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STUDY THE ANTIOXIDANT ACTIVITY OF RUTIN NANOPARTICLES IN RAT MODELS

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Abstract:

Controlled drug release system is one of the most favorable technique of novel drug delivery system owing to its reproducibility and ease of formulation. Nanotechnology is very useful for controlling the drug release and thus improving the pharmacokinetic and pharmacodynamic properties of the drug. The technique improves patient compliance by reducing both dose and the frequency of administration and thus minimizing the local as well as systemic toxic effects. The aim of the present research work was to formulate and evaluate nanoparticles of Rutin by using the Emulsion solvent evaporation method. Sustained release nanoparticles of Rutin were prepared to increase the drug residence time in gastrointestinal tract and thus improving the bioavailability of drug. The nanoparticles were prepared by using Chitosan and Carbopol 940 as polymers. Different formulations were prepared with varying concentrations of Chitosan and Carbopol 940 in order to achieve the optimum particle size and maximum encapsulation efficiency. The particle size of nanoparticles was found to be in the range of 0.181±0.051 nm to 0.390±0.101 nm. Drug encapsulation efficiency ranged between 58.1±0.651 percent to 82.9±1.216% with controlled drug release up to 99.29% in phosphate buffer pH 6.8, 12 hrs. FT-IR studies showed that the drug and polymers were compatible. The results of Nanoparticles indicated that optimized formulation exhibited excellent properties.

Nanoparticles in the doses of 50 mg/kg, 100 mg/kg, and 200 mg/kg were used in wistar rats of either sex. The oxidative stress was produced by overdose of acetaminophen and estimation of serum concentration of various enzymes such as malonaldehyde (MDA), superoxide dismutase (SOD), reduced glutathione (GSH), and catalase (CAT) were measured by standard biochemical methods. Silymarin (100 mg/kg) was used as a standard drug for assessment of antioxidant status. When compared with the standard antioxidant silymarin, Nanoparticles did not exhibit antioxidant activity in terms of MDA level reduction. However, it significantly increased serum levels of the antioxidant enzymes (SOD, GSH, and CAT) exerting a potent antioxidant effect in a graded manner. The observed results suggest that Rutin loaded Nanoparticles could be a potential source of antioxidants. However, further studies are required to explore this therapeutic property of Rutin.

Keywords: *- Nanoparticles, Chitosan, Carbopol 940, Rutin and Emulsion solvent evaporation method and antioxidant.*

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INTRODUCTION:

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. They may protect cells from damage caused by unstable molecules known as free radicals. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. Free radicals are fundamentals to any biochemical process and represent an essential part of aerobic life and metabolism. Majority of the diseases are mainly linked to oxidative stress due to free radicals [1,2]. Our body is rich in endogenous antioxidants, the substances that have the ability to stop free radicals formation or to limit the damage they cause[3]. The effectiveness of current used exogenous antioxidants arises most probably from the increase of the endogenous free radical scavengers as enzymes (superoxide dismutase and selenium-dependent glutathione peroxidase), vitamins (alpha tocopherol and ascorbic acid). Many plants have been also found to posses free radical scavenging activity (Polyphenols, alkaloids and terpenoids). Low levels of one or more of the essential antioxidants have been shown to be associated with many disorders including cancer, inflammation, atherosclerosis, coronary heart disease and diabetes. Thus, in such cases, the administration of exogenous antioxidants seems to be salutary. Nowadays, a great deal of effort being expended to find effective antioxidants for the treatment or prevention of free radical-mediated deleterious effects[4].

Oxidative stress is characterized as an imbalance between the production of reactive species and antioxidant defense activity, and its enhanced state has been associated with many of the chronic diseases such as cancer, diabetes, neurodegenerative and cardiovascular diseases[5]. Based on that, many research groups have driven efforts to assess the antioxidant properties of natural products. These properties have been investigated through either chemical (in vitro) or biological (in vivo) methods, or both [6]. The results of these researches have led some to suggest that the long-term consumption of food rich in antioxidants can retard or avoid the ocurrence of such diseases [7,8]. According to Brewer, the effectiveness of a large number of antioxidant agents is generally proportional to the number of hydroxyl (OH) groups present in their aromatic ring(s). Based on that, the natural compounds would seem to have better antioxidant activity than the currently used synthetic antioxidants, making them a particularly attractive ingredient for commercial foods. Despite the large number of natural products that are currently consumed as antioxidant agents, the search for new chemical entities with antioxidant activity still remains a burgeoning field. In this context, the lichens have played an important role as a source for new antioxidant agents. Lichens are symbiotic organisms consisting of a fungus and one or more photosynthetic partners, the latter usually being either a green alga or a cyanobacterium [11,12]. They are found in a wide variety of natural habitats or in places with low temperatures, prolonged darkness, drought and continuous light. Lichens produce characteristic and unique secondary metabolites, and most of them occur exclusively in these symbiotic organisms. The most common lichen compounds are aromatic polyketides, particularly depsides, depsidones, depsones, dibenzofurans, and chromones. Lichens have been used in the folk medicine for numerous purposes, among them as astringents, laxatives, anticonvulsives, antiemetics, antiasthmatics, anti-inflammatories, antibiotics, and also for the treatment of cardiovascular, respiratory, and gastric disorders. Furthermore, pharmacological and biotechnological studies have been carried out in order to test and to develop biomaterials containing lichen-isolated natural compounds for humans use [13,14].

Medicinal plants with antioxidant potential

1. Rhizophora mangle is a plant from Rhizophoraceae family. The bark extract of the plant showed scavenging activity of hydroxyl radicals and the extract contained polyphenols, carbohydrates and sterols[15].

2. Diospyros malabarica is a plant from Ebenaceae family. The bark is used for the treatment of fever and fruit juices for healing of wound ulcer5 . The stem extract of the plant competes with oxygen to react with nitric oxide and thus, inhibits the generation of anions. The main phytoconstituents in the extract are phenolic compounds[16].

3. Asparagus racemosus is a tree from Liliaceae family. It shows antioxidant activity through the free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, nitric oxide scavenging, metal chelation, reduction power and inhibition of lipid peroxidation in rats. The main phytoconstituents are saponins, alkaloids and flavonoids[17].

4. Auricularia auricular is a tree and known as _tree ear or wood ear‘ from Auriculaceae family: It has shown a potent hydroxyl radical scavenging and lipid per-oxidation inhibitory activities. The main phytoconstituents are flavonoids [18].

5. Eucalyptus globules is a tree and known as ―Karpura maram‖ from Myrtaceae family. The antioxidant activity of Eucalyptus oil was estimated by two in vitro assays namely diphenyl picryl hydrazyl radical scavenging activity and inhibition of ascorbate induced lipid peroxidation method[19].
6. Acacia arabica is a plant from Mimosae family. The antioxidant assays were carried out in vivo and in vitro experimental models. In vitro, lipid peroxidation was carried out by tertiary butyl hydroperoxide (TBH) induced lipid peroxidation. In vivo, experiments were carried out in CCl4-induced hepatotoxicity in rats. The bark of the plant contained quercetin, (+) catechin, (-) epicatechin and gallic acid. The polyphenol rich active fraction of Acacia arabica is a potent free radical scavenger and protects TBH induced lipid peroxidation and CCl4-induced hepatic damage. The bark is used in the treatment of asthma, bronchitis, diabetes, dysentery and skin diseases[20].

7. Ligustrum vulgare is a plant from Oleaceae family. The leaves antioxidant activity was evaluated using DPPH test. The main phytoconstituents are flavonoids, iridoids, coumarins and essential oil, where flavonoid aglycones are responsible for the antioxidant activity and it shows a potent free radical scavenging activity[21].

8. Terminalia chebula is a tree and known as Myrobalanus chebula. Combretaceae family. The main phytoconstituents are tannins, chebulinic and gallic acids. The extract was tested by studying the inhibition of radiation induced lipid peroxidation in rat liver microsomes. It shows free radical scavenging activity due to presence of tannins and also It inhibits the development of duodenal ulcer and so the extract has appeared to show a cytoprotective effect on the gastric mucosa [22].

9. Lobelia nicotianaefolia is a plant from Campanulaceae family. The chemical constituents are alkaloids as lobeline and also it contains volatile oil, resin, gum and fixed oil. It is mainly used in the treatment of asthma and as respiratory stimulant[19].
10. Citrus lemon is a tree from Rutaceae family. The antioxidant activity was estimated by two in vitro assays, DPPH radical scavenging activity and inhibition of ascorbate induced lipid peroxidation (LPO) method. The main phytoconstituents are citral and limonene. The antioxidant property is shown due to the presence of citral.

MATERIALS AND METHODS:

Analytical Method Development

Determination of absorption maxima: Absorption maxima are the wavelength at which maximum absorption takes place. For accurate analytical work, it is important to determine the absorption maxima of the substance under study.

Procedure: For the preparation of calibration curve stock solution was prepared by dissolving 100 mg of accurately weighed drug in 100ml of methanol (1mg/ml). Further 1ml of the stock solution was pipette out into a 100 ml volumetric flask and volume was made up with phosphate buffer (6.8pH). From this stock solution pipette out 1ml and dilute to 10 ml with phosphate buffer and subject for UV scanning in the range of 200-400 nm using double beam UV spectrophotometer. The absorption maxima were obtained at 252 nm with a characteristic peak.

Preparation of calibration curve: It is soluble in Methanol; hence Methanol was used for solubilizing the drug. Stock solution (1 mg/mL) of Rutin was prepared in methanol and subsequent working standards (5, 10, 15, 20 and 25 µg/mL) were prepared by dilution with phosphate buffer of pH-6.8. These solutions were used for the estimation Rutin by UV method. The whole procedure was repeated three times and average peak area was calculated. Calibration plot was drawn between concentrations and peak area. Calibration equation and R^2 value are reported.

Preparation of nanoparticles

Preparation of Rutin loaded nanoparticles : Polymeric Nanoparticles were prepared by Emulsion solvent evaporation method. Required quantity of polymer and drug were weighed & dissolved in 12ml of dichloromethane. Quantity of Tween 80 and Span 60 was mixed with of water & this solution was kept in another beaker. Both the phases were kept for sonication for 15 min. until it become clear. Solution containing drug and polymer were added drop wise to aqueous phase under continues stirring. The formed nanoparticles suspension were homogenized at 18000 rpm for 15min then followed by magnetic stirring for 3hr. The suspension was centrifuged at 9,000 rpm for 45 min. The samples were added to glass vials & freeze-dried with mannitol 2% (w/v) as cryprotectant in a lyophilizer.

Table 1: Composition of nanoparticles formulations (F1 to F8)

Excipients	F1	F2	F3	F4	F5	F6	F7	F8
Rutin	100	100	100	100	100	100	100	100
Chitosan	100	200	300	400	-	-	-	-
Carbopol 940 %	-	-	-	-	100	200	300	400
Tween 80 (mL)	5	5	5	5	-	-	-	-
Span 60 (mL)	-	-	-	-	10	10	10	10
Distilled water (ml)	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s
Dichloromethane (ml)	10	10	10	10	10	10	10	10
Methanol	10	10	10	10	10	10	10	10

Characterization of nanoparticles:

Particle Sizes, PDI, Zeta Potential: The mean particle length and polydispersity index (PDI), that's a degree of the distribution of nanoparticles population, was decided the usage of dynamic light scattering (Delta Nano C, Beckman counter), and Zeta capability becomes anticipated on the premise of electrophoretic mobility under an electric powered field, the use of zeta Sizer Nano ZS (Malvern Instruments, UK). Samples had been diluted with the distilled water before measurement and measure at a hard and fast angle of 1650c for the particle size and poly dispersity index (PDI) analysis. For the Zeta ability measurement, Samples have been diluted as 1;40 ratio with filtered water (v/v) before analysis. Average particle size, PDI, and zeta potential have been then measured in triplicate.

Drug content: Rutin content in nanoparticles was assayed by an UV-visible spectrophotometer. Nanoparticles (100mg) were dissolved in 10ml methanol by shaking the mixture for 5 mins. One ml of the resultant solution was taken and diluted to 10ml with methanol. Then, aliquots were withdrawn and absorbance was recorded at 252 nm using UV-visible spectrophotometer (Lab India 3200).

Yield of nanoparticles: After complete drying the nanoparticles powders were collected and weighed accurately. The yield of nanoparticles was calculated using the formula.

$$Percentage\ yield = \frac{Total\ weight\ of\ nanoparticles}{\text{total weight of drug} + \text{weight of added materials}} \times 100$$

Entrapment Efficiency:

Entrapment Efficiency (EE) of the Rutin loaded nanoparticles changed into determined by measuring the awareness of uninterrupted drug in an aqueous medium by centrifugation method. The nanoparticles had been centrifuged during a high-space cooling Centrifuge (C-24.Remi) the usage of nano step centrifuge tubes with ultra-filter out having a relative molecular mass cutoff 100KD (Pall existence sciences-India) at 5000rpm for 15min at 4oc, and therefore the supernatant was separated. The amount of Rutin inside the supernatant changed into determining the usage of a UV-Visible spectrophotometer (U-1800, Hitachi) at lambda max 252nm after suitable dilution.

The percent entrapment efficiency (%) changed into calculated by means of the usage of the subsequent formula:

$$\%EE = \frac{\text{Total drug content-Free drug}}{\text{Total drug content}} \times 100$$

Percent amount of drug release from semi permeable membrane: Franz diffusion cell was used for the in vitro drug release studies. Semi permeable membrane was placed between donar and receptor chamber of diffusion cell. Receptor chamber was filled with freshly prepared 30ml 6.8 pH phosphate buffer. Nanoparticles equivalent to 1gm was placed on semi permeable membrane. The Franz diffusion cell was placed over magnetic stirrer (REMI 1ML) with 500rpm and temperature was maintained at 37 ± 1^0C. 5ml of samples were withdrawn periodically and replaced with fresh buffer. The withdrawn samples were periodically diluted and analysed for drug

content using UV visible spectrophotometer (Lab India 3200) at 252 nm.

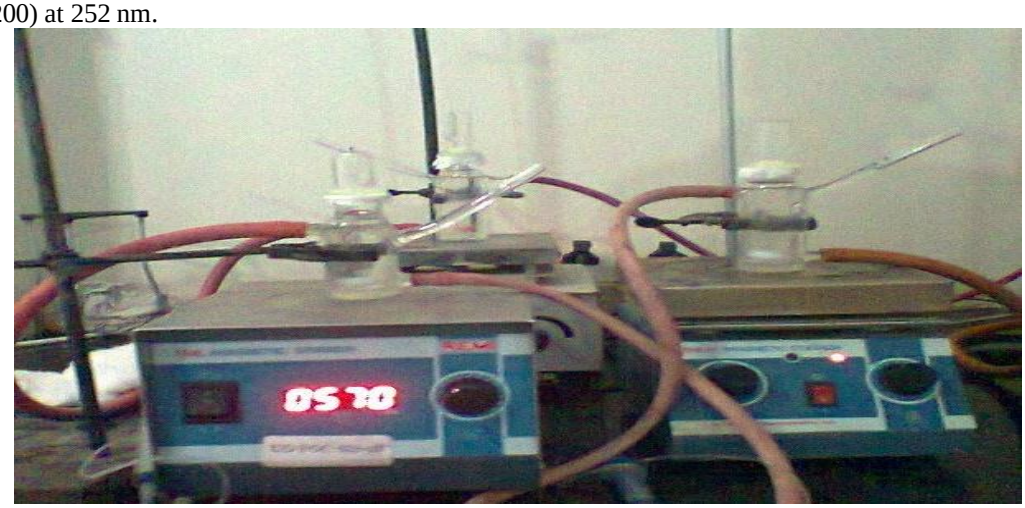

Fig 1: Drug release from semi permeable membrane

Powder X-ray Diffraction (PXRD) Studies: The prepared mixtures were also analyzed using X-ray powder diffractometer (PXRD) which confirms the formation of the new solid phases. The difference in the 2 theta lines confirms the formation of the new solid phases as no two solids have same 2 theta lines, thus revealing the formation of new solid phases. It also reveals the information about the crystal structure, chemical composition, and physical properties of the material and also helps in structural characterization. This technique detects changes in the crystal lattice and is therefore a powerful tool for studying polymorphism, pharmaceutical salts, and cocrystalline phases. Spectra of PXRD were taken on a sample stage Spinner PW3064. The samples were exposed to nickel filtrate Cukœ radiations (40 KV, 30 mA) and were scanned from 10° to 40°, 2Θ at a step size of 0.045° and step time of 0.5 s.

Fourier Transform Infrared (FTIR) spectroscopy: The formulations were subjected to FTIR studies to find out the possible interaction between the drug and the excipients during the time of preparation. FTIR analysis of the pure drug and optimized formulation were carried out using an FT IR spectrophotometer (Bruker FT-IR - GERMANY).

SEM (Scanning Electron microscope) studies: The surface morphology of the layered sample was examined by using SEM (Hitachi, Japan). The small amount of powder was manually dispersed onto a carbon tab (double adhesive carbon coated tape) adhered to an aluminum stubs. These sample stubs were coated with a thin layer (30Å) of gold by employing POLARON-E 3000 sputter coater. The samples were examined by SEM and photographed under various magnifications with direct data capture of the images onto a computer.

ANTIOXIDANT ACTIVITY

Reagents: Sodium hydroxide (Analytical grade, FisherChemicals Inc., Fair Lawn, NJ), citric acid (analytical grade), hexanes (HPLC grade, EMD Chemicals Inc., Gibbstown, NJ), methanol (HPLC grade, EMD Chemicals Inc., Gibbstown, NJ), ethyl acetate (HPLC grade, EMD Chemicals Inc., Gibbstown, NJ), BCL3-methanol (Supelco Inc., Belletonte, PA), 98% 2, 2- Dimethoxypropane (Sigma-Aldrich Inc., St. Louis, MO), Anhydrous sodium sulfate (10-60 mesh, Fisher Chemicals Inc., Fair Lawn, NJ), cholesterol (Aldrich Chem. Co., Milw., WI), 5α- cholestane (Sigma-Aldrich Co., St. Louis, MO), heptadecanoic acid (Sigma chemical Co., St.Louis, MO), DHA (cis-4, 7, 10, 13, 16, 19-Docosahexaenoic acid, Sigma-Aldrich Inc., St. Louis, MO) The solvents were stored at room temperature (20-25ºC) and other reagents were storedat -20ºC freezer.

PHYTOCHEMICAL EVALUTION

The powdered drug was and subjected to qualitative chemical tests.

- **Detection of Carbohydrates:** Small quantities of powdered drug and different extracts were dissolved in distilled water separately and filtered. The filtrates were taken for Molisch's Test, Fehling's Test, Benedict's Test, Barfoed's Test, Test for starch tests to detect the presence ofcarbohydrates.
- **Test for Gums and Mucilages:** The powdered drug and extracts were treated with absolute alcohol stirred and filtered. The filtrate was dried and examined for its swelling properties.

- **Test for Proteins and Amino Acids:** Small quantities of powdered drug and different extracts were dissolved in few ml of distilled water and subjected to Ninhydrin, Biuret, Million, Xanthoproteic test, test with tannic acid and heavy metals.
- **Test for Fixed Oils and Fats:** The powdered drug and extracts were subjected for Spot Test, Saponification Test.
- **Test for Alkaloids:** Small amount of powdered drug and solvent free various extracts were separately stirred with a few ml of dilute hydrochloric acid and filtered. The filtrates were tested with various alkaloidal reagents such as Mayer's, Dragendroff's, Wagner's and Hager's reagent and Tannic acid.
- **Test for Glycosides:** A small amount of powdered drug and different extracts were dissolved separately in 5ml of distilled water and filtered. Another portion of the extracts were hydrolyzed with hydrochloric acid for one hour on a water bath and hydrolysate was subjected to Legal's, Baljet's, Borntrager's, KellerKilliani'stests and for the presence of Cyanogenetic glycosides.
- **Test for Phytosterols:** The powdered drug and extracts were refluxed with 0.5N alcoholic potassium hydroxide until the saponification was complete. The saponification mixture was diluted with distilled water and extracted with petroleum ether. The ethereal extract was evaporated and unsaponification matter

Methods

Acute oral toxicity study: Acute oral toxicity study for the test extract of the plant was carried out as per the guidelines set by Organization for Economic Co-operation and, revised draft guidelines 425 and by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India.

Methods for evaluation of antioxidant activity in rats: Antioxidant activity of nanoparticles was evaluated by measurement of oxidative stress markers and antioxidant enzymes in serum of albino rats after the induction of oxidative stress by the procedure described by Galal *et al*.

The animals were divided into five groups of six animals each.

Group I received normal saline orally and served as control group.

Group II received silymarin (100 mg/kg) orally and served as a standard group.

Group III, IV, and V received nanoparticles at 50 mg/kg, 100 mg/kg, and 200 mg/kg orally and served as test groups. The animals were orally treated with respective drugs for 7 successive days. On the 6th day (i.e., 1-day before the last treatment) animals of all groups were fasted for 18 h. On the 7th day, 1 h after the last dose of agents given, all the animals were given acetaminophen 2 g/kg per for the induction of oxidative stress. After 24 h of administration, all the animals were sacrificed by ether overdose. Blood samples were collected by cardiac puncture and used for further laboratory investigations.

Estimation of malondialdehyde: Malondialdehyde in plasma is one of the aldehyde products of lipid peroxidation which react with TBA to form a colored product, the absorbance of which is measured spectro-photometrically at 530 nm. In the test tubes 0.5 ml of serum from test samples were taken, and 3 ml of 10% TCA was added to it, mixed well and the tubes were left to stand for 10 min at room temperature, and then centrifuged for 15 min at 5000 rpm. Two sets of test tubes were taken marked as blank and test. For a test sample, 2 ml of supernatant fluid was taken and added to 1.5 ml of 0.67% TBA. For a blank sample, 2 ml of distilled was added in 2 ml of 0.67% TBA. After mixing well and keeping in the boiling water bath for 10 min, they were cooled under tap water. A pale pink color developed, the color intensity was measured at 530 nm by colorimeter. Using the molar extension coefficient (1.5×10^5) and result was expressed as n moles of malonaldehyde (MDA)/100 ml of serum.

1.5 = 100 µmol/L (here, 100 is for conversion from ml to dl).

Then MDA = 100 × O.D. of unknown/1.5.

Estimation of superoxide dismutase: This method utilizes the inhibition of auto-oxidation of pyrogallol by superoxide dismutase (SOD) enzyme. The assay mixture in a 3 ml volume consisted of 100 µL each of 0.2 mM pyrogallol, 1 mM EDTA, 1 mM DTPA, and varying concentrations of standard SOD enzyme or 100 µL of serum in air equilibrated tris-HCl buffer (50 mM; pH 8.2). The reaction mixture prepared in 3 sets includes standard, test and control. Pyrogallol was added after the addition of all other reagents to start the reaction. Initial 10 s period was considered as induction period of the enzyme. So after 10 s, change in absorbance at 420 nm at 10 s intervals was recorded to a period of 4 min. The average change in the absorbance per minute was calculated.

Estimation of reduced glutathione: The method described is based on the development of a yellow color when DTNB (Ellman's Reagent) is added to sulphydryl compounds due to redox reaction between GSH and DTNB. The color which develops is fairly

stable for about 10 min, and the reaction is little affected by variation in temperature. The reaction is read at 412 nm. GSH in red cells is relatively stable and venous blood samples anticoagulated with ACD maintain GSH levels up to 3 weeks at 4°C GSH is slowly oxidized in solution, so only fresh lysates should be used for the assay.

Estimation of catalase: The method is based on the fact that dichromate in acetic acid gets reduces to chromic acetate when heated in the presence of H_2O_2 with the formation of perchromic acid as an unstable intermediate. The chromic acetate thus produced is measured colorimetrically at 570 nm. The catalase (CAT) preparation allows splitting of H_2O_2 for different periods of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture, and the remaining H_2O_2 is determined by measuring chromic acid colorimetrically after heating the reaction mixture.

Statistical analysis: The data are presented as a mean ± standard error mean. The data were analyzed by one-way analysis of variance followed by Student's unpaired *t*-test by using Graph Pad Prism 6.03 version. *P* values of <0.05, <0.0,1 and <0.001 were considered to be significant, very significant, and highly significant, respectively.

RESULTS AND DISCUSSION:

Preparation of Standard Graph:

a. Determination of absorption maxima: The standard curve is based on the spectrophotometry. The maximum absorption was observed at 252nm.

b.Calibration curve: Graphs ofRutin was taken in 6.8 Phosphate buffer

Table 2: Calibration curve data for Rutin at 252nm

Concentrations [µg/mL]	Absorbance
0	0
5	0.158
10	0.304
15	0.449
20	0.587
25	0.717

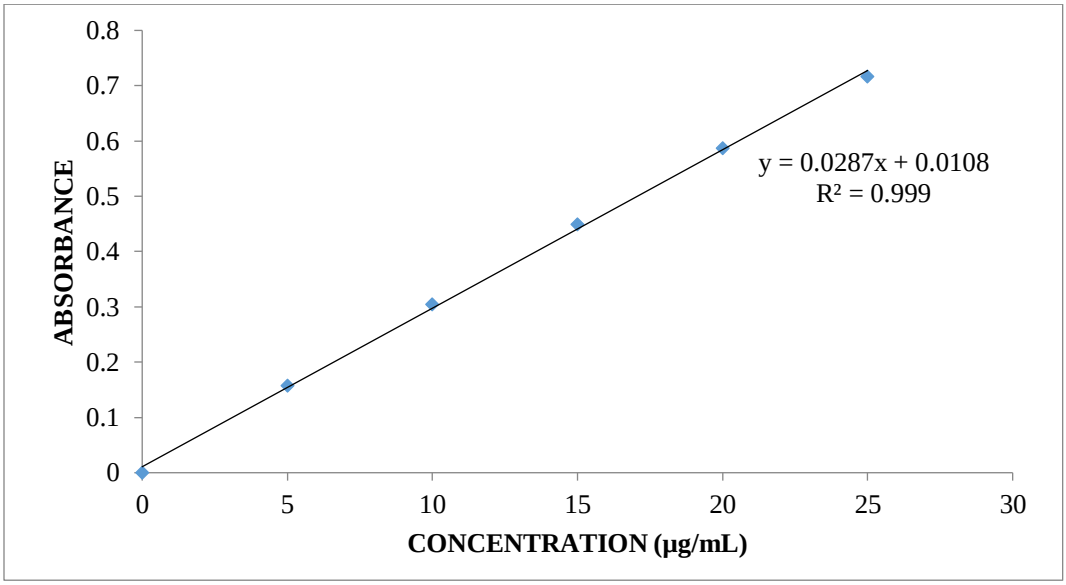

Fig 2: Standard graph of Rutin in pH 6.8 Phosphate buffer

Standard graph of Rutin was plotted as per the procedure in experimental method and its linearity is shown in Table 8.1and Fig 8.1. The standard graph of Rutin showed good linearity with R^2 of 0.999, which indicates that it obeys "Beer- Lamberts" law.

EVALUATION OFRUTINLOADED NANOPARTICLES:

Table 3: Evaluation of Nanoparticles

Batch No	Mean Particle size(nm)	%Yield	Drug Content	Drug encapsulation efficiency	PDI	Zeta Potential(mV)
F1	0.164±0.09	62.14	93.69	58.1±0.651	0.280±0.036	-25.1±0.301
F2	0.171±0.025	78.92	95.33	61.6±0.215	0.293±0.028	-23.5±0.810
F3	0.290±0.061	81.64	97.62	63.1±5.621	0.241±0.040	-23.0±8.641
F4	0.181±0.051	90.94	98.99	82.9±1.216	0.421±0.015	-35.9±1.824
F5	0.230±0.089	64.82	90.44	81.1±2.356	0.432±0.021	-32.7±0.581
F6	0.376±0.101	72.61	92.65	84.9±2.306	0.380±0.035	-30.6±0.301
F7	0.223±0.081	81.94	93.55	73.9±3.219	0.590±0.031	-42.4±0.302
F8	0.390±0.101	86.39	94.05	75.6±2.603	0.643±0.025	-36.2±0.392

Percentage yield of formulations F1 to F8 by varying drug was determined and is presented in Table. Highest drug content, Highest Entrapment efficiency observed for F4 formulation.

PDI observed in the F4 formulation i.e., 0.403 respectively. The Zeta potential range from -23.5±0.810mV to -42.4±0.302 mV to all the formulations.

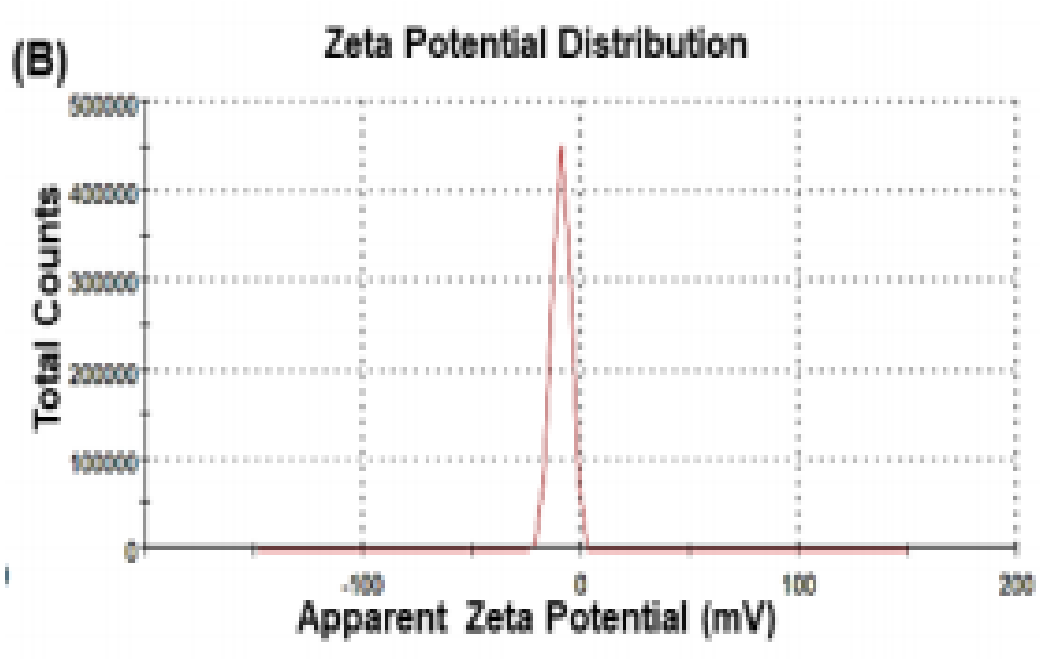

Fig 3: Zeta Potential of F4 Formulation

***In vitro* Drug release studies:**

Table 4:*In vitro* Drug release studies of Selegiline

TIME (hr)	CUMULATIVE PERCENT OF DRUG RELEASED							
	F1	F2	F3	F4	F5	F6	F7	F8
0	0	0	0	0	0	0	0	0
1	36.15	29.10	24.98	21.57	45.63	35.96	27.56	23.61
2	49.62	34.53	28.60	27.49	50.75	45.48	35.43	30.26
3	56.98	48.86	33.54	32.26	67.14	56.41	42.29	37.14
4	60.83	57.54	49.72	40.52	73.60	63.24	52.59	43.11
5	76.47	65.99	55.34	56.14	77.59	68.67	65.63	50.14
6	89.68	77.42	60.75	65.38	80.37	73.68	70.15	54.33
7	97.89	84.27	76.18	78.89	95.10	86.11	85.66	68.91
8		91.38	82.26	91.14		96.93	90.34	79.14
10		95.18	95.74	95.72			95.24	88.63
12			97.51	99.29			97.14	93.25

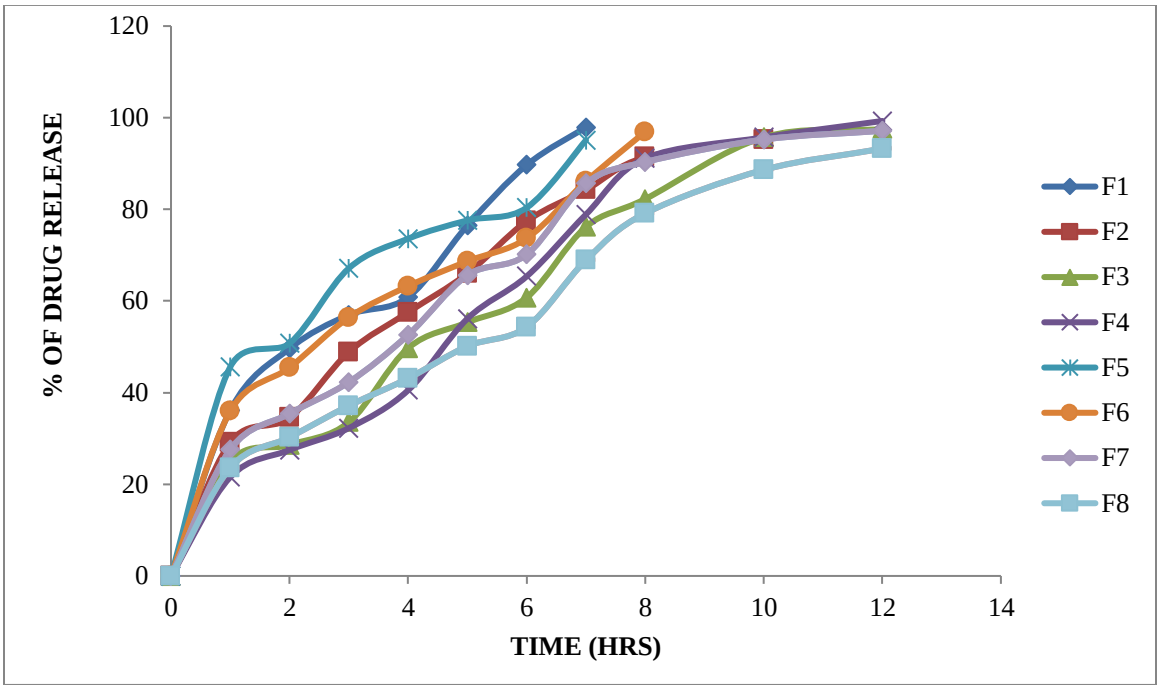

Figure No 4: Dissolution study of Rutin Nanoparticles

Hence based on dissolution data of 9 formulations, F4Chitosan**(400mg)**formulation showed better release (99.29%) up to 12 hours. So F4 formulation is optimised formulation.

Application of Release Rate Kinetics to Dissolution Data: Data of *in vitro* release studies of formulations which were showing better drug release were fit into different equations to explain the release kinetics of drug release from Nanoparticles. The data was fitted into various kinetic models such as zero, first order kinetics; higuchi and korsmeyer peppas mechanisms and the results were shown in below table it follows the zero order kinetics.

Table 5: Release kinetics data for optimized formulation (F4)

CUMULATIVE (%) RELEASE Q	TIME (T)	ROOT (T)	LOG (%) RELEASE	LOG (T)	LOG (%) REMAIN	RELEASE RATE (CUMULATIVE % RELEASE / t)	1/CUM % RELEASE	PEPPAS log Q/100	% Drug Remaining	Q01/3	Qt1/3	Q01/3 - Qt1/3
0	0	0			2.000				100	4.642	4.642	0.000
21.57	1	1.000	1.334	0.000	1.894	21.570	0.0464	-0.666	78.43	4.642	4.280	0.361
27.49	2	1.414	1.439	0.301	1.860	13.745	0.0364	-0.561	72.51	4.642	4.170	0.472
32.26	3	1.732	1.509	0.477	1.831	10.753	0.0310	-0.491	67.74	4.642	4.076	0.565
40.52	4	2.000	1.608	0.602	1.774	10.130	0.0247	-0.392	59.48	4.642	3.904	0.738
56.14	5	2.236	1.749	0.699	1.642	11.228	0.0178	-0.251	43.86	4.642	3.527	1.115
65.38	6	2.449	1.815	0.778	1.539	10.897	0.0153	-0.185	34.62	4.642	3.259	1.382
78.89	7	2.646	1.897	0.845	1.324	11.270	0.0127	-0.103	21.11	4.642	2.764	1.878
91.14	8	2.828	1.960	0.903	0.947	11.393	0.0110	-0.040	8.86	4.642	2.069	2.572
95.72	10	3.162	1.981	1.000	0.631	9.572	0.0104	-0.019	4.28	4.642	1.624	3.018

99.29	12	3.464	1.997	1.079	-0.149	8.274	0.0101	-0.003	0.71	4.642	0.892	3.749

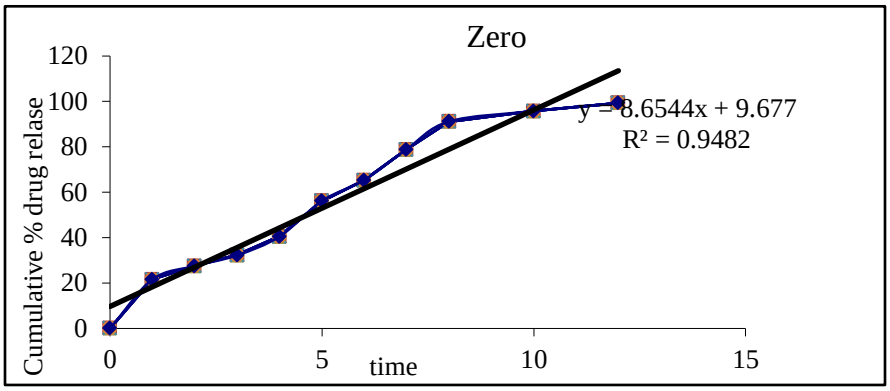

Figure 5: Graph of zero order kinetics

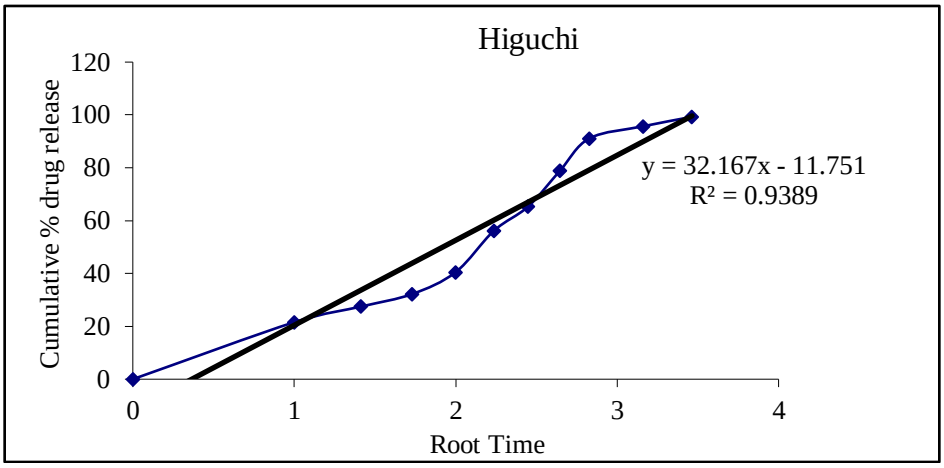

Figure 6: Graph of higuchi release kinetics

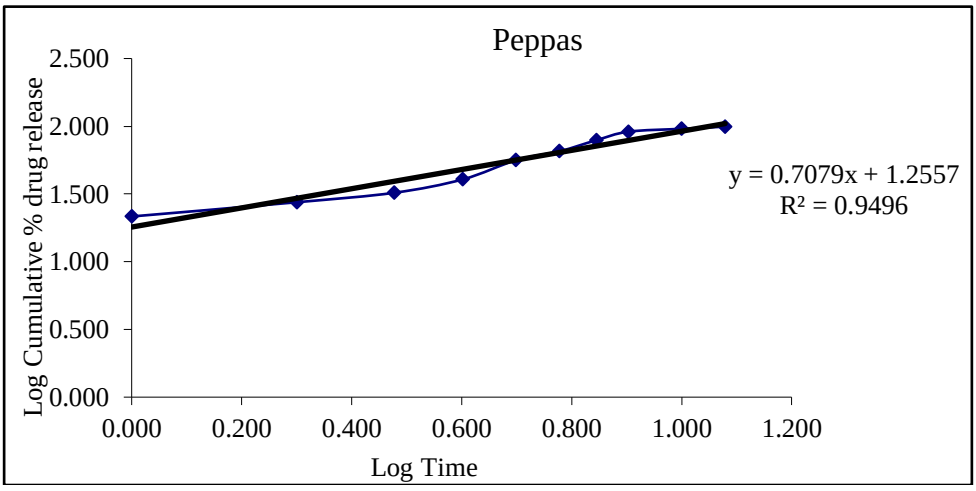

Figure 7: Graph of peppas release kinetics

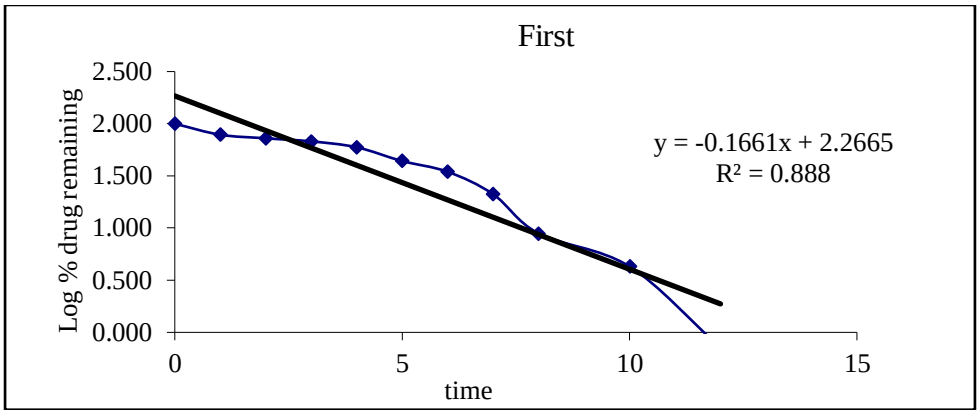

Figure 8: Graph of first order release kinetics

Based on the data above results the optimized formulation followed **Peppas release** kinetics.

Drug – Excipient compatibility studies

Fourier Transform-Infrared Spectroscopy:

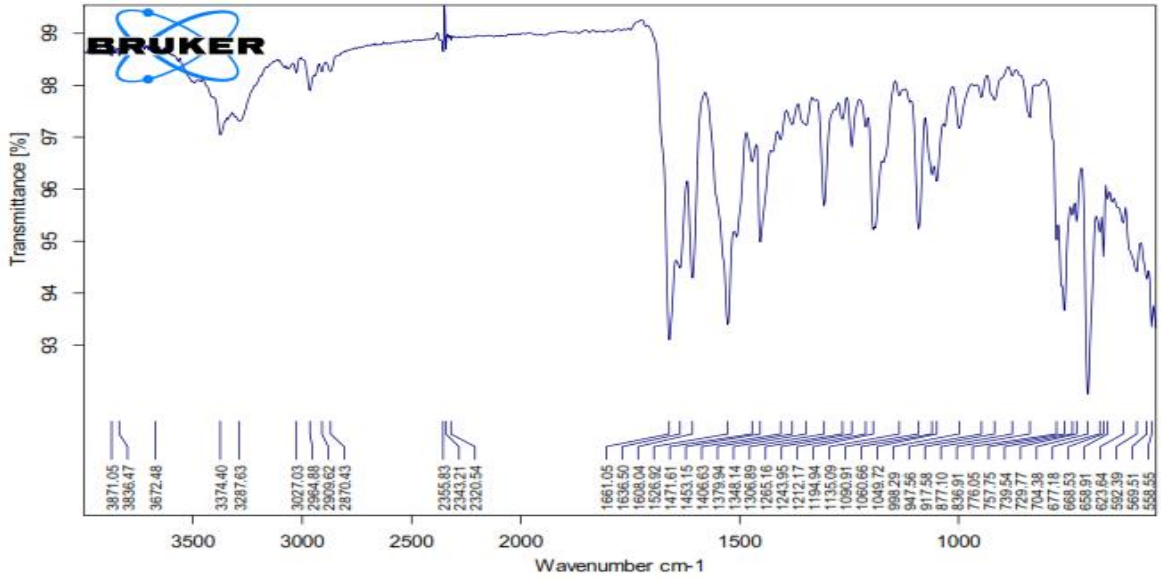

Figure 9: FT-TR Spectrum of Rutinpure drug

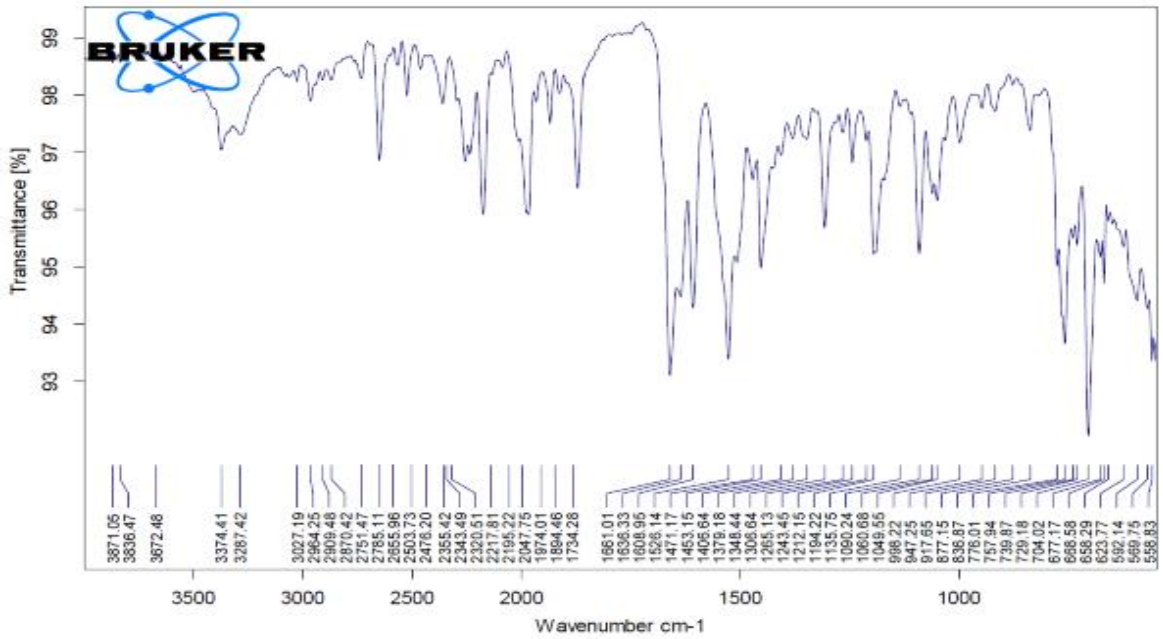

Figure 10:FT-IR Spectrum of Optimised Formulation

There is no incompatibility of pure drug and excipients. There is no disappearance of peaks of pure drug and in optimised formulation.

SEM

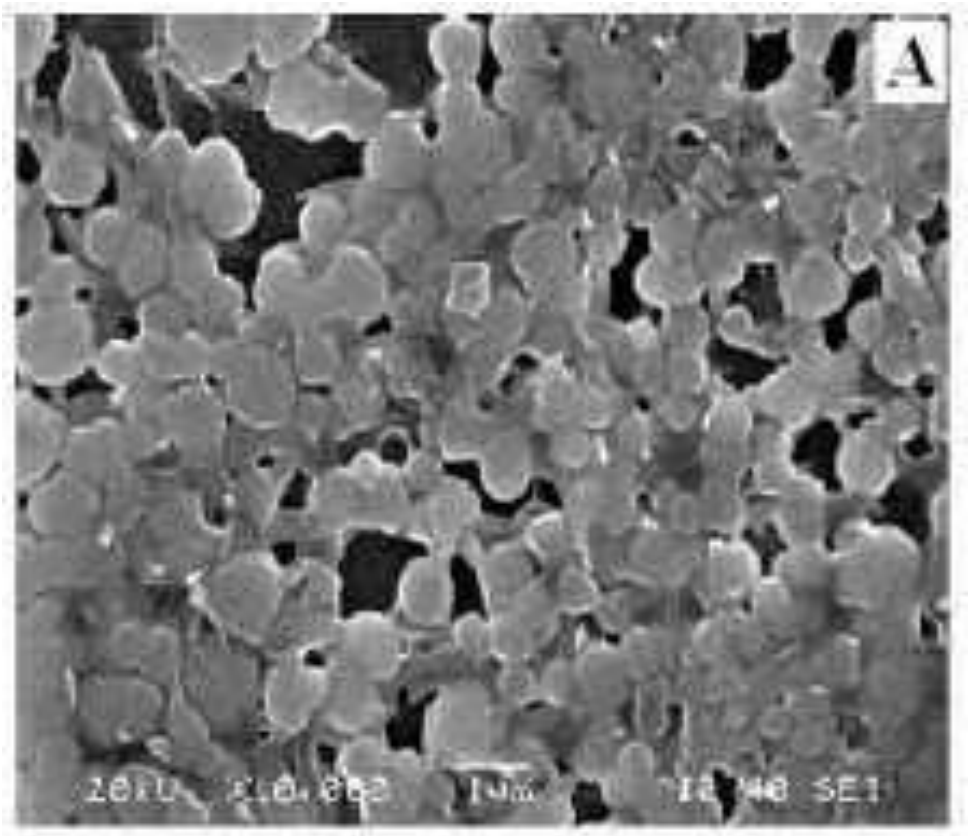

Figure 11: SEM graph of optimized formulation

SEM studies showed that the Rutin- loaded nanoparticles had a spherical shape with a smooth surface as shown in Figure.SEM image revealed that the Rutinnanoparticles were in nano size rangeand smooth spherical in shape in this F4 Formulation.

XRD

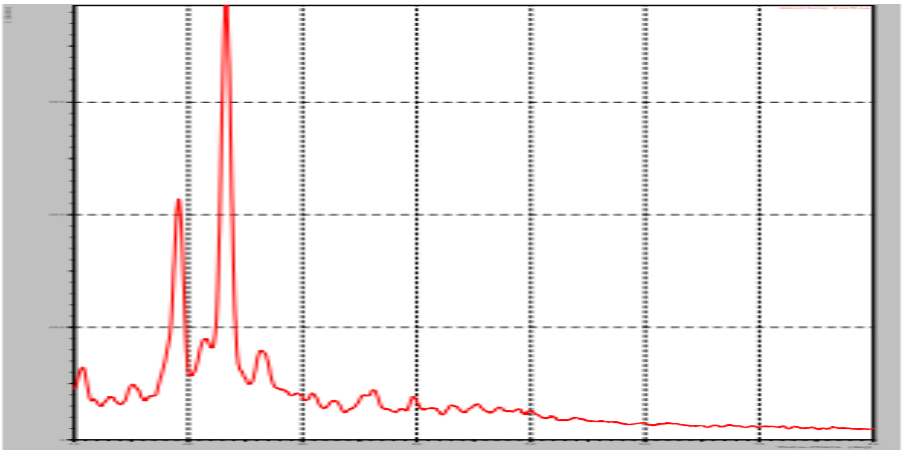

Figure 12: RutinF4 optimized formulation

The zeta potential of nanoparticles is commonly used to characterize the surface charges property of nanoparticles. It reflects the electrical potential of particles influenced by the composition of the particles band the medium in which it is dispersed. Hence, to increased minimize the opsoniazation and to prolong the circulation of nanoparticles *invivo*. The zeta potential of the nanoparticle formulation with Chitosan(Formulation F4) particles which present in the formulation are de-aggregated and remain same and more stable in the substance and zeta potential (mV) is -35.9±1.824mv. So this polymer is more suitable for nanoparticles preparation and the result shows smooth surface character and efficient repelled action and it decreases the opsoniazation.

ANTI-OXIDANT ACTIVITY: In this method, antioxidant activity was measured by the estimation of serum concentrations of various enzymes by standard biochemical methods. Silymarin (100 mg/kg) was used as a standard drug for assessment of antioxidant status. Silymarin is obtained from the plant sliybon marinum, and it has already proven as an antioxidant.

Table 6: Effect of Rutin nanoparticles on serum antioxidant enzyme levels

Group	Drug Treatment	Dose	MDA (nmole/ml)	SOD (units/mL)	GSH (mg/dl)	Cat (units/mL)
I	Normal saline (Control)	5mL/kg po	3.91	1.82	3.81	14.36
II	Silymarin (Standard)	100mg/kg PO	3.01	6.52	6.96	61.89
III	Nanoparticles	50mg/kg PO	4.35	2.71	3.75	14.25
IV	Nanoparticles	100mg/kg PO	3.36	2.92	3.86	25.99
V	Nanoparticles	200mg/kg	3.21	6.02	5.26	46.36

Standard increased the serum SOD level, and the difference was highly significant ($P < 0.001$) when compared with control group indicating potent antioxidant capacity. Oral administration of graded doses of nanoparticle also increased serum SOD levels when compared with control group. The highest increase was found in nanoparticle (200 mg/kg) group ($P < 0.001$), while MEC (100 mg/kg) showed very significant increase ($P < 0.01$) in comparison with control group. In nanoparticle (50 mg/kg) group, serum SOD level was increased but the difference was statistically nonsignificant in comparison with control group. In this aspect, nanoparticle (200 mg/kg) was comparable with the standard silymarin.

Serum CAT level was found to be increased in standard and all three extract groups in a graded manner in comparison with control group. This increase was statistically very significant ($P < 0.001$)

in the standard group, Nanoparticle(100 mg/kg) and Nanoparticle(200 mg/kg) groups in comparison with control groups. The increase in Nanoparticle(50 mg/kg) groups was statistically nonsignificant in comparison with control group. In this aspect, Nanoparticle(200 mg/kg) was relatively comparable with the standard silymarin.

Discussion: For assessing antioxidant activity of Prepared Nanoparticles, the oxidative stress was produced in rats by an overdose of acetaminophen and estimation of serum concentration of various enzymes by standard biochemical methods. Serum levels of MDA, SOD, reduced GSH, and CAT were measured. Our results showed that standard drug silymarin lowered serum MDA level in comparison with control. The graded extract doses increased serum MDA levels but not significantly as compared to control. This shows that the extract does not have antioxidant activity in terms of MDA level in the reduction of peroxidation level. Standard increased serum levels of all the antioxidant enzymes (SOD, GSH and CAT) significantly exerting a potent antioxidant effect. Nanoparticles (100 mg/kg) and Nanoparticles (200 mg/kg) also showed similar results in a graded manner and thus exerted a potent antioxidant effect. However, Nanoparticles (50 mg/kg) was totally ineffective in this regard. Hence, it can be concluded that Nanoparticles exert potent antioxidant activity at higher doses. Especially, Nanoparticles (200 mg/kg) is comparable with standard silymarin in this aspect. MDA is a stable secondary aldehyde degeneration product of lipid peroxidation and is used as a biological marker for the assessment of lipid peroxidation. The relative increase in serum MDA levels in all extract groups proved that extract was not as an effective antioxidant as standard.

An antioxidant works by retarding the process of oxidation by free radicals and further damage. Increased levels of measured antioxidant enzymes clearly envisaged the antioxidant potential of this plant. SOD is an important endogenous antioxidant enzyme acting as the first line defense system against reactive oxygen species (ROS) which scavenges superoxide radicals to H_2O_2 and thus provide protection against the deleterious effects of radicals. H_2O_2 accumulated by this reaction leads to the formation of hydroxyl radicals which can be harmful too. GSH and CAT work as antioxidant enzymes by virtue of scavenging these hydroxyl radicals. GSH is a tripeptide and a powerful antioxidant present in the cytosol of cells and is the major intracellular nonprotein thiol compound. SH groups present in GSH reacts with H_2O_2 and the hydroxyl radical and prevent tissue damage, and it is also capable of scavenging ROS directly or enzymatically. The antioxidant effects observed in our study can be attributed to many phytochemicals in the experimental plant leaves as they are reported to possess the antioxidant activity. Among these, flavonoids have been very frequently correlated with the antioxidant potential of any plant extract. It has been proposed by Ye *et al*. Those Flavonoids have the very strong capacity to eliminate free radicals in the blood and promotes the activities of antioxidant enzymes such as SOD, GSH, and CAT. These actions of Flavonoids are also dose dependent. Hence, an increase in the serum concentrations of antioxidant enzymes in our study can be explained by this background. A low dose of Nanoparticles extract 50 mg/kg) was unable to increase these enzyme levels, hence failed to exert antioxidant activity. Antioxidant potential of the Flavonoids has also been suggested by various other researchers in past. They are effective scavengers of various types of free radicals. Apart from these, the NanoparticlesRutin have shown the presence of other constituents such as polyphenols, tannins, anthocyanins, alkaloids, glycosides, saponins, and steroids, which have been linked with the antioxidant potential by many researchers.

CONCLUSION:

The method used for preparation of nanoparticles of Rutin was found to be simple and reproducible. The slow and constant release of Rutin from nanoparticles maintain constant drug plasma concentration thereby increasing therapeutic efficacy. The developed formulation overcome and alleviates the drawbacks and limitations of Rutin sustained release formulations. The development of effective nano delivery systems capable of carrying a drug specifically and safely to a desired site of action is one of the most challenging tasks of pharmaceutical formulation investigators. On the basis of different parameters i.e. physicochemical and *in-vitro* release study, nanoparticles of batch F4 are concluded as optimum formulations. Further, it can be concluded that the nanoparticulate formulation can be an innovative and promising approach for the delivery of Rutin.

The nanoparticles demonstrated significant antioxidant properties in experimental animals in this study. These activities may be attributed to the various phytoconstituents of nanoparticles such as flavonoids, tannins, saponins, alkaloids, anthocyanins, glycosides, polyphenols, steroids, iron, and vitamins such as A, C, E. However, further experimental studies are required to explore the exact mechanism of actions and next level of clinical trials to generate novel drugs. This

might prove helpful to use its immense therapeutic efficacy as a potent antioxidant phytomedicine.

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