, is granulation, a method of particle enlargement by agglomeration.[36] The wet granulation method uses ethanol as a granulation fluid to help create the granules.[37]

2. MATERIALS & METHODS:

2.1 MATERIALS

Nimesulide, Microcrystalline cellulose (MCC), Magnesium stearate and talc, Polyvinylpyrrolidone K30, Ethanol was received by Sanskar college of Pharmacy & Research, Ghaziabad and all chemicals purchased from Central Drug House Pvt. Ltd. Dariyaganj, New Delhi. All other solvent and reagent are used was of analytical grade.

2.2 EXPERIMENTAL

Identification of drug

2.2.1 By UV Spectroscopy

Nimesulide was identified using the UV Spectrophotometric technique with a Shimadzu Spectrophotometer UV-1800 (Shimadzu Corp. Japan).[38] A 100 ml volumetric flasks was filled with precisely weighed 100 mg of Nimesulide. It was dissolved in enough methanol to reach a volume of 100 milliliters. After pipetting off precisely 1 ml of the stock solution, 10 ml of methanol (10µg/ml) was added to dilute it. The spectra were captured between 200 and 400 nm. Spectrum was captured.

2.2.2 Melting point determination

The melting point of drug sample was determined by using melting point apparatus.[39]

2.2.3 Preparation of standard Calibration curve of Nimesulide in methanol (λ max 300nm)

Nimesulide calibration curve was created in methanol at 300 nm. For methanol, the absorbance values (average of three measurements) and their standard deviation at various concentrations between 5 and 30 µg/ml are tabulated. In the concentration range, the medication complies with Beer's Lambert law. Nimesulide calibration curve's linear regression analysis is shown in the table 1. Therefore, the drug's solubility in various solvents, drug content, and drug release were calculated using this equation.[40,41]

2.2.4 Formulation and Evaluation of Sustained release matrix tablet

The wet granulation process was used to formulate the sustained release matrix tablet. Release rate retardant, polymer, binder, granulation solution, diluent, lubricant, and glidants are all chosen.[42] Ocimum Sanctum Linn as a release rate retardant, Acacia as a polymer, Polyvinylpyrrolidone K30 as a binder, Ethanol as a granulation solution, Micro Crystalline Cellulose as a diluent, Magnesium stearate as a lubricant, and Talc as a guide were used to create the sustained release matrix tablet. Variable medication concentrations in Ocimum Sanctum Linn (Release rate retardant) with variable binder to lubricant ratios were used to create a number of formulations. The following are included in the sustained release matrix tablet formulation.

Figure 2: Granules of Tulsi and Acacia

Nimesulide matrix tablets with four distinct natural polymer compositions As indicated in Table 1, Ocimum Sanctum Linn and acacia powders were made using wet granulation techniques. Weighing and passing through sieve no. 30-mesh were Nimesulide, natural polymers, diluents, binders, lubricants, and glidants. After mixing Nimesulide, polymers, diluents, and binders, a sufficient amount of granulating agent (ethanol) was gradually introduced to create a cohesive mass in a stainless steel container by rotating the wet material with a stainless

wire rod. Wet granules were obtained by passing the resulting wet mass through sieve number 16-messh. After 30 minutes of drying at 40 degrees Celsius, the produced wet granules were resized by passing them through a 16-mesh filter. Once more, the dried granules were passed through sieve number 16 and dried for five to ten minutes at 40°Celsius, which helped to remove any remaining moisture from the granules. Then, for each recipe, talc and magnesium stearate were added and thoroughly mixed as lubricants and glidants.

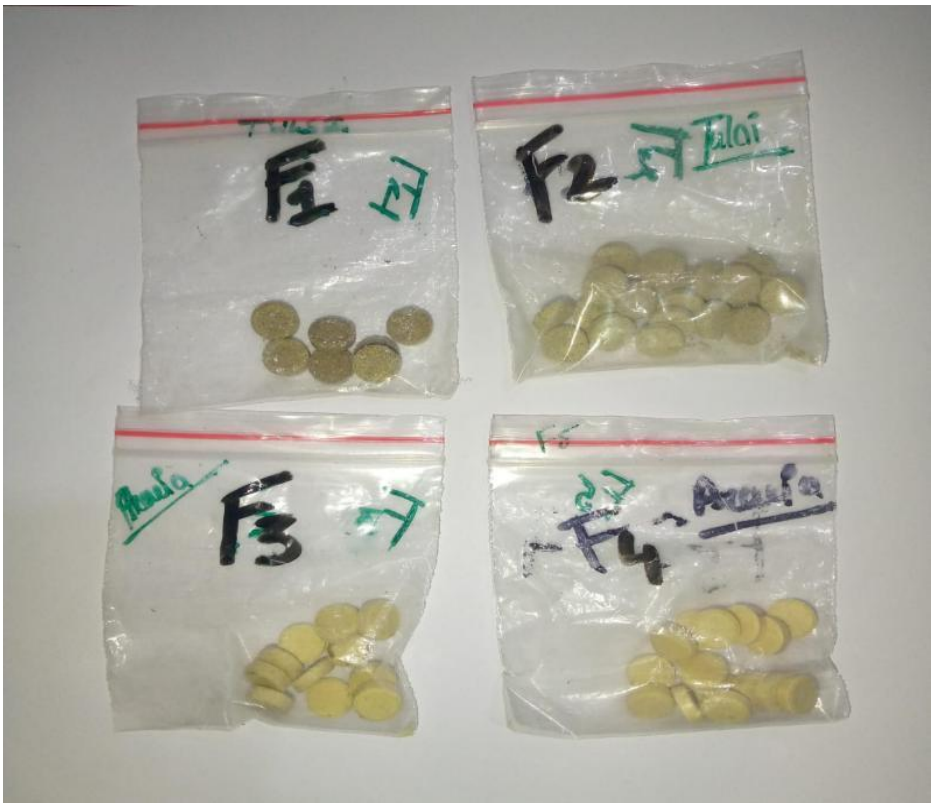

Figure 3: Preparation of sustained release matrix tablet

Table 1: Composition of Nimesulide matrix tablets

Ingredients (mg)	F1	F2	F3	F4
Nimesulide	**100**	**100**	**100**	**100**
Ocimum sanctum linn	**70**	**60**	–	–
Acacia	–	–	**70**	**60**
Microcrystalline cellulose	**15**	**25**	**15**	**25**
Polyvinyl pyrolidine K30	**10**	**10**	**10**	**10**
Ethanol	**4ml**	**4ml**	**4ml**	**4ml**
Magnesium stearate	**0.5**	**0.5**	**0.5**	**0.5**
Talc	**0.5**	**0.5**	**0.5**	**0.5**
Total	**200**	**200**	**200**	**200**

2.2.5. Evaluation of Granules

2.2.5.1. Angle of Repose

The angle of repose of granules was determined by the funnel method. The granules were allowed to flow through the funnel freely onto the surface. The diameter of the powder cone was measured and angle of repose was calculated using the following equation.[43]

$$\text{Tan } \theta = h/r$$

Where 'h' and 'r' are the height and radius of the powder cone, respectively.

2.2.5.2. Bulk Density

Tapped density (TBD) and loose bulk density (LBD) were also calculated. A measuring cylinder was filled with a calculated amount of 2 gm of powder from each

formula, and it was tapped for a certain amount of time until the volume did not change any more. The following formula was used to determine LBD and TBD.[44]

LBD= Weight of the powder/ Volume of the packing

TBD = Weight of the powder/ Tapped Volume of the packing

2.2.5.3. Compressibility (Carr's) index

The compressibility index of the granules was determined by Carr's compressibility index.[45]

Carr's index (%) = [TBD-LBD] X 100 / TBD

2.2.5.4. Tapped Density

Tapped density was determined by digital bulk density apparatus. A known amount of granules was transferred into the measuring cylinder and tapped upto 100 times and measure the tapped volume.[46] The tapped density was determined by using the formula:

Tapped Density = Weight of granules / tapped volume

2.2.5.5. Hausner's Ratio

Hausner's ratio was determined by following formula

Hausner's ratio = Tapped density / Bulk density[46]

2.2.6. Evaluation of Tablets

2.2.6.1. Thickness

Ten tablets from each batch were used to measure the thickness and diameter of the tablets using a Vernier Caliper, and average values were computed.[47]

2.2.6.2 Friability

After being weighed, ten tablets are put in the friabilator. The chamber rotates at a speed of 25 rpm for four minutes. After being taken out of the chamber, the tablets are weighed once again. Weight loss is a sign of friability. If the weight loss is less than 0.8%, the tablets are deemed to be of good quality.[48]

2.2.6.3 Hardness

The definition of tablet hardness is "the force required to break a tablet in a diametric compression test." A Monsanto hardness analyzer is used to measure the hardness of five tablets for each formulation; the tablet is broken to determine the end point.[49]

2.2.6.4 Weight variation

Twenty tablets of each formulation were chosen at random, and their average weight was calculated in order to examine the weight fluctuation. None of the individual weights should differ from the average weight by more than twice that percentage (the limit for not more than 130 to 324 mg is 7.5%). Only two of the individual weights may differ from the average weight by more than the percentage deviation.[50,51]

2.2.6.5 In Vitro Dissolution studies

Using apparatus no. 2 or paddle equipment, the dissolving investigation was carried out for every batch of commercially available conventional tablet formulation.[52] The in vitro drug release characteristics of tablet.[53] were evaluated using equipment with 900 cc of pH 7.2 phosphate buffer as the dissolution medium, kept at 37±0.5 °C, and stirred at 100 rpm. Ten milliliter aliquots were taken out at predetermined intervals, filtered, and the absorbance was measured at 300 nm using a Shimadzu Spectrophotometer UV-1800 (Shimadzu Corp. Japan).[54] every sample was gathered in a precisely calibrated 10 ml volumetric flask of A grade purity.

2.2.6.6 Drug content

The drug content of all the formulation were determined as per Indian Pharmacopoeia 1996 (i.e. 90-110% w/w).[55]

2.2.7. Permeation study by Franz diffusion cell

The transdermal transport of a product across a biological or synthetic barrier is assessed by a Franz diffusion cell permeation study.[56] To ensure equal distribution, the receptor chamber is filled with an appropriate receptor medium and kept at 32 ± 0.5°C with continuous stirring.[57] To ensure that there are no air bubbles on the receptor side, we installed a hydrated membrane between the donor and receptor compartments using an egg membrane with a diameter of 3.2 cm. Samples are taken out of the receptor chamber at prearranged intervals and replaced with new medium after a fixed volume of the test formulation is applied to the donor chamber. A proven analytical technique is used to determine the drug concentration in samples, and cumulative permeation is computed by adjusting for dilution following each sampling.[53,58]

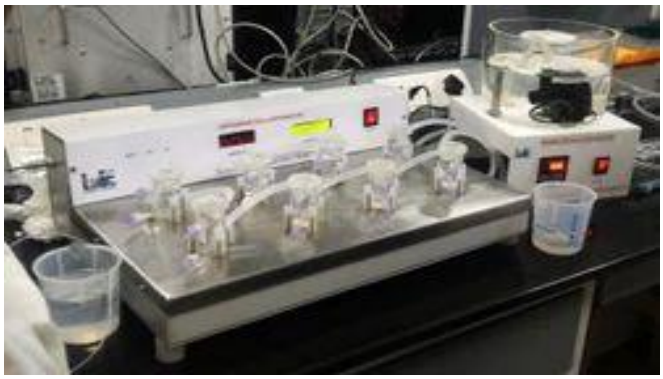

Figure 4: Franz Diffusion Cell apparatus

3. RESULT:

3.1Identification of drug

The λ max of Nimesulide was obtained at 300 nm. The UV spectrum of Nimesulide drug is shown in the fig 5.

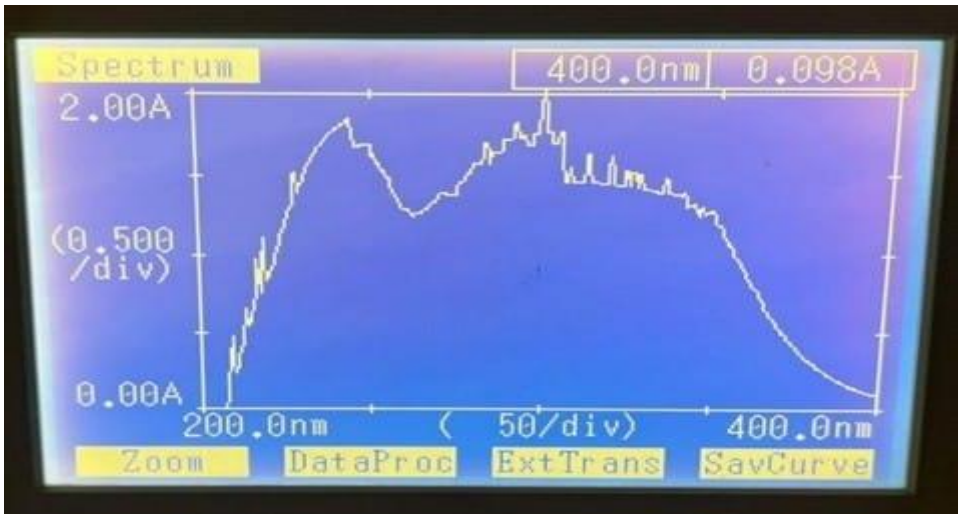

Figure 5: Spectrum of Nimesulide by UV Spectroscopy

3.2 Melting Point

The melting point was found to be in the range of 142-145 °C.

3.3 standard Calibration curve of Nimesulide in methanol

The calibration curve of Nimesulide is shown in Fig 6.

Table 2: Standard calibration curve of Nimesulide in Methanol

S. N.	Concentration (µg/ml)	Absorbance (nm)
1.	0	0
2.	5	0.561
3.	10	1.197
4.	15	1.567
5.	20	2.101
6.	25	2.674
7.	30	4.291

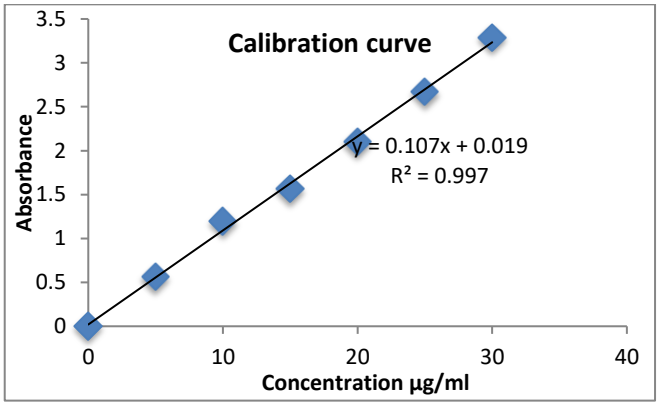

Figure 6: Calibration curve of Nimesulide in methanol

3.4Evaluation of Granules

The produced granules were suitable for additional tablet compression due to their good micromeritic properties, which included ideal flowability, bulk density, tapped density, Carr's index, and Hausner's ratio as shown in table 3.

Table 3: Determination of flow properties of granules

Formulation Code	Angle of Repose	Bulk Density	Tapped Density	Carr's Index	Hausner;sRation
F1	20.52	14.5	13.5	7.40	0.93
F2	18.43	13	12	8.33	0.92
F3	19.21	14	13	7.69	0.92
F4	18.43	12.5	11.5	8.69	0.88

[Bulked density and tapped density in (g/ml)*]

3.5 Evaluation of Tablets

Pharmacopoeial standards were confirmed by the examined tablets' acceptable physicochemical characteristics, which included uniform weight variation, sufficient hardness, thickness and low friability as shown in table 4.

Table 4: Evaluation of Prepared sustained release matrix tablets

Parameters	F1	F2	F3	F4
Thickness (mm)	0.35	0.3	0.34	0.3
Friability (%)	0.62	0.64	0.54	0.52
Hardness (kg/cm2)	4-5	4-5	8-9	8-9
Weight Variation (mg)	pass	Pass	Pass	pass

3.6 In Vitro Dissolution studies

The formulation met pharmacopeial dissolve requirements by attaining the appropriate percentage drug release within the allotted time, according to the in vitro dissolution investigations, which showed a consistent and regulated drug release profile.

Table 5: In- Vitro drug release rate of Formulations.

Time (hrs)	F1 %CDR	F2 %CDR	F3 %CDR	F4 %CDR
0	0	0	0	0
1	19.8	19.0	15.5	9.6
2	25.2	25.8	24.2	18.7
3	33.8	28.2	38.4	34.9
4	43.8	38.6	47.0	42.5
5	50.9	44.9	51.3	48.4
6	63.2	52.1	59.0	55.8
7	71.4	62.1	71.3	67.3
8	79.6	73.0	82.9	78.5
9	87.9	83.3	89.3	85.9
10	94.1	92.5	93.2	92.9

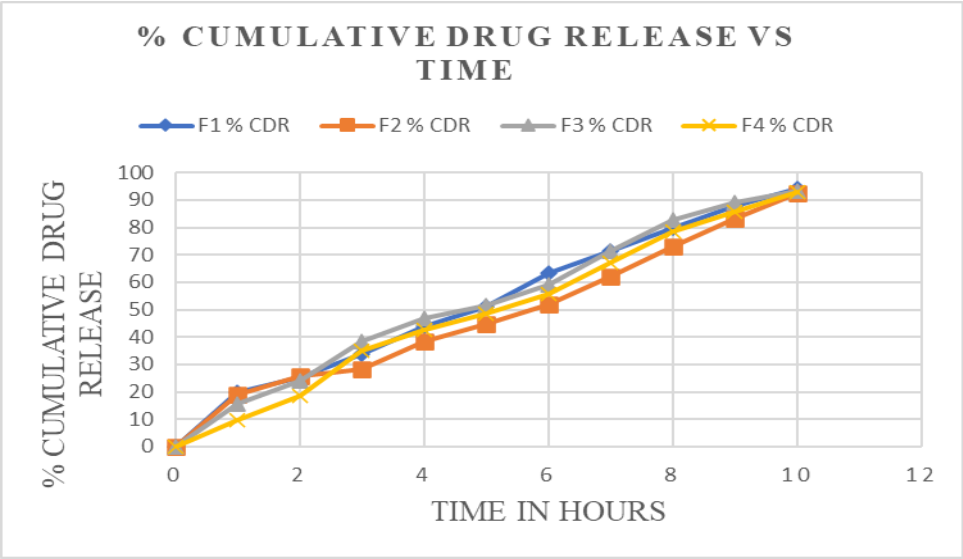

Figure 7: Percent cumulative drug release of Formulations (F1, F2, F3 & F4)

3.7 Drug content

Good content uniformity was indicated by the drug content analysis, which revealed a uniform distribution of the active pharmaceutical ingredient within the formulation with values falling within the permissible pharmacopeial limits.

Table 6: Drug content of various formulations

S. No	Formulations	% Drug content
1	F1	98.54
2	F2	96.76
3	F3	97.82
4	F4	95.91

3.8 Permeation study by Franz diffusion cell

Consistent and notable drug permeation across the membrane was shown in the Franz diffusion cell permeation investigation, showing efficient drug release and advantageous permeability properties of the formulation.

Table 7: Cumulative permeation table

Time (hrs)	cumulative amt.(µg/cm^2)	% Release (of 100,000 µg)	SD (± µg)
0	0	0 %	0
0.5	4,000	4.0 %	±120
1	8,500	8.5 %	±150
2	18,000	18.0 %	±260
3	28,500	28.5 %	±320
4	40,000	40.0 %	±410
5	52,000	52.0 %	±520
6	62,000	62.0 %	±590
7	69,000	69.0 %	±630
8	75,000	75.0 %	±700

4. DISCUSSION:

The current study highlights the potential of natural polymers *Ocimum sanctum* (Tulsi) and Acacia in controlled drug delivery systems by focusing on the formulation and comparative assessment of sustained release matrix tablets of Nimesulide.[20,26,27] Granules with adequate micromeritic qualities, such as good flowability, ideal bulk and tapped density, low compressibility index, and acceptable Hausner's ratio, were evaluated prior to compression.[43-46] These features guaranteed regular die filling and effective tablet compression, both of which are necessary for consistently high-quality tablet production.[36,37] All created formulations met pharmacopeial requirements, according to post-compression examination.[47-51] The tablets demonstrated good mechanical strength and integrity, with uniform weight fluctuation, sufficient hardness, low friability, and constant thickness.[47-50] These findings imply that the wet granulation technique and the chosen excipients were suitable for the creation of sustained release matrix tablets.[36,42]

For every formulation, the *in vitro* dissolution experiments showed a regulated and extended drug release profile over a ten-hour period.[52-54] Formulations with higher natural polymer concentrations were found to exhibit longer-lasting medication release.[42,44] This is explained by the hydrophilic properties of Acacia and *Ocimum sanctum*, which swell when hydrated to create a gel-like barrier around the tablet matrix.[21,26,28] This barrier controls the release rate by regulating matrix degradation and drug diffusion.[28] F1 and F3 showed relatively superior sustained release behavior among the formulations, demonstrating the useful impact that polymer composition and concentration play in modifying drug release kinetics.[42,44] Nimesulide was uniformly distributed throughout the matrix, as confirmed by the drug content analysis, which revealed that all formulations contained drug within acceptable pharmacopeial limits (95–99%).[55] This guarantees the formulation's dependability and dosage precision. Additionally, the Franz diffusion cell permeation research showed consistent and substantial drug penetration through the membrane, suggesting effective drug release and advantageous permeability properties.[56-58]

The sustained release matrix tablets showed better potential for delivering long-lasting and reliable therapeutic benefits when compared to the commercial Diclofenac ge.[56,57] Gels and other topical formulations are helpful for localized activity, but they might not be able to sustain systemic medication levels.[57] Oral sustained release tablets, on the other hand, provide regulated medication distribution over a prolonged duration, lowering the frequency of administration and enhancing patient compliance.[6,14,15] Overall, the results of this study support the successful use of natural polymers like Acacia and *Ocimum sanctum* in the creation of sustained release matrix tablets. These polymers offer benefits like biocompatibility, biodegradability, and cost-effectiveness in addition to being an efficient way to regulate drug release.[20,21,26] In the long-term treatment of inflammatory diseases like arthritis, the proposed formulations may improve patient adherence, reduce side effects, and increase therapeutic efficacy.[6,14,15]

CONCLUSION

Using the natural polymers Ocimum sanctum and Acacia, the current work effectively demonstrated the formulation and assessment of Nimesulide sustained release matrix tablets. The developed formulations demonstrated regulated drug release over a 10-hour period and good pre- and post-compression properties. The findings verified that drug release behavior is strongly influenced by polymer type and concentration.

The new tablets showed greater potential for a long-lasting therapeutic effect than the commercial Diclofenac gel. All things considered, natural polymers were shown to be efficient, affordable, and biocompatible excipients for sustained release systems, improving patient compliance, lowering the frequency of doses, and providing a viable strategy for the long-term treatment of inflammatory diseases.

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Data Availability: The data generated during this study are available from the corresponding author upon reasonable request.

Author Contributions: Madhur Yadav contributed to the experimental work, formulation development, data collection, and initial drafting of the manuscript. Deepak Jaiswal assisted in performing evaluation studies, data analysis, and interpretation of results. Babita Kumar supported literature review, data compilation, and manuscript editing. Dr. Swati Mittal conceptualized and supervised the study, guided the research methodology, and critically reviewed and finalized the manuscript. All authors have read and approved the final version of the manuscript.

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