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Withania somnifera nanoparticulate drug delivery system for rheumatoid arthritis: design, development, characterization, and preclinical investigation

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Abstract

The study's objective is to evaluate the improved therapeutic efficacy and decreased toxicity of *Withania somnifera* nanoparticulate drug administration for rheumatoid arthritis. The active chemical components of *Withania somnifera* were initially separated via extraction and column chromatography.The acute toxicity study was performed, and it suggested that the prepared nanoparticles had less toxicity. According to the stability study, they had a 3-month shelf life and a 90% drug entrapment rate.After 60 minutes, all 12 batches of Tablet formulations released more than 70% of the medication they contained, while five batches released more than 90%.According to the accelerated stability study, the initial drug release from the best batch was 92.5%, and six months later, it was 91.1%. The result of preclinical research revealed that prepared nanoparticulate formulations responded better than standard and control formulations. According to anti-rheumatoid arthritis activity tests, the produced formulation had improved therapeutic efficacy and decreased toxicity. Patients with rheumatoid arthritis can benefit more from this formulation.

Keywords: Rheumatoid arthritis, *Withania somnifera* nanoparticles, drug delivery, preclinical study, drug development.

Full length article **Corresponding Author*, e-mail:chetana_frs@yahoo.com

1. Introduction

A series of chronic illnesses known as arthritis are characterized by joint inflammation and stiffness. It can result in discomfort, edema, limited movement, and a lower quality of life and affects millions of individuals worldwide. There are various kinds of arthritis, including psoriatic, rheumatoid, gouty, and osteoarthritis (the most prevalent variety)[1][2][3]. There are some medications available to treat this medical condition, however many of them have toxicity, drug delivery, and pharmacokinetic problems[4][5]. In other hand a dose-dependent method of incorporating bioactive chemical components demonstrated remarkably high efficacy for the treatment of various illnesses[6].It was shown that several phytoconstituents have the capacity to target a variety of inflammatory mediators, including those that are actively implicated in the pathogenesis of rheumatoid arthritis, such as nitric oxide (NO), cytokines, chemokines, adhesion molecules, NF-k, lipoxygenase (LOXs), and arachidonic acid (AA)[7].Different bioactive components found in *Withania somnifera* root may be crucial for tracking rheumatoid arthritis. Every disorder must be treated with an efficient drug delivery system and with an effective drug treatment plan. These kinds of difficulties are easily overcome by making herbal nanoparticulate medication delivery[8][9]. Numerous benefits of using nanoparticulate drug delivery were demonstrated, including improved drug targeting, sustained drug release, drug protection and stability, greater drug solubility, decreased systemic toxicity, etc[10]. The study of phenomena and the manipulation of materials at the atomic, molecular, and macromolecular scales where their properties are very different from those of larger scales is known as nanoscience [11,12,13]. The design, characterization, manufacture, and use of structures, devices, and systems using nanoscale shape and size-control constitutes nanotechnology. The physical, chemical, and biological characteristics of materials at the nanoscale are fundamentally and significantly different from those of discrete atoms and molecules or bulk matter [14,15]. Research and development in nanotechnology is focused on comprehending these novel features and developing better materials, tools, and systems to take advantage of them. Because of its distinct size (1-100 nm) and high surface-to-volume ratios, nanotechnology has the potential to provide solutions to the existing challenges in cancer therapy. Because of the makeup of their materials, nanotechnologies have characteristics such as self-assembly, stability,

specificity,drug encapsulation, and biocompatibility [16,17,18]. The use of nanotechnology in cancer prevention, detection, diagnosis, imaging, and treatment has significant potential [19,20]. Nanotechnology involves viewing, measuring, modeling, and manipulating materials at this scale and encompasses nanoscale science, engineering, and technology. Aerospace, agriculture, biotechnology, homeland security and national defense, energy, environmental improvement, information technology, medicine, and transportation are just a few of the industries and technology sectors that nanotechnology has the potential to transform and revolutionize[21][22][23]. It is now possible to spot applications that will have an influence on the world we live in thanks to advances in some of these fields of discovery(**Fig. 1**).In this investigation, we used column chromatography, TLC detection, and standard extraction methods to identify and separate certain active chemical compounds found in *Withania somnifera* roots. The produced extract-loaded chitosan nanoparticles were then used to make tablets. The manufactured tablets were assessed using several assessment criteria before being consumed by rats to determine their anti-arthritis activity.

2. Materials and methods

2.1. Collection, authentication and drying of plant material

The *Withania somnifera* roots were taken from the district of Pune (Maharashtra). The plant was authenticated by D. L. Shirodkar, Botanist, Botanical Survey of India, Pune by comparing morphological features. The herbarium of the plant specimen was deposited at Botanical Survey of India, Pune; with the Voucher specimen number CDP-01 (Ref. No. BSI/WRC/IDEN.CER. /2021/H3 Dated 04/06/2021). The plant material was dried in the shade at room temperatureground into grinder and powder material was passed into 120 mesh size.

2.2 Chemicals, reagents & solvents

Petroleum ether, Ethanol, Methanol, Dichloromethane, n-hexane, Ethyl acetate, Glacial acetic acid, N-Butanol, Chloroform, Acetone, Formic acid, Benzene, Dimethyl sulfoxide (DMSO), conc. Sulphuric acid, Hydrochloric acid, Benzene, pyridine, toluene, anisaldehyde, calcium chloride, copper sulphate, Ferric chloride, Follin's reagent, Iodine, Lead acetate, Magnesium chloride, Mercuric chloride, Ninhydrin, Nitric acid, Phloroglucinol, Potassium iodide, Potassium Dichromate, Potassium sodium Tartarate, Ruthenium red, Sodium acetate, Sodium iodide, Sodium hydroxide, Sodium nitroprusside, Hide powder, Folin Ciocalteu reagent, Sodium bicarbonate, Gallic acid and all the chemicals and reagents are analytical grade (Research lab Fine Chemicals Pvt. Ltd Mumbai, SD Fine Chem Mumbai, and Merck, India) were purchased from local suppliers.

2.3Standardization of plant material

Pharmaceutical evaluation is crucial for standardizing plant medicines. The Indian Pharmacopoeia 2014 and other standard reference books were used to standardize the *withania somnifera* roots. The standardization of herbal products is done in accordance with WHO guidelines and involves measuring the importance of the ash, the extractive value, and the amount of drying that is lacking. It is highly helpful to understand the morphology, microscopy, and physical characteristics of herbal remedies. Experiments in pharmaceuticals provide important research insights on the purity, consistency, and identification of plant drugs[24,25,26,27].The following study was performed to standardize the *Withania somnifera* roots: A) Pharmacognostic Study, B) Physical Evaluation. The physical evaluation contains Determination of foreign organic matter, moisture content, Ash value, Total ash,Water-soluble ash,and Acid -insoluble ash. Finally, the study contains evaluation of extractive values like water-soluble and Alcohol-soluble extractive value.

2.3.1 Extraction

The targeted steroidal and alkaloid components of *Withania somnifera* were extracted utilizing the continuous Soxhlet extraction method with hydroalcoholic solvent. 150 grams of the crude drug were extracted in a single stage of reflux using ethanol and water in a 70:30 v/v ratio at 60 degrees Celsius. The extract from the two batches was then filtered through a cotton cloth to remove the marc. To obtain the constituent extract, the filtrate was heated to a temperature of 45°C and dried out there. In accordance with the methods described above, an extract was evaluated for preliminary phytochemical screening and thin-layer chromatography for the identification of separated components.

2.3.1.1 Thin layer chromatography of extract

Following a preliminary phytochemical analysis of the extract, thin layer chromatography was used to further assess it. **Table1** lists the various solvent systems that were employed for the identification of chemical constituents and their detection.

2.3.1.2 Column chromatography of Withania somnifera root extract

Additionally, column chromatography was used to separate the desired chemical components of the *Withania somnifera* root extract. The chromatographic column in question had a diameter of 3 cm and a height of 40 cm. The stationary phase utilized was Silica Gel 60 (Mesh 230-400), while the solvent for packing the columns used was n-Hexane. Gradient solvent systems range from non-polar to highly polar solvents. Different ratios of n-hexane, ethyl acetate, methanol, and water were utilized. Each portion had a 20 ml volume.

2.3.1.3 Acute toxicity study

Guidelines for oral acute toxicity research are governed by the Organization for Economic Co-operation and Development (OECD). Nine adult albino rats were separated into three groups of three for the acute toxicity study. For the evening, all the animals were fasting. Separately diluted in 1% CMC, the separated extracts of two different plants were administered orally at doses of 300, 1000, and 2000 mg/kg body weight, respectively. The animals were watched for any symptoms of mortality for two hours, and then again for four hours. The animals were kept under close observation for a further 14 days after the initial 72 hours of monitoring for gross behavior, pupil size, general motor activity, convulsion, water intake, feces output, writhing, and any other hazardous indications.

2.4 Preparation of Withania sominifera extract loaded chitosan nanoparticles

Sodium tripolyphosphate (TPP) was used as a cross linker with a small modification to create the chitosan nanoparticles in accordance with the ionic gelation procedure. Chitosan was fully dissolved in acetic acid prior to the addition of the *Withania sominifera* extract (5%) and magnetic stirring of the mixture. TPP (0.5%) was then gradually administered via syringe at a consistent pace. In this procedure, glacial acetic acid (1.6%) was dissolved in distilled water, and chitosan (0.5–1) was added as a concentration. After 2 hours of additional stirring, it was centrifuged for 10 minutes at 10,000 rpm. The remainder was re-dissolved in phosphate buffer saline (PBS) after supernatant was discarded. They gathered the nanoparticle. Before being used, the prepared nanoparticle was lyophilized and kept at 40°C.Particle size, polydispersity index, UV-visible spectroscopy, X-ray powder diffraction (XRD), Zeta potential, % Yield of nanoparticles, Drug entrapment efficiency, In-vitro drug release, and stability were studied to characterize the produced nanoparticles.

2.5 Herbal tablet formulation

2.5.1.Pre-compression study

Pre-compression testing was done on the powder before it was compressed into tablet form. Pre-compression parameters attest to the final dosage form's high quality. For the analysis of the desired powder's quality, the ensuing parameters were put to the test.

2.5.1.1. Angle of Repose

The angle of repose can be used to calculate the frictional forces present in loose powder or granules. This is the greatest angle that can be formed between a pile of powder or grains' surface and the horizontal plane.

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

θ = tan-1 (h/r) Formula 1

Where, θ is the angle of repose, h is the height, r is the radius.

2.5.1.2. Bulk density (BD)

The ratio of a powder's mass to its bulk volume is known as bulk density. The distribution of particle size, shape, and the propensity of the particles to stick to one another are the main determinants of a powder's bulk density.

Bulk density = weight of powder / Bulk volume.

Db = M/V_0

M = mass of the powder; V_0 = bulk volume of the powder.

2.5.1.3. Tapped density (TD)

It is the proportion of the powder's total mass to its tapped volume.

Tapped density = Weigh of powder / Tapped volume

Dt = (*M*) / (Vt*)*

M = mass of the powder; Vt = tapped volume of the powder.

Carr's Index

Evaluation of a powder's BD, TD, and rate of packing down is a straightforward test. The following is the formula for Carr's index:

$$\text{Compressibility index} = 100 \times \frac{\text{density Tapped} - \text{Bulk denisty}}{\text{density Tapped}}$$

2.5.1.4. Hausner's ratio

The Hausner's ratio is a proximate indicator of powder flow simplicity. The formula used to calculate it is as follows.

Hausner's ratio = tapped density / bulk density.

2.5.2. Tablet Evaluation

For the manufacture of compressible tablets, previously prepared *Withania sominifera* nanoparticles were lyophilized. The 200 mg *Withania sominifera* nanoparticles with varying composition ratios of HPMC K4M, HPMC K15M, MCC PH102, magnesium state, and Talc were used to make the 300 mg tablet utilizing the direct compression method. Total F1 to F12 (12 batches) were created utilizing various **Table2** compositions.

2.5.3. Post compression study

Compressible *Withania somnifera* tablets(batches F1–F12) are analyzed for post–compression characteristics after production in order to assess tablet quality. The post-compression evaluation research was conducted using the aforementioned criteria.

2.5.3.1. General appearance

The formed tabletsgeneral appearance was evaluated, and observations of shape, color, and texture were made.

2.5.3.2. Weight Variation test

For more than 324 mg of tablets, no more than two of the individual weights departed from the average weight by more than 5.0%.

Average weight = weight of 20 tablets/20

$$\text{\% Weight variation} = \frac{\text{Average waight - Weight of each tablet}}{\text{Average weight}} \text{ x 100}$$

Formula 3

2.5.3.3. Thickness

Thickness of the tablets(n=3) was determined using a Vernier Caliper.

2.5.3.4. Hardness test

Using the Monsanto hardness tester (n=3), the lower plunger was brought into contact with the tablet, and a reading of zero was taken to determine the tablets hardness. The tablet eventually broke when the plunger was turned by a threaded bolt against a spring. A pointer rode along a gauge in the barrel to show the force when the spring was squeezed.

2.5.3.5. Friability test

This test is run to see whether tabletscan tolerate abrasion during handling, packing, and transportation. It should ideally range from 0.5 to 1.0%.

%Friability = [(W1-W2)/W1] × 100

Where, W1= weight of tabletsbefore test, W2 = weight of tabletsafter test

2.5.3.6. Assay of Tablet (Drug content)

First 20 tablets, weighted and finely powdered. The powder was then properly measured and transferred into a 100ml volumetric flask, equaling around 10mg of an herbal tablet. After that, a small amount of methanol was added, and the solution was sonicated for 30 minutes. Then, using methanol, the volume was adjusted to the mark. The clear solution (100ppm Stock solution) was obtained by filtering the solution via Whatmann filter paper. To make 8ppm, 10ppm, and 12ppm solutions, 0.8ml, 1.0ml, and 1.2ml of the stock solution (100ppm solution) were withheld. Utilizing methanol as a blank, the absorbance was determined spectrophotometrically by sweeping wavelengths between 400nm and 200nm. To help with the analysis, the absorbance was noted.

2.5.3.7. In vitro Dissolution Study

According to USP, dissolving research was carried out on the prepared batches F1–F12. The USP dissolving Device II was used to determine the dissolving profile (F1-F12) in 900 ml of simulated fluid (7.4 pH Phosphate buffer) at a stirring speed of 50 rpm. At 0.5, 1, 2, 6, 8 and 12 hours, various aliquot samples were obtained with simulated substitute fluid in the same amount. A UV visible spectrophotometer was used to measure absorbance in order to determine how much medicine was discharged.

2.5.3.8. In-vivo Anti-arthritis Study

In this investigation, Wistar albino rats were utilized, and they were purchased from Crystal Biological Solution in Pune, India. When the rats were treated at the age of seven weeks, their weight variance did not surpass 20% of the mean body weight for each sex. Rats were given the prescribed dosage orally.The Akindele and Adeyemi approach was initially used to create the arthritis syndrome, but with a few minor alterations. On the first and third days of the experiment, Wistar albino rats were subcutaneously injected with 0.1 ml (2.5% v/v in normal saline) formaldehyde solution in the subplantar region of the right hind paw.

Therats weredividedintofivegroups(n=5in each group)asfollows:

Group I: Normal

GroupII:controlwhichreceivedaqueoussolution.

GroupIII:received10mg/kgDiclofenacsodium(PO)

GroupIV:receivedCMN(PO)

GroupV:receivedWSN(PO)

All groups received oral medication every day for 10 days, starting an hour before formaldehyde injection. Rats' paw thicknesses were measured using a digital caliper on days 0, 2, 4, 6, and 8. Last but not least, radiograph recording and estimate of hemoglobin, C-reactive protein, and rheumatoid factor were carried out.

3 Result and discussion

3.1 Standardization of Plant Material

3.1.1. Pharmacognostic Study and Physical characteristics

Selected *Withania somnifera* plant parts underwent examination for their organoleptic characteristics, additional characteristics, and macroscopical details. (C. K. Kokate's Practical Book) *Withania somnifera* is an evergreen shrub that can reach heights of 45 to 95 cm. All branches radiate forth from a single stem. The leaves are oval, dull green, and often 10–12 cm (3.9–4.7 in) long. Small, bell-shaped, and green, the flowers are. The mature roots are smooth and branching, and the ripe fruit is an orange-red color. Collection and drying of roots measuring 4 to 8 cm for further analysis (**Fig.2**).The TS of *Withania somnifera* thick root was treated with Phluroglucinol and hydrochloric acid (1:1) to stain and analyze under microscope. The microscopic study of root showed: 1) Cork cell, 2) Cortex, 3) Medullary rays, 4) Vessels, and 5) Phloem fibers (**Fig.3**).

3.1.2. Physicochemical Evaluation

The foreign organic matter, moisture content, total ash value, water-soluble ash value, acid-insoluble ash value, water-soluble extractive value, and alcohol-soluble extractive value of *Withania somnifera* roots were all evaluated. The results are shown as mean SEM in **Table2** and are based on the observations.The amount of foreign stuff was found to be extremely minimal in the current study. For the purpose of obtaining desired quality phytocomponents, the moisture content present in crude medicine must be consistent and within the established range because it might alter microbial growth, enzyme activity, and the quality of plant material. The plant material's moisture content was fixed after the removal of the watery quantity shown in **Table2**.The selected plant portions' moisture content was below the permissible level of 5%, which would prevent the growth of bacteria and fungi.When assessing the quality and purity of pharmaceuticals, such as the presence or absence of foreign inorganic materials like silica, ash value is very crucial. The weight of ash in some medications varies very little, in terms of percentage, from sample to sample; any significant variation denotes a change in quality. The metrics that can be used to validate and standardize the characteristics of *Withania somnifera* roots are their water solubility, acid-insolubility, and sulphated ash value. The chemical components of powdered medications are evaluated using their extractive values, which also aids in determining which chemical components are soluble in each solvent. More components are found to be soluble in alcohol when the alcohol soluble extractive value is greater than the water-soluble extractive value.

3.2 Extraction

Withania somnifera roots were extracted using a hydro-alcoholic solvent, and the extracts contained 20.77% of the required components by weight (**Table 3**). Therefore, we can draw the conclusion that the majority of phytocomponents may exhibit improved solubility in pertinently chosen solvents. Purification of isolated chemicals necessitates further separation.

3.2.1. Preliminary Phytochemical Screening of Extract

Qualitative analysis of *Withania somnifera* roots extract was performed to identify different phytoconstituents by using different qualitative tests and the results are depicted

in **Table4**.*Withania somnifera* hydroalcoholic extracts revealed the presence of steroids, flavonoids, tannins, glycosides, alkaloids, amino acids, and proteins. To further identify and separate out the steroidal and alkaloid components, a TLC and column chromatography analysis is required.

3.2.2. Thin layer chromatography

After extracts underwent a preliminary phytochemical examination, thin layer chromatography was used to record additional findings. The various extract elements were identified using various mobile phase percentages. **Table5** provided the details.

3.2.3 Fractionation of withania somnifera roots hydroalcoholic extract by column chromatography

After column chromatography was used to separate the various chemical components of *Withania somnifera* roots that had been identified by TLC. Most non-polar n-Hexane solvent was used to start the constituent separation process, and water was used to finish it. Ethyl acetate and methanol were used for the separation between them. Ten fractions in all, each with a capacity of 100 ml, were collected. All fractions were concentrated, and their steroid and alkaloid content were assessed using thin layer chromatography. **Table6** lists the various mobile phases, fraction codes, colors, and yield percentages for each fraction.

3.3 Characterizations of Nanoparticles

The prepared nanoparticles of *Withania somnifera* extract *(fraction 6)* were subjected for different evaluations parameters.

3.3.1 Particle size determination by Zeta sizer

The size of the nanoparticles created from *Withania somnifera* extract was measured. The size of the nanoparticles' particles was estimated to be between 1 and 100 nm. The tested nanoparticles' average particle size was discovered to be 42.5 nm. These nanoparticles' zeta potential was discovered to be -29.12 mV (**Table7**). The produced nanoparticles appear to be stable, according to this. **Fig.4** and **5** displayed the peak of the zeta particle size distribution and the particle density index.

3.3.2. Scanning electron microscopy

In order to analyze the surface morphology of silver nanoparticles, scanning electron microscopy was employed. The investigation helped the researchers better understand the morphological characteristics of the nanoparticle. There were several roughly spherical nanoparticles present, and they will tear apart from one another. A tiny, spherical nanoparticle with a small size can be seen in the SEM image of a freeze-dried silver nanoparticle with a longer cross-linking duration.Almost all the nanoparticles were sphere-shaped. *Withania somnifera* extract produced nanoparticles with average diameters of 47.2 nm. After being freeze-dried, the nanoparticle dispersion formed sponge-like structures. The morphology of the sponge was ascertained by SEM (**Fig.6**.).

3.3.3. Production yield of nanoparticles

The Production yield of prepared nanoparticles was calculated, and it was found 69.10 %.

3.3.4. In-vitro release study

Drug release from Withaniasomnifera extract-loaded nanoparticles was investigated in vitro. The maximum drug release was determined to be between 90 and 95 percent for all nanoparticles. It was investigated how generated nanoparticles released in vitro at 37 0C in phosphate buffered saline (PBS) (PH 7.4). By measuring absorbance at 440 nm with a UV-visible spectrophotometer, the amount of medicine released was calculated. From the initial 0 to 60 min, the drug release was examined at various time intervals (**Table9**). At the 60th minute, a drug release of 94.76% was discovered. It implies that the formulation's oral in vitro drug release profile is favourable. **Fig. 7** displayed the cumulative medication release as a percentage.

3.3.5. Drug entrapment efficiency

For formulation, the *Withania somnifera* extract-loaded nanoparticles entrapment efficiency was found to be between 80% and 90%, showing higher drug entrapment efficiency (**Table10**).

3.3.6. Transmission electron microscopy

Fig.8displayed a TEM micrograph of nanoparticles. According to the results of the TEM study, the average particle size was 45 nanometers, falling between the ranges of 30 and 60 nanometers (**Fig.8**). The particles are round in shape. To confirm that no further types of metal oxide were present, the same sample was also examined using electron diffraction. Overall, the findings imply that the nanoparticles possess desirable qualities.

3.3.7. Stability of Nanoparticles

By examining the silver nanoparticles' absorption spectra after 12 weeks, the stability of the particles was assessed. The nanoparticles did not agglomerate and saw no significant changes during storage, suggesting that they were more stable.For prepared nanoparticles, the pattern of change in entrapment effectiveness, particle size, and zeta potential was the same. After three months of storage at 4°C, there was a modest (1%) increase in the size of the nanoparticles. Zeta potential was found to have decreased by 4%, whereas the entrapment efficiency of nanoparticles decreased by roughly 1% to 2%. The storage-related modifications that were noticed are insignificant (**Table11**). The overall findings imply that the required nanoparticles' stability was adequate.The prepared lyophilized nanoparticle powder blends and herbal formulation (F1-F12) taken to formulate the Tablet and to carry out the pre formulation study.

3.4 Pre-formulation study

3.4.1. Organoleptic studies

Powder of blend was found to be off-white.

3.4.2. Precompression parameters

The details of different Pre compression evaluation parameters studied were reported in **Table12**.

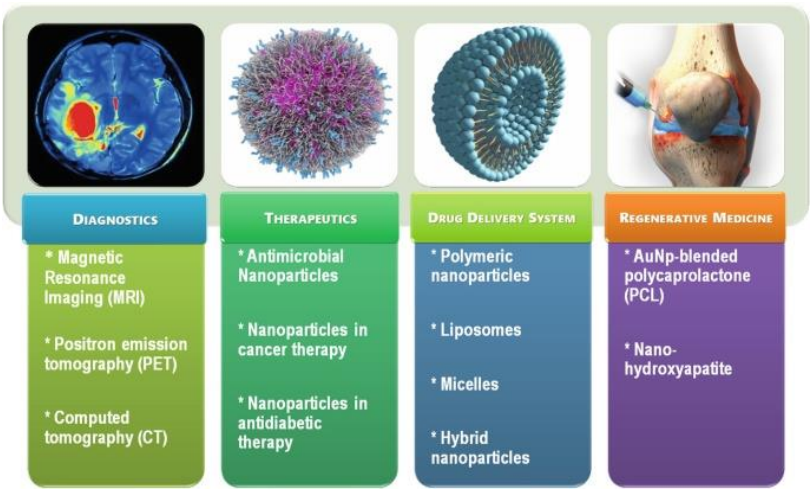

Figure 1: Current advances in Nanotechnology

Table 1: Chemical constituents, solvent system, and their detection by TLC.

Sr. no.	Chemical constituent	Mobile Phase	Detection
1.	Alkaloids	n- butanol: Ethyl acetate: Formic acid: Water (30:50:10:10) Toluene: Ethyl acetate: Formic acid (50:40:10)	UV -365nm
2.	Glycoside	Ethyl acetate:Methanol: Water (100:16.5:13.5)	UV -365nm
3.	Flavonoid	Toluene: Ethyl acetate: Glacial acetic acid: Water (100:11:11:26)	Anisaldehyde – Sulfuric acid. UV -365nm
4.	Steroids	Toluene: Ethyl acetate (9: 1) Ethyl acetate:Methanol: Water (70:20: 10)	Vanillin – Sulfuric acid. Anisaldehyde-Sulphuric acid reagent
5.	Terpenoids and Carotenoids	Cyclohexane: Ethyl acetate (75: 25)	UV- 268nm
		Petroleum ether: Benzene (9: 1)	UV- 254nm

Table2: Batch F1 to F12 with different composition of tablet.

Ingredients / Batch	Withaniasomnifera (mg)	HPMC K4M (mg)	HPMC K15M (mg)	MCC PH102 (mg)	Magnesium stearate (mg)	Talc (mg)
F1	200	10	-	77	5	8
F2	200	20	-	67	5	8
F3	200	30	-	57	5	8
F4	200	-	10	77	5	8
F5	200	-	20	67	5	8
F6	200	-	30	57	5	8
F7	200	40	-	47	5	8
F8	200	50	-	37	5	8
F9	200	60	-	27	5	8
F10	200	-	40	47	5	8
F11	200	-	50	37	5	8
F12	200	-	60	27	5	8

#Total weight of compressed tablet- ±300mg

Figure 2: *Withania somnifera* plant and roots

Figure 3: Transverse section of *Withania somnifera* thick root

Table 2: Physicochemical analysis observations.

Evaluation parameters	**Observations**
Foreign organic matter	0.4±0.03
Moisture content	4.15 ±0.64
Ash values	
Total ash value	11.71±0.51
Water-soluble ash value	1.01± 0.45
Acid-insoluble ash value	2.48±0.36
Extractive values	
Water soluble extractive value	4.99±0.55
Alcohol soluble extractive value	20.87± 0.48
Values are mean ± SEM, (n=3)	

Table 3: Characteristics of *Withania somnifera roots* extract.

Sr. No.	Extraction solvent used	Label	Percent Yield (% W/W)	Colour
1.	Hydroalcoholic extract	WSE	20.77%	Brown

Table 4: Different qualitative tests performed to identify phytoconstituents in extract.

Sr. No.	Tests	WSE	Sr. No.	Tests	WSE
1	**Test for carbohydrate**		5	**Test for Alkaloids**	
	Molish's test	-		Dragondorff's test	+
	Benidicts test	+		Mayer's test	+
	Fehling test	+		Hager's test	-
	Barfoed test	-		Wagner's test	+
2	**Test for Proteins**		6	**Test for Glycosides**	
	Biuret Test	+		Anthraquinone glycoside test	+
	Millions Test	-		Cardiac glycoside test	-
3	**Test for amino acids**		**7**	**Test for Saponin**	
	Ninhydrine test	+		Foam test	+
4	**Test for Steroids**		8	**Test for Flavonoids**	
	Salkowski test	+		Shinoda test	+
	Libermann test	+		Lead acetate test	+
	Libermann-Burchard reaction	-		Sodium hydroxide test	-
9	**Test for tannins and phenolics**				
	Test for Tannins and Phenolic				+
	Test for Tannins and Phenolic				-

Table 5: TLC-Characterization used mobile phase and observations.

Sr. No.	Chemical Constituent	Mobile Phase	Rf Valve
1	Alkaloids	Toluene: Ethyl acetate: Formic acid (50:40:10)	0.45
2	Glycoside	Ethyl acetate: Methanol: Water(100:16.5: 13.5)	0.60
3	Flavonoid	Toluene: Ethyl acetate: Glacial acetic acid:Water (100:11:11:26)	0.42
4	Steroids	Ethyl acetate: Methanol: Water (70:20: 10)	0.55
5	Terpenoids and Carotenoids	Petroleum ether: Benzene (9: 1)	0.55

Table 6: Separation of chemical constituents of *Withania somnifera* roots extract with different solvent system.

Solvent used	Ratio	Fraction Code	Colour	% Yield
Hexane	100%	A1	Dark brown	1.4
Hexane: Ethyl acetate	7:3	A2	Brown	1.6
Hexane: Ethyl acetate	5:5	A3	Brown	1.6
Hexane: Ethyl acetate	2:8	A4	Brown	1.7
Ethyl acetate	100%	A5	Brown	2
Ethyl acetate: Methanol	7:3	A6	Yellowish-brown	2.1
Ethyl acetate: Methanol	5:5	A7	Yellowish-brown	2
Methanol	100%	A8	Yellow	1.7
Methanol: Water	5:5	A9	Yellow	0.7
Water	100%	A10	Faint yellow	0.5

Table 7: Particle size and zeta potential of extract of *Withania somnifera*.

Sr. No	Sample	Nanoparticle Size (nm)	Zeta Potential (mV)
1	*Withania somnifera* nanoparticles	42.5 ± 12	-29.12

Values are shown as the mean ± standard deviation; n=5.

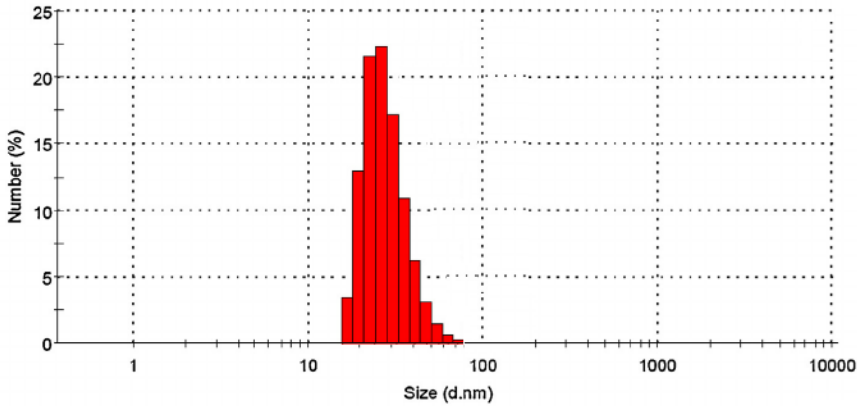

Figure 4: Results of Particle Density Index of extracts derived extract of *Withania somnifera* loaded nanoparticles.

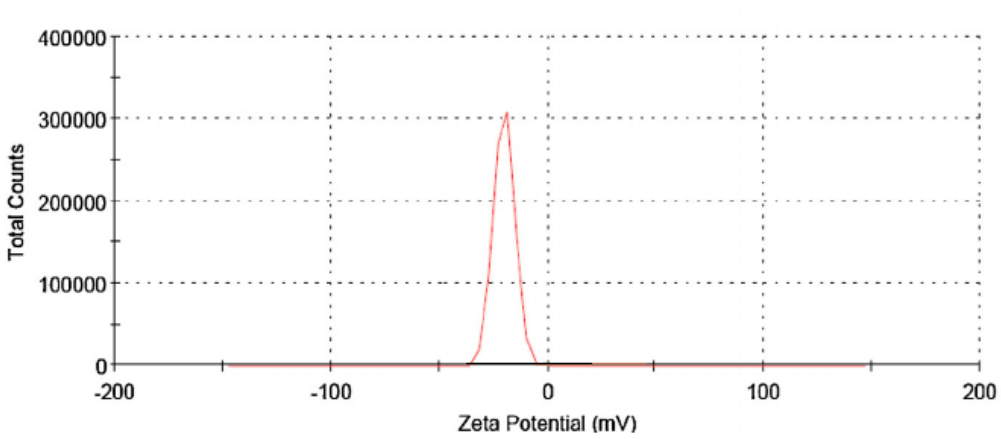

Figure 5: Zeta particle size distribution peak of nanoparticles of extract of *Withania somnifera*

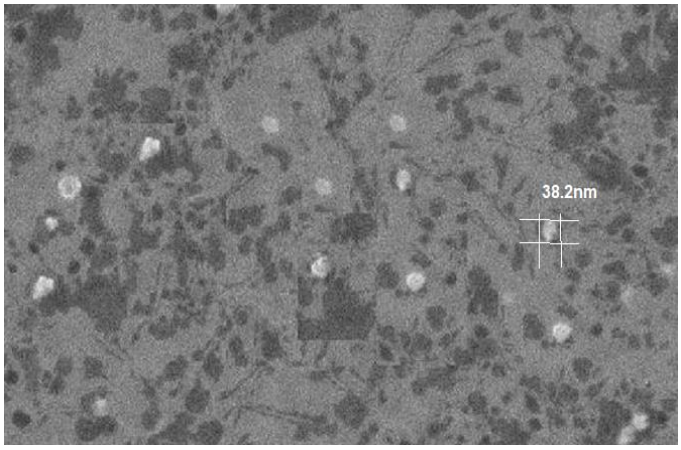

Figure 6: Scanning electron micrograph of nanoparticles obtained by extract of *Withania somnifera*

Table 8: Production yield of all nanoparticles

Formulation	**Production yield (%)**
Extract of *Withania somnifera*	69.10

Table 9: In-vitro % drug release study.

Time (In Min)	% Drug Release
0	0
10	16.10±0.01
15	30.16±1.02
30	57.23±0.13
45	80.43±1.28
60	94.76±0.25

Table 10: % Entrapment efficiency of formulation.

Formulations	**Entrapment efficiency (%)**
Extract of *Withania somnifera*	88.56

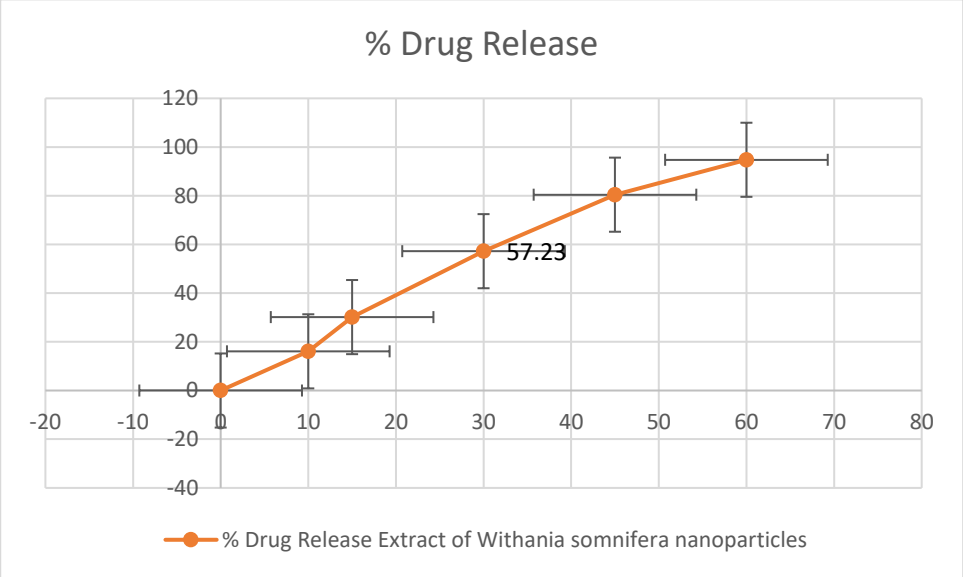

Figure 7: %Cumulative drug release

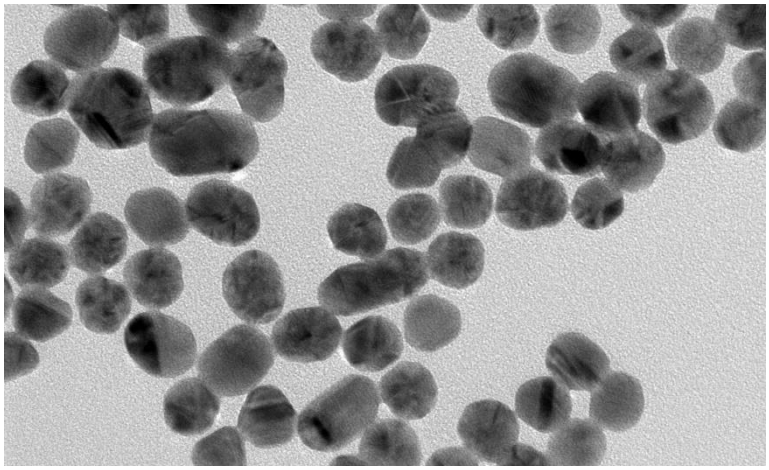

Figure 8: TEM of *Withania somnifera* nanoparticles

Table 11: Effect of storage on particle size, zeta potential and entrapment efficiency of Nanoparticles (n=3). Values are expressed as mean ±SD.

Storage time		"0" Month	"1" Month	"2" Month	"3" Month
Particle size (nm)	Extract F1	44.2 ± 10	44.5 ± 11	44.2 ± 09	43.1 ± 11
	Extract F3	44.3 ± 12	45.4 ± 12	45.6 ± 12	46.2 ± 12
Zeta potential (mV)	Extract F1	-29.32±0.8	-28.58±0.4	-27.88±0.4	-26.17±0.5
	Extract F3	-50.25±0.5	-48.75±0.8	-45.69±0.5	-43.26±0.7
Entrapment efficiency (%)	Extract F1	70.45±0.3	71.24±0.7	70.21±0.7	69.54±0.08
	Extract F3	74.25±0.8	73.21±0.3	72.45±0.8	74.25±0.5

(n=3). Values are expressed as mean ±SD

Table 12: Different Pre compression evaluation parameters.

Batch	Angle of Repose (θ)	Bulk Density (BD)	Tapped Density (TD)	Carr's Index	Hausner's Ratio
F1	28.21	0.41	0.42	2.38	1.02
F2	31.10	0.40	0.42	4.76	1.05
F3	27.01	0.41	0.42	2.38	1.02
F4	28.80	0.41	0.42	2.38	1.02
F5	30.24	0.41	0.43	4.76	1.05
F6	28.21	0.41	0.42	2.38	1.02
F7	30.81	0.41	0.42	2.38	1.02
F8	26.84	0.41	0.42	2.38	1.05
F9	26.56	0.4	0.42	2.38	1.05
F10	27.21	0.41	0.42	2.38	1.05
F11	27.32	0.41	0.42	2.38	1.02
F12	26.44	0.41	0.43	2.38	1.05

Table 13: Different Pre compression evaluation parameters.

Batch	Weight Variation	ThicknessTest	Hardness Test	Friability Test	Disintegration Test
F1	3.12	4.49	5.5	0.64	12.15
F2	3.56	4.50	5.3	0.99	13.45
F3	3.15	4.50	5.5	0.66	12.12
F4	3.56	4.50	5.3	0.98	13.41
F5	3.25	4.49	5.4	0.66	12.20
F6	3.30	4.49	5.4	0.66	11.50
F7	3.41	4.50	5.4	0.66	11.12
F8	3.85	4.49	5.3	0.99	12.03
F9	3.45	4.50	5.5	0.66	11.56
F10	3.15	4.50	5.4	0.33	11.45
F11	3.69	4.50	5.3	0.33	11.42
F12	4.01	4.50	5.5	0.66	11.05

Table 14: Cumulative drug release (F1-F12).

Time	F1	F2	F3	F4	F5	F6	F7
0	0	0	0	0	0	0	0
10	22.12±0.12	18.66±0.09	8.45±0.15	11.25±0.19	25.05±0.25	26.1±0.16	12.39±0.27
15	42.15±0.18	38.82±0.16	16.57±0.18	23.12±0.14	32.69±0.21	32.99±0.24	23.6±0.15
30	71.45±0.11	66.47±0.22	40.21±0.15	43.51±0.15	65.57±0.11	67.51±0.19	69.25±0.26
45	87.68±0.21	74.21±0.21	64.54±0.26	69.35±0.10	70.62±0.09	71.26±0.17	87.68±0.02
60	88.31±0.10	81.33±0.02	78.68±0.23	72.84±0.11	85.11±0.10	74.56±0.18	92.5±0.16

Time	F8	F9	F10	F11	F12
0	0	0	0	0	0
10	20.12±0.36	22.36±0.63	24.56±0.48	23.14±0.63	26.32±1.63
15	44.15±0.48	43.12±1.05	48.74±1.52	45.78±1.59	47.88±0.85
30	68.59±0.89	65.25±1.15	66.53±1.05	67.89±1.56	68.95±0.48
45	75.62±1.05	81.15±0.59	82.56±1.04	75.66±0.54	72.15±0.47
60	89.56±1.03	90.56±0.14	91.14±0.63	90.21±1.04	92.74±0.52

± S.D. n=6

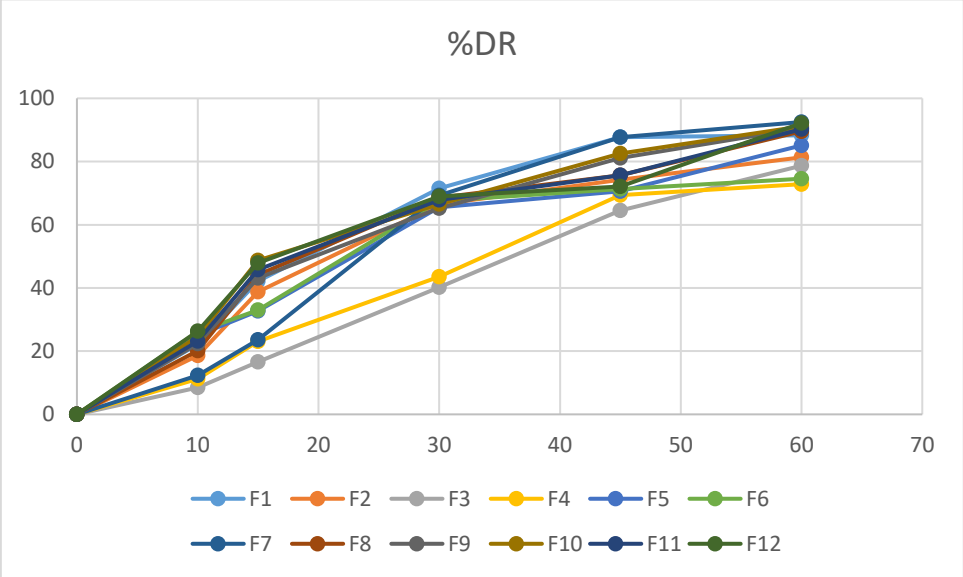

Figure 9: % Cumulative drug release (F1-F12)

Table 15: Dissolution Study Data of stability study.

Time in Min	Initial	First month	Two month	Third month	Sixth month
0	0	0	0	0	0
10	12.39±0.27	12.10±0.24	12.16±0.95	13.19±0.14	12.24±0.65
15	23.6±0.15	23.63±0.59	22.86±0.49	23.01±0.19	23.46±0.79
30	69.25±0.26	70.11±0.14	69.16±0.74	70.66±0.64	69.29±0.64
45	87.68±0.02	86.36±0.36	86.48±0.76	86.76±0.16	87.32±0.66
60	92.5±0.16	92.9±0.14	91.01±0.58	91.17±0.23	91.10±0.14

Table 16: Other parameters data of stability study.

Physical Parameter	Accelerated Stability Testing				
	Initial	First month	Two month	Third month	Sixth month
Appearance	Off white	Off white	Off white	Off white	Off white
Wt variation (%)	4.71	4.50	4.25	4.35	4.40
Hardness	5.4±0.13	5.4±0.23	5.3±0.16	5.4±0.21	5.4±0.18
Thickness	4.50±0.03	4.35±0.03	4.38±0.03	4.50±0.03	4.20±0.05
%Friability	0.10±0.01	0.11±0.01	0.10±0.01	0.12±0.01	0.10±0.02

Table 17: Paw Volume on 0^{th} to 10^{th} day.

Groups	Treatment andDose	PawVolume (mm)					%Inhabitation of paw volumeon 10^{th}Day
		0Day	2^{nd}day	4^{th}Day	6^{th}Day	8^{th}Day	
1	**Normal**	2.97±0.05	2.97±0.05	2.97±0.05	2.97±0.05	2.97±0.05	0
2	**Control**	2.65±0.08	5.92±0.17	8.621±0.34	8.54±0.19	7.57±0.34	-
3	**Standard (Diclofenac)**	2.79±0.18	5.31±0.26	7.72±0.34	5.5±0.35	4.91±0.42	21.03%
4	**WSN**	2.59±0.22	6.27±0.18	7.18±0.28	7.33±0.48	4.23±0.47	17.11%

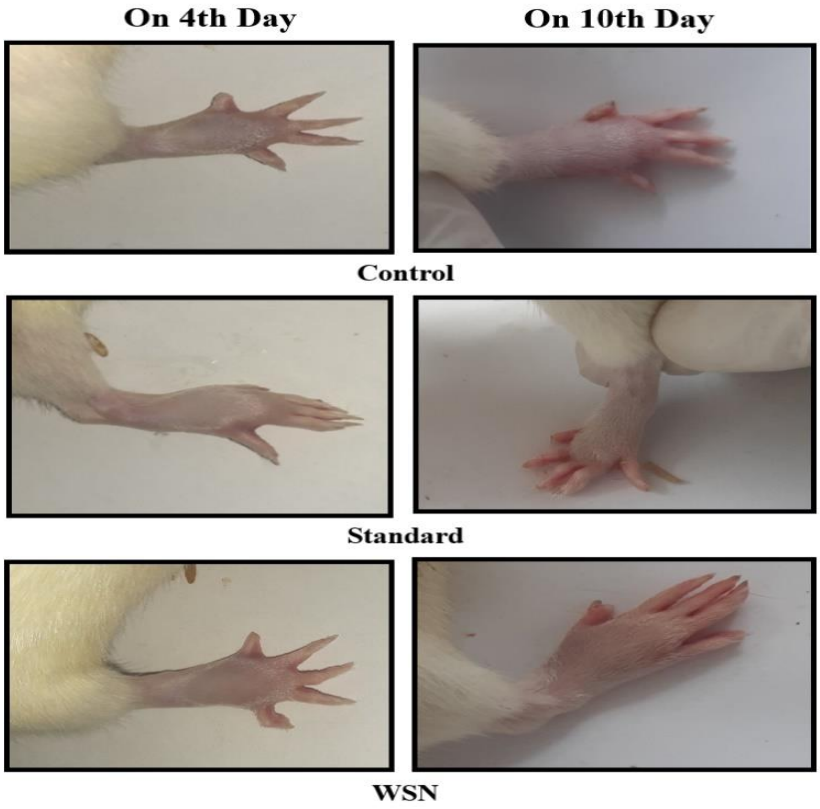

Figure 10: Response of Control, standard and desired Withaniasomnifera nanoparticles against animal groups

Table 18: HemoglobinEstimation in different groups of animals.

Groups	Treatment andDose	Hbmg/dl
1	**Normal**	14.83±0.568
2	**Control**	10.5±0.856
3	**Standard (Diclofenac)**	16.03±0.254
4	**WSN**	15.26±0.404

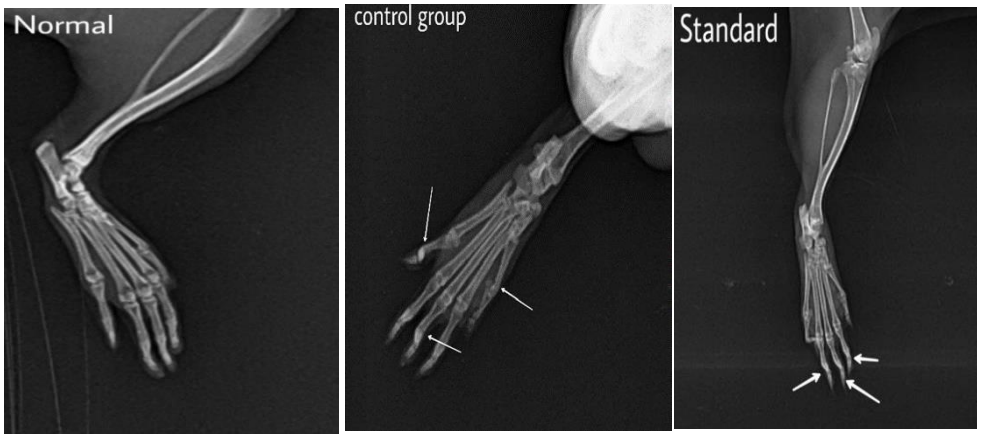

Figure 11: Radiograph of animals after and before the treatment

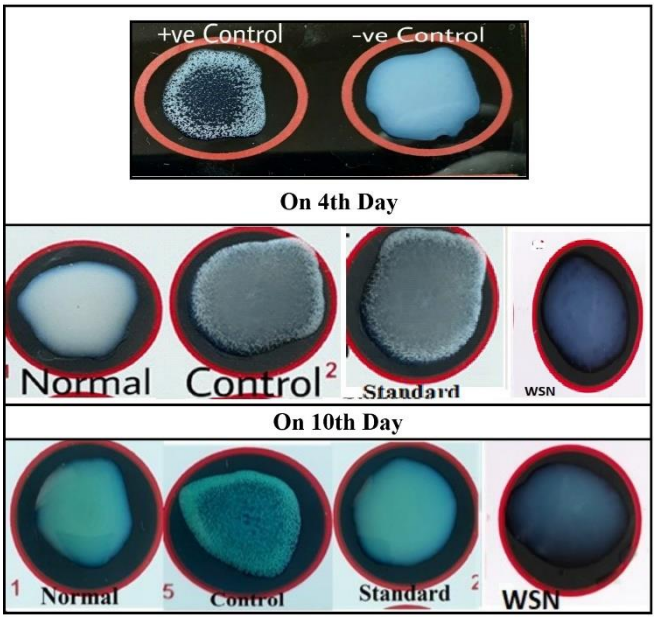

Figure 12: Effect of formulation dose on C-reactive protein(CRP)

Table19: Effect of treatment dose on different animal groups.

Groups	Treatment and Dose	Arthritic Index	
		On 4thDay	On 10thDay
1	Normal	Negative	Negative
2	Control	Positive	Negative
3	Standard Diclofenac	Positive	Negative
5	WSN	Positive	Negative

3.5 Evaluation of Tablets (Post compression parameters)

3.5.1 Organoleptic properties

All batches **(**F1-F12) were assessed for organoleptic properties like color, odor, and taste and found to be acceptable in all aspects.

3.5.2 General appearance

The formulated tablets were assessed for its general appearance and observations were made for shape, colour and texture (**Shape-** Round, **Colour-** off white, **Texture-** smooth). From the results obtained it was found that F1-F12 formulations have hardness, weight variation & friability within IP limit (**Table13**).

3.5.3 Dissolution Study

The corresponding Tables below contain information on the matrix tablets rates of dissolution. Each formulation's dissolution research was conducted in triplicate in 7.4 pH Phosphate Buffer.

3.5.4 Stability Study (Accelerated study)

An accelerated stability study was carried out for Formulation F12 in compliance with ICH stability requirements. Hardness, overall drug release percentage, and weight variance. Parameters such as friability were examined. The appearance, feel, and color of the produced tabletsfrom batch F12 up until the stability period remained consistent. During the stability analysis, other parameters were found to be good. Investigation results revealed that the optimized formulation (F12) was stable.

3.6 Anti-arthritic activity

Withania somnifera nanoparticles were administered to normal, control, standard, and treated animals to determine the anti-arthritic activity in terms of paw volume. **Table17** summarized the study's specifics. On days 0 through 10, the paw volume was documented in various groups, and responses were gauged. The percentage of paw volume inhibition was also measured on the tenth day, and it was discovered that the produced nanoparticle demonstrated 17% inhibition while the standard medication demonstrated 21%. According to the findings, the produced nanoparticles are more effective than conventional drugs.

3.6.1 HemoglobinEstimation

The findings of the hemoglobin estimation suggested that the usual medication and our nanoparticles produced quite comparable types of results. Both instances demonstrated that the animals' elevated hemoglobin levels were higher than those of the normal and control groups. **Table18** contains information about it in depth.

3.6.2 Radiograph

On the basis of radiographs and coned-down views of the lower limbs, radiographic examination was carried out. In order to take radiographs, GE 500 mA, 40 kvp, and 4 MAS were used. Radiological analysis revealed that the conventional and WSN treated animals had improved conditions.

3.6.3 Estimation of C - reactive protein

A diagnostic kit is used to estimate the biochemical parameter. C-reactive protein (CRP) test kit estimation. To measure the degree of inflammation, C-reactive protein (CRP) in serum was detected in vitro. The C-reactive protein (CRP) levels in the WSN-treated animals were lower than those in the normal, control, and standard. **Fig.12** depicted the effects of C-reactive protein (CRP) in more depth. **Table19** displayed the effects of dosage therapy on several animal categories.

4. Conclusions

In this investigation, we used traditional extraction using hydro-alcoholic solvents, TLC detection, and column chromatography technique to identify and isolate certain chemical compounds contained in *Withania somnifera* roots. The resulting extract-loaded chitosan nanoparticles were prepared and successfully validated by evaluation parameters. Different composition batches were designed, and herbal tabletswere manufactured. *In vitro* drug release suggested that batch F12 is the best amongst all and has good stability over six months. The initial% drug release from the best batch F12 was 92.5, and six months later, it was 91.1. Other factors, such as thickness, weight fluctuation, hardness, and friability, continue to vary little and fall within acceptable limits. Preclinical studies showed that produced nanoparticle formulations responded more favorably than standard and control formulations. Tests on the production formulation's anti-rheumatoid arthritis activity revealed that it had increased therapeutic efficacy and decreased toxicity. This formulation is better for rheumatoid arthritis patients.

Conflict of interest

No conflict of interest is claimed by the authors.

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