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African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Preparation of magnetic microspheres of mesalamine by phase separation emulsion polymerisation technique

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The study involved magnetic microspheres of mesalamine prepared by phase separation emulsion polymerization (PSEP) method technique. Magnetic microspheres were prepared by PSEP method to target them to the colon. Three polymers namely Eudragit S 100, ethylcellulose and chitosan were used for the preparation of magnetic microspheres. Magnetite content and entrapment of mesalamine was evaluated. Eudragit S 100, ethylcellulose and chitosan were used as polymers. Fourier transform infrared spectroscopy (FTIR) spectrum of drug and polymer was taken to visualize the compatibility of drug and polymer. Scanning electron microscope (SEM) images show the uniformity and particle size of the microspheres formed. The *in vitro* release study was carried out in phosphate buffer pH 6.8. The various results obtained were fit into the mathematical models and the Higuchi model was found to be most suitable for the formulations. Chitosan magnetic microspheres prepared by phase separation emulsion polymerization were found to be best in all the evaluation parameters (practical yield, magnetite content, magnetic responsivity of microspheres, particle size, *in vitro* release studies). They contain maximum magnetite content which is the utmost feature for the magnetic microspheres. Microspheres can be targeted by the external magnetic field applied due to magnetite entrapped. Thus toxicity and reticuloendothelial clearance can be minimized.

Key words: Chitosan, Eudragit S 100, ethylcellulose, magnetic, phase separation emulsion polymerization (PSEP)

INTRODUCTION

Magnetic microspheres play a compromising role in controlled and novel drug delivery. Polymeric controlled drug delivery systems have evolved as one of the most attractive areas in drug delivery research. The drug release is controlled by properties of the polymer-drug system and also by other factors like pH, enzymes etc (Khar and Diwan, 2001). Despite several advantages offered by the controlled drug release, one important problem pertinent to the entire field is that all the systems so far developed give release rates that are constant or decrease with time. Increased delivery on demand will be very beneficial in certain situations. This increased delivery

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on demand can be achieved by using external feedback control systems such as magnetic control.

Tyle (1988) proposed the concept of magnetic drug targeting. Magnetic fields are believed to be harmless to biological systems and adaptable to any part of the body. Magnetic microsphere is a newer approach in pharmaceutical field. Traditional radiation methods use highly penetrating radiation that is absorbed throughout the body, thus causes harm to the body. Its use is limited by toxicity and side effects. The aim of the specific targeting is to enhance the efficiency of drug delivery and to reduce the toxicity and side effects. Localization of the drug to the localised disease site is the important feature of this delivery system. This larger amount of freely circulating drug can be replaced by smaller amount of magnetically targeted drug (Vyas and Khar, 2004).

Techniques used in preparation of magnetic microspheres

Given the enormous advantage of multiparticulate system over single-unit oral dosage forms, extensive research has focused recently on refining and optimizing existing techniques for the formulation of magnetic microspheres as well as on the development of novel manufacturing approaches that use innovative formulation and processing equipment (McBride et al., 2013). Magnetic microspheres are prepared mainly by two methods namely: phase separation emulsion polymerization (PSEP) and continuous solvent evaporation (CSE), by using mixture of water soluble drugs (for lipophilic drugs, along with the dispersing agent) and 10 nm magnetite (Fe_3O_4) particles in an aqueous solvent of matrix material, which are about 1.0 µm in size that is small enough to allow them to be injected intravenously without any occlusion in the micro vascular. These microspheres are nontoxic and nonreactive with blood components (Salim et al., 2010). They can be stabilized by heating or chemically cross linking albumin to achieve a wide spectrum of drug release kinetics. These are infused into an artery, supplying a given target site. A magnet of sufficient field strength is then placed externally over the target area to localize the microspheres at the capillary bed in this region. In order to localize microspheres in a fast-moving arterial system, generally greater field strength is required. There are mainly two techniques, which are commonly employed for microspheres preparation (Ishida et al., 1983). Preparation of magnetic microspheres by phase separation emulsion polymerization method is shown in Figure 1.

MATERIALS AND METHODS

Mesalamine was obtained as a gift sample from IPCA Laboratories Ltd. Chitosan (Sigma Aldrich), Ethylcellulose (CentralDrugHouse, NewDelhi), Eudragit S 100 (Alphachemika), goethite (Shree Surya minerals), nitrogen gas (Deluxe industrial gases, pune). All the reagents were of analytical grade.

Preparation of magnetite

Figure 2 shows the procedure for preparation of magnetite (Kahani et al., 2009).

Formulation of magnetic microspheres

Drug polymer interaction studies

Compatibility of drug with polymers was checked by Fourier transform infrared (FTIR) and differential scanning calorimetry (DSC) studies. The FTIR spectrum of mesalamine is shown in Figure 3. Figures 4, 5, 6 and 7 show that there is no interaction between drugs and polymers used. DSC studies were done to estimate the compatibility. Melting point of the drug was found to be 280°C. Microspheres were prepared by phase separation emulsion polymerization method. Several batches of magnetic microspheres were prepared in different drug: polymer ratio using three polymers separately namely chitosan, eudragit S 100, ethylcellulose (Kakar et al., 2013). The scheme for preparation has been summarized in Table 1.

Characterization of magnetic microspheres

Determination of percentage yield

Magnetic microspheres prepared by PSEP were dried and weighed (Lalit and Tapar, 2011).

Percentage yield = Practical yield / Theoretical yield × 100

Figure 8 and Table 2 shows the representation of percentage yield.

Flow properties of magnetic microspheres

Carr's index, angle of repose and Hausner's ratio were evaluated by fixed funnel method and compared with the standard values (Table 3) (Vimal et al., 2009).

Drug content and entrapment efficiency

Weighed amount of microspheres were digested with phosphate buffer and analyzed for the drug content (Kahani et al., 2009). Figure 9 and Table 4 shows the entrapment efficiency.

Entrapment efficiency = Experimental drug content / Theoretical drug content × 100

Particle size determination

Particle size was determined by SEM. Particle size is represented in Table 5. SEM pictures are shown in Figures 10, 11 and 12.

Determination of magnetite content

Determination of magnetite content in prepared magnetic microspheres was conducted by employing a conventional titrimetric method using thiosulphate and potassium iodide for quantitative analysis. It was observed that the entrapment of magnetite increased

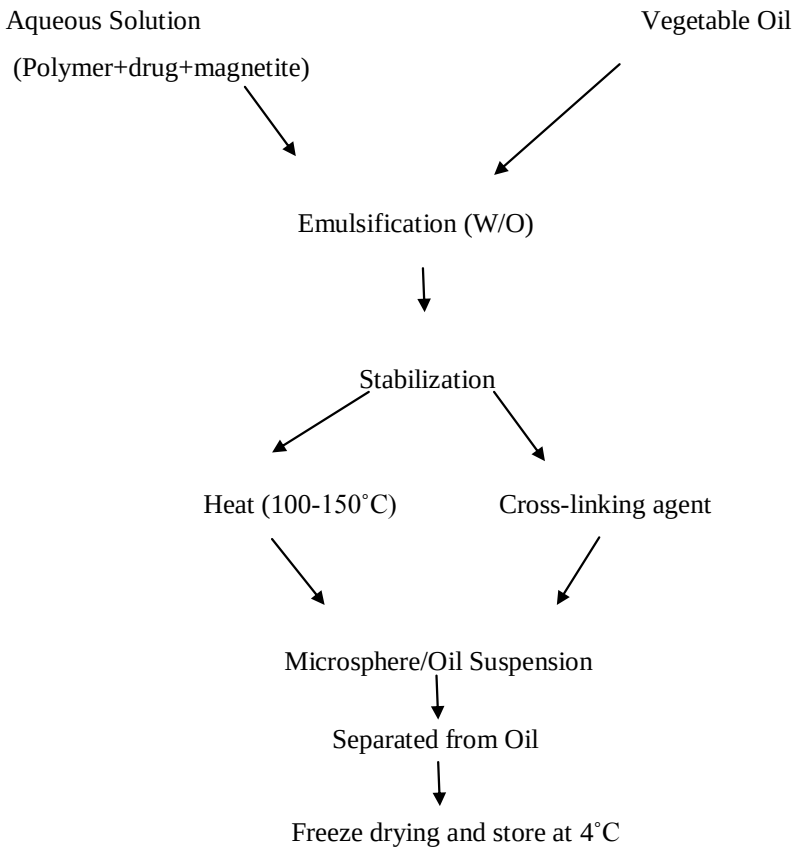

Figure 1. Preparation of magnetic microspheres by phase separation emulsion polymerization.

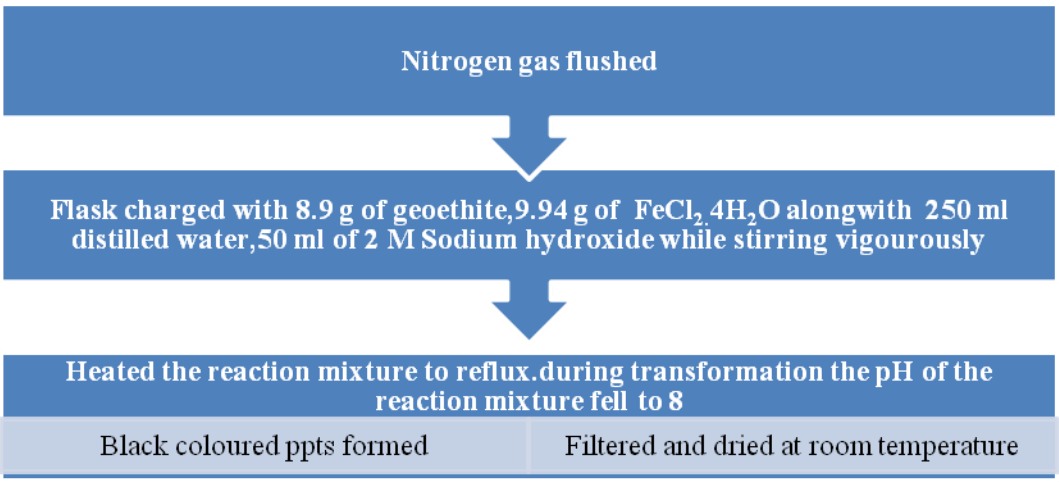

Figure 2. Procedure for preparation of magnetite.

with increase in concentration of polymer added in consecutive formulations. Maximum magnetite content was found for formulation F 3 (Vyas et al., 2013). Representation of percentage magnetite content is shown in Figure 13 and Table 6. Each ml of 0.1 N sodium thiosulphate is equivalent to 0.005585 g of ferric ion.

Dissolution studies

The dissolution studies were carried out in basket type apparatus. Phosphate buffer pH 6.8 was used. Accurately weighed 100 mg microspheres were introduced in phosphate buffer solution 900 ml.

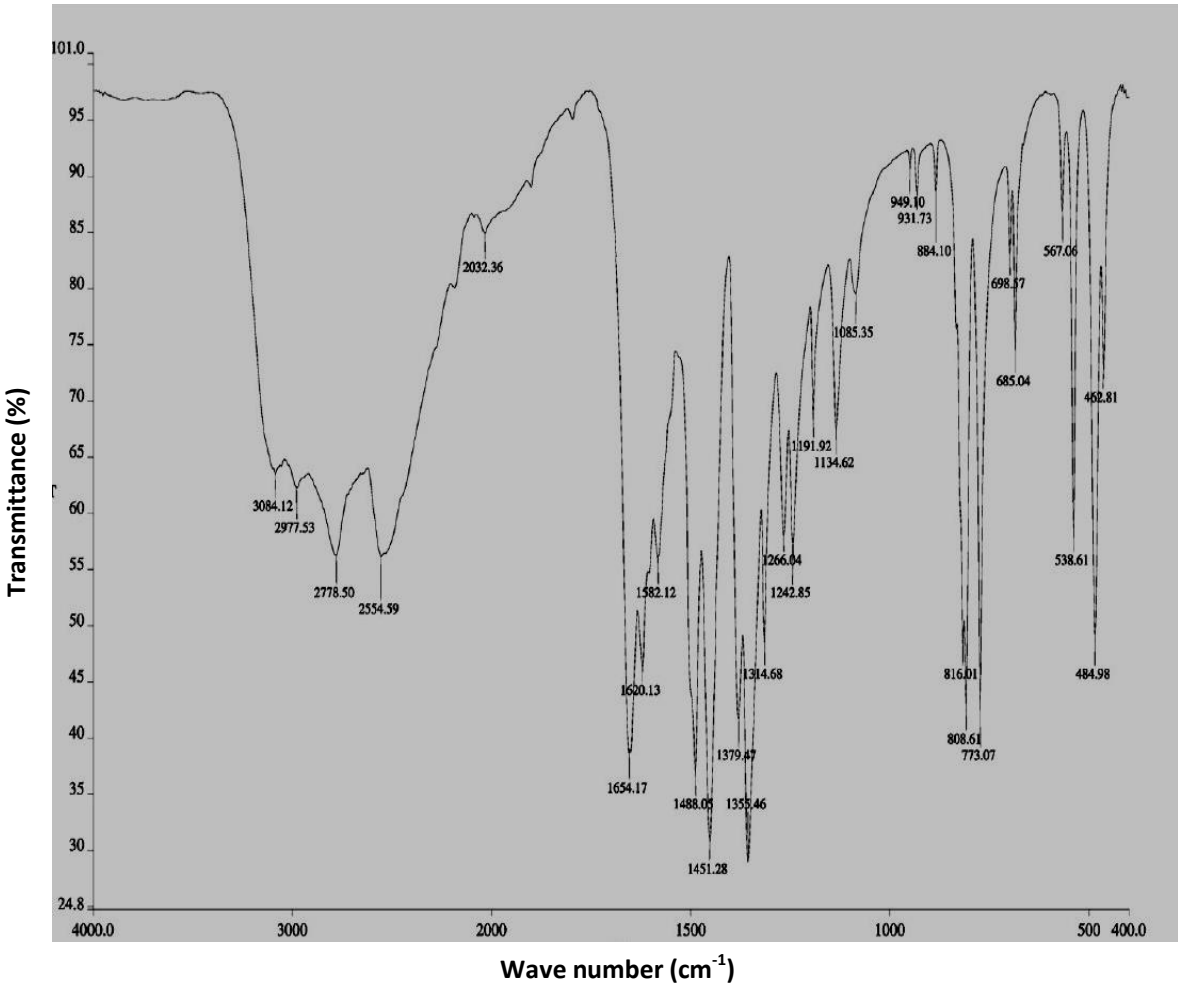

Figure 3. FTIR spectrum of mesalamine.

Aliquots were taken at different time intervals and percentage drug release analysed by UV Spectrophotometer at 230 nm (Zhang et al., 2007). Figure 14 represents the comparison of dissolution studies conducted on formulations F1, F2 and F3. Table 7 shows the percentage release of formulations.

In vitro study of release kinetics of magnetic microspheres

In the present study the raw data obtained from *in vitro* drug release study was analyzed wherein data were fitted to different equations in kinetic models to study the release kinetics of the optimized formulation. The kinetic models used were zero order, first order, Higuchi's equation, Hixon Crowell and Korsemeyer Peppas model. F 3 formulation best suits Higuchi model.

Zero order release kinetics

The zero order graphs were plotted between % cumulative drug release (CDR) versus time and are presented in Figure 15.

First order release kinetics

The first order graphs was plotted between log cumulative percent-tages of drug remaining versus time and are represented in Figure 16.

Higuchi model release kinetics

The Higuchi model graph was plotted between % cumulative drug release (CDR) versus square root of time and are shown in Figure 17.

Korsemeyer Peppas model release kinetics

The Korsemeyer Peppas model graph was plotted between log of time versus % cumulative drug release (CDR) and is shown in Figure 18.

Hixon Crowell model

The Hixon Crowell model graph was plotted between time vs. cube root of amount remaining and is shown in figure 19.

RESULTS

Magnetic microspheres could be better retained due to its

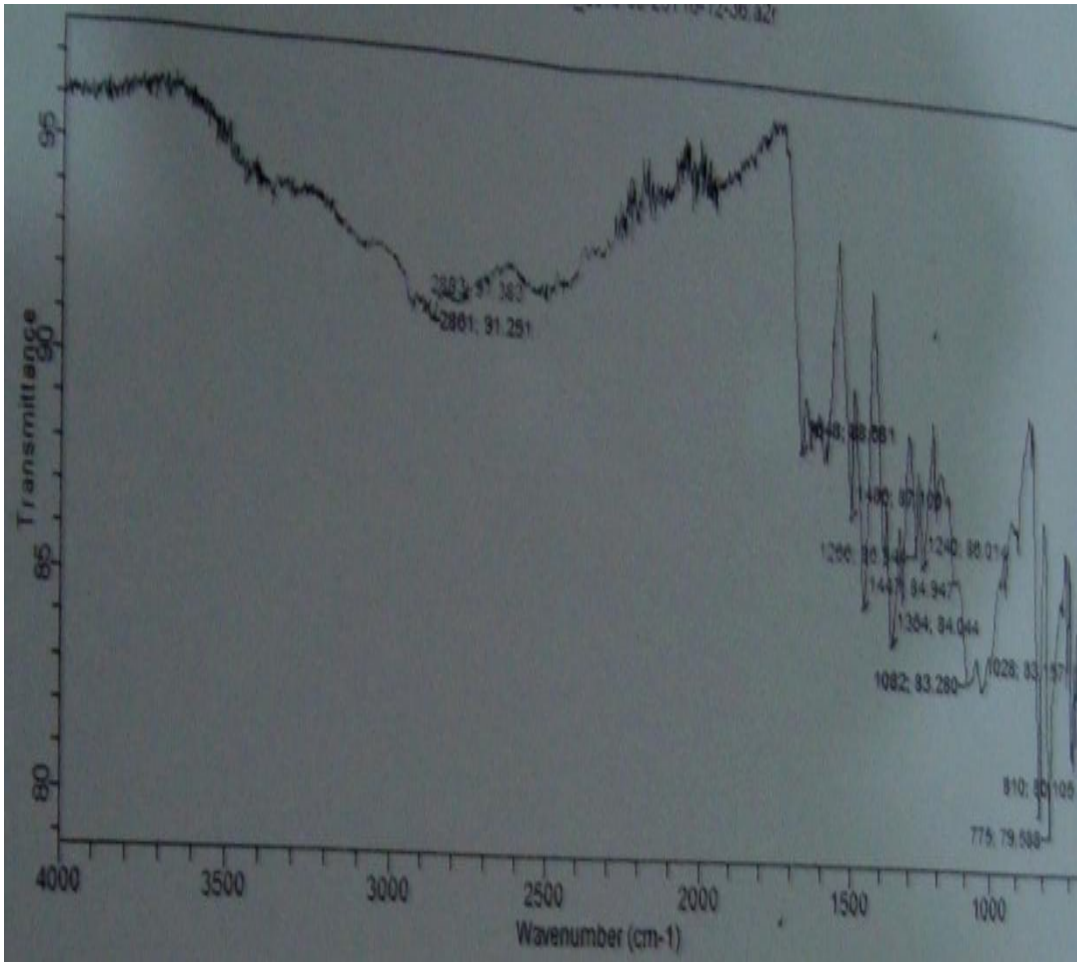

Figure 4. FTIR spectrum of physical mixture of chitosan and mesalamine.

Table 1. Composition of Magnetic microspheres.

Formulation code	Magnetite (mg)	Polymer (mg)	Drug (mg)	Polymer: Drug ratio	Method
F1	50	Chitosan (125)	125	1:1	PSEP
F2	50	Chitosan (84)	166	1:2	PSEP
F3	50	Chitosan (63)	187	1:3	PSEP
F4	50	Ethylcellulose (125)	125	1:1	PSEP
F5	50	Ethylcellulose (84)	166	1:2	PSEP
F6	50	Ethylcellulose (63)	187	1:3	PSEP
F7	50	Eudragit (125)	125	1:1	PSEP
F8	50	Eudragit (84)	166	1:2	PSEP
F9	50	Eudragit (63)	187	1:3	PSEP

more magnetite content. Chitosan microspheres were found to be best. The better sustained release was found for formulation F3.

1. 5-ASA was found to be compatible with chitosan, ethylcellulose, eudragit S 100 for the preparation of magnetic microspheres.

2. Solubility of 5-ASA was found to be optimum at neutral pH.

3. Chitosan is a most suitable and compatible polymer for the preparation of magnetically responsive polymers of 5-aminosalicyclic acid.

4. Percentage practical yield increases as the ratio of polymer to the drug added increased.

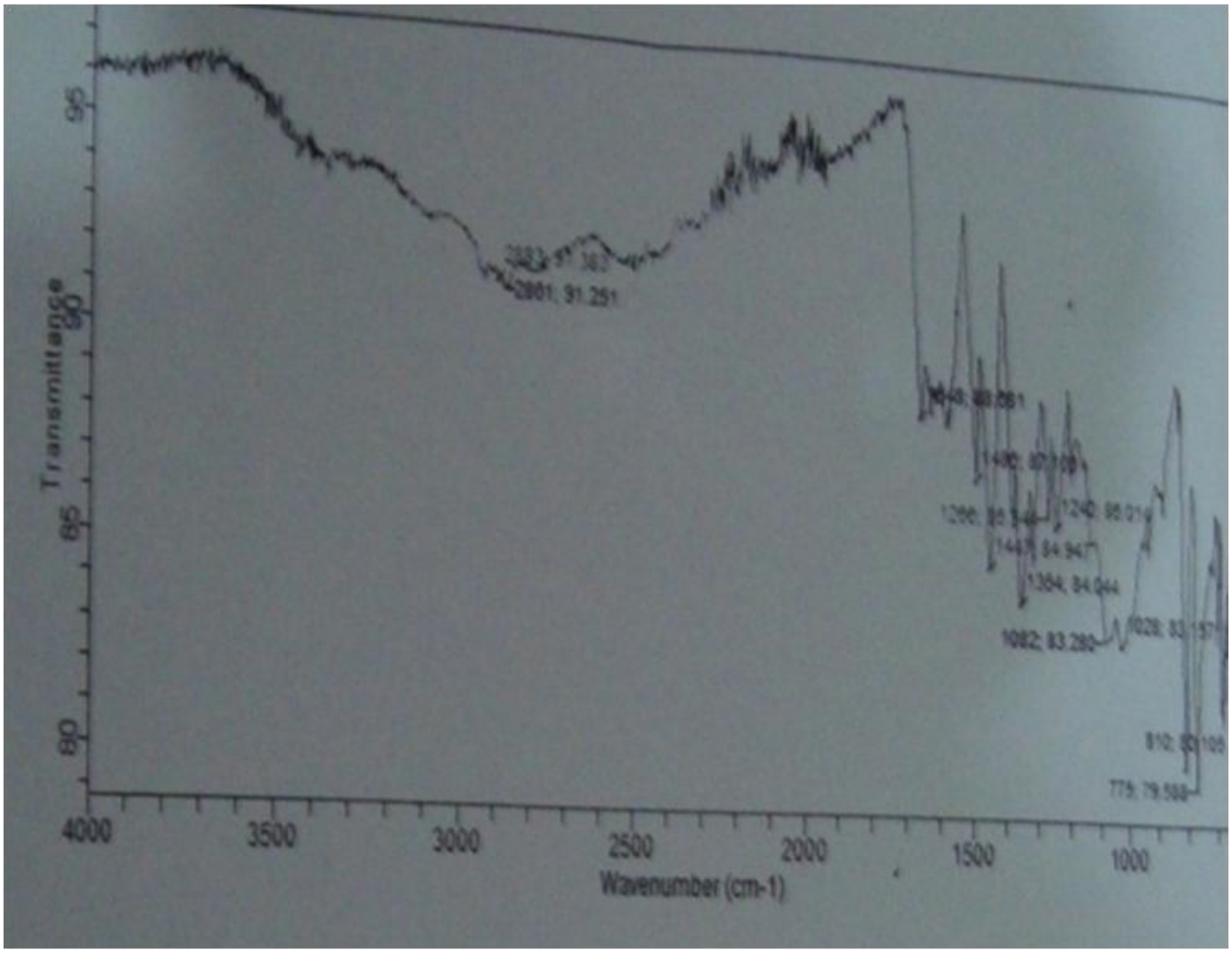

Figure 5. FTIR spectrum of physical mixture of ethylcellulose and mesalamine.

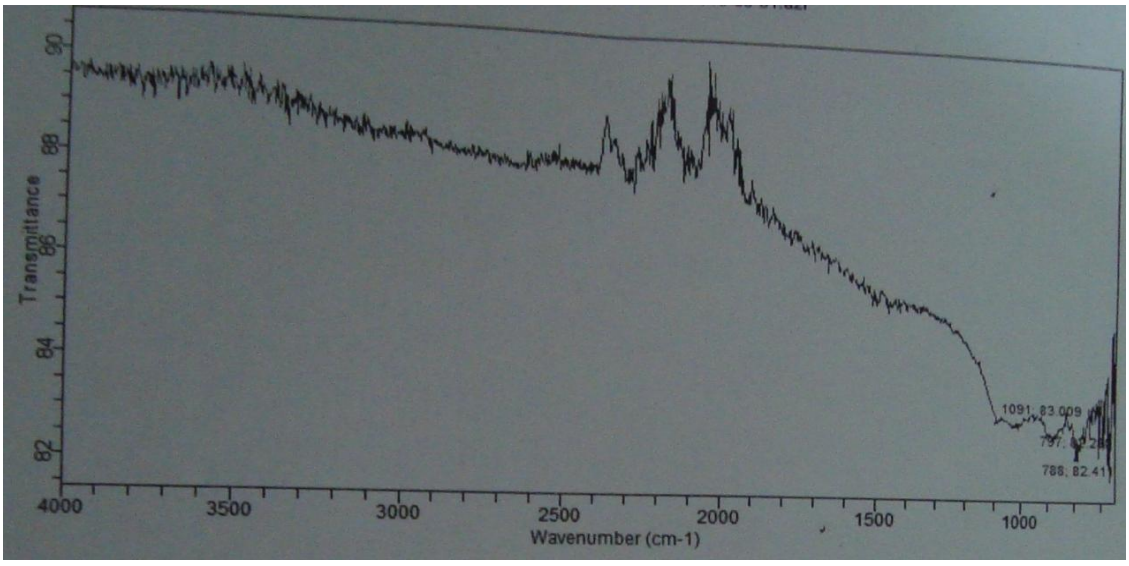

Figure 6. FTIR spectrum of physical mixture of Eudragit S 100 and mesalamine.

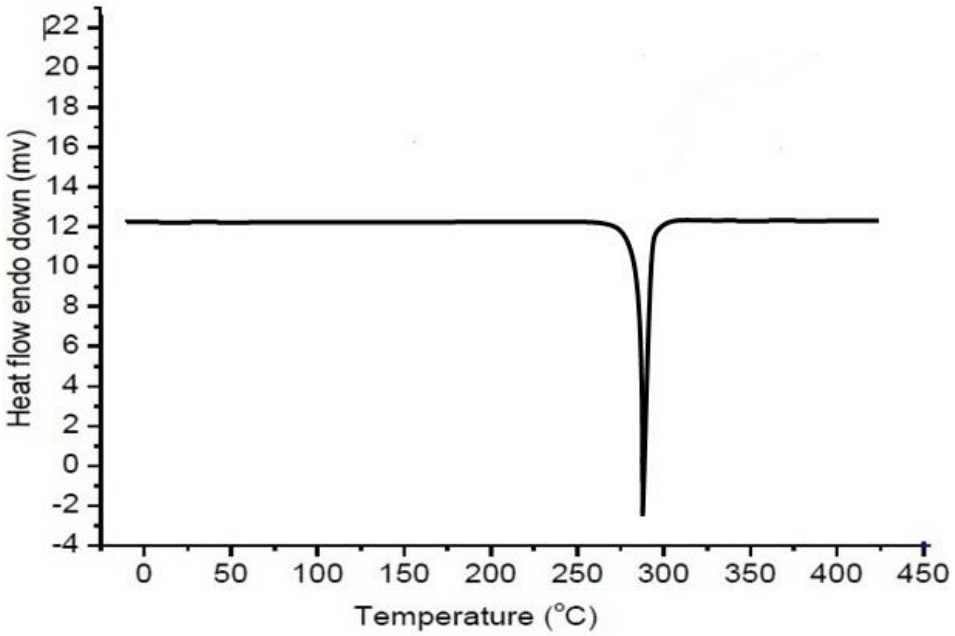

Figure 7. DSC thermogram of physical mixture of drug and chitosan.

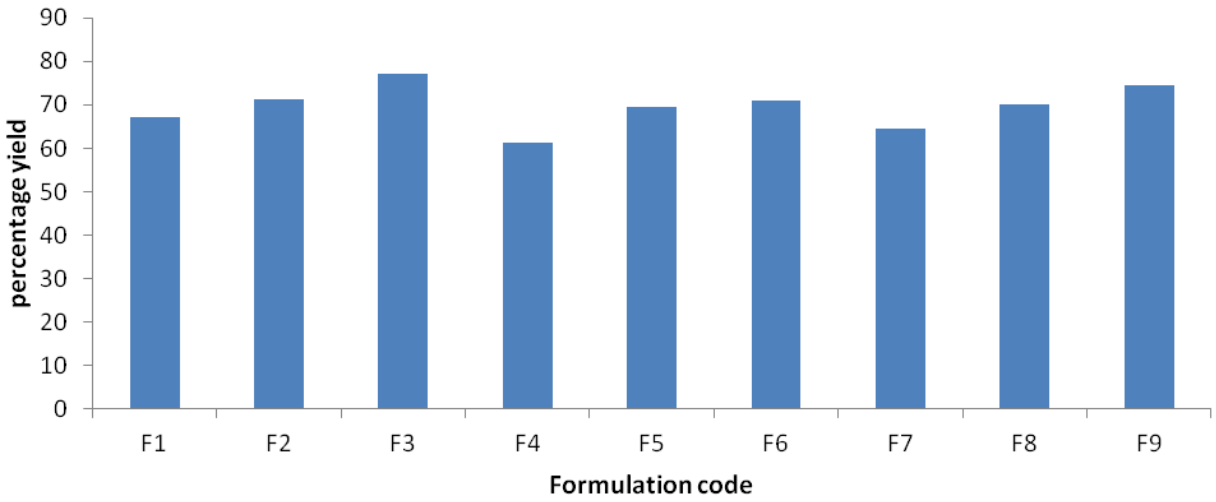

Figure 8. Percentage yield of formulations.

Table 2. Percentage yield of magnetic microspheres.

Formulation code	Percentage yield
F1	67.16
F2	71.14
F3	77.18
F4	61.27
F5	69.58
F6	71.09
F7	64.40
F8	70.23
F9	74.45

5. Particle size revealed that microspheres were in size ranging from 10 to 100 µm.
6. Increase in the amount of polymer added to the formulation increases the entrapment efficiency of both the drug and magnetite.
7. Increase in swelling ratio of microspheres was reported with increase in concentration of polymer with time.
8. The prepared magnetic microspheres of 5-amino-salicyclic acid were found to be magnetically responsive. The magnetic responsiveness increases with increase in entrapped magnetite content.
9. Overall, the curve fitting into various mathematical model

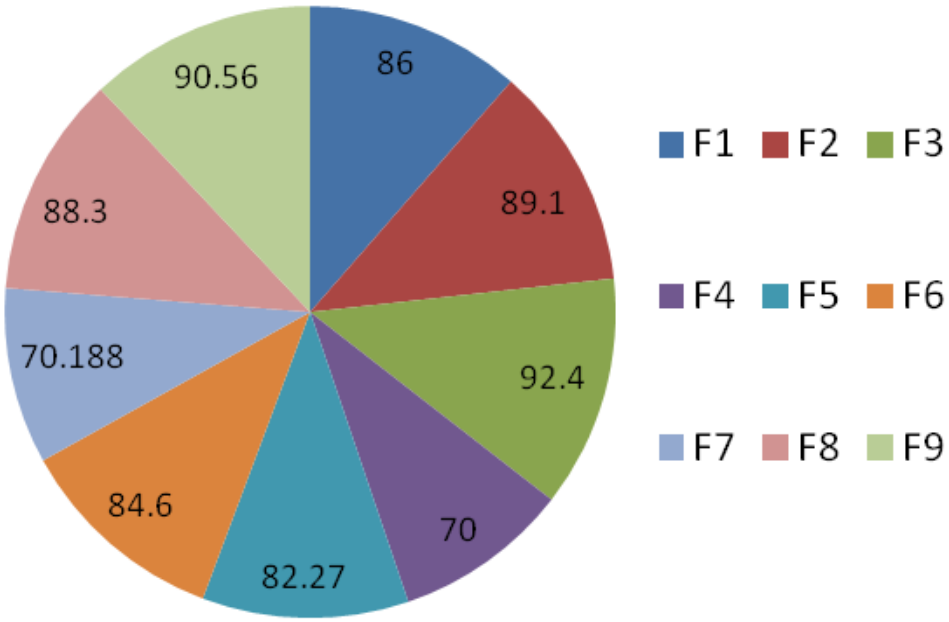

Figure 9. Entrapment efficiency of magnetic microspheres.

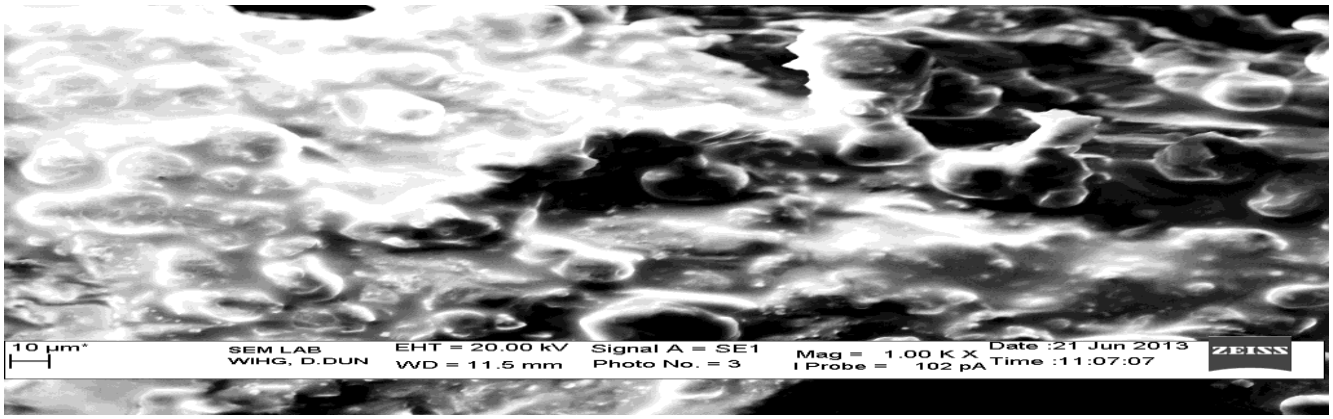

Figure 10. SEM image of F1 formulation.

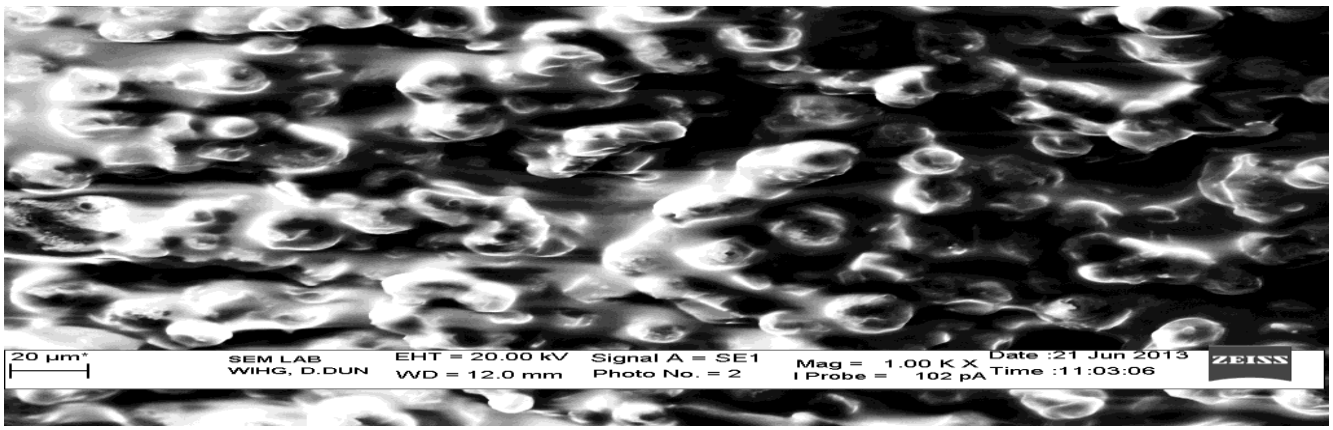

Figure 11. SEM image of Formulation F2.

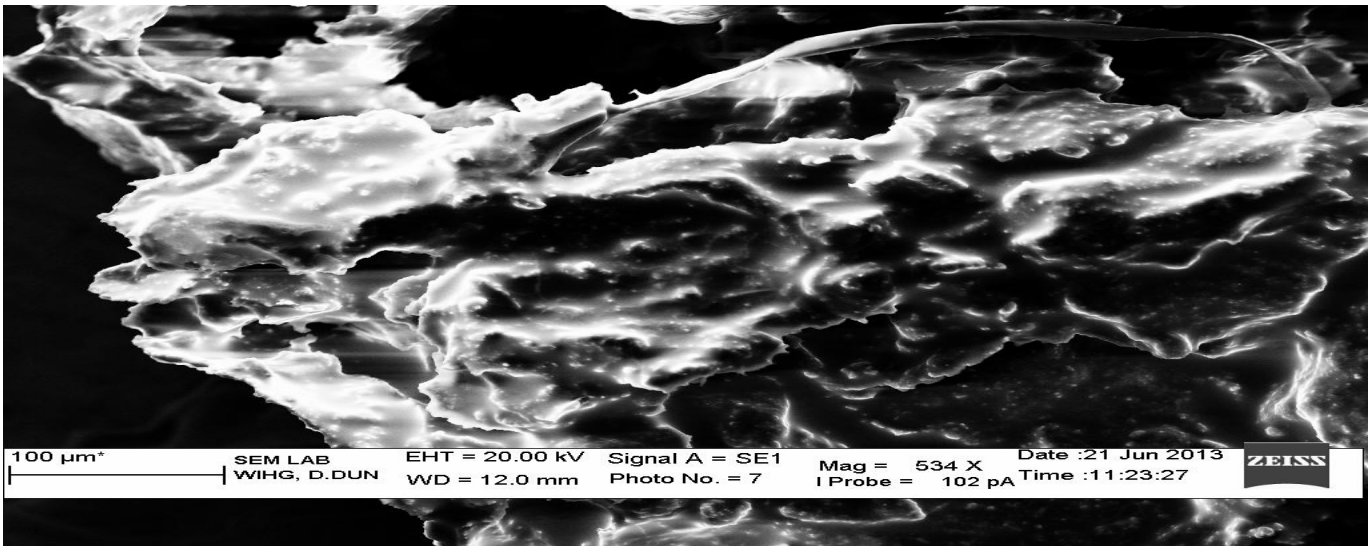

Figure 12. SEM image of formulation F3.

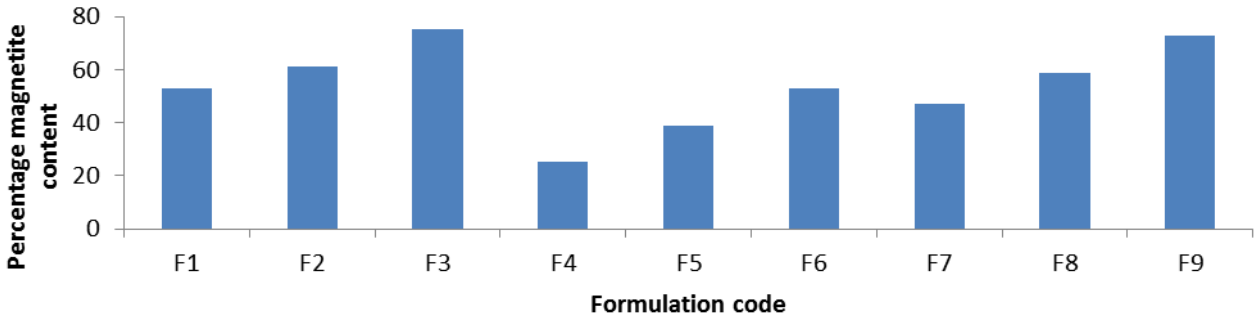

Figure 13. Percentage magnetite content entrapped.

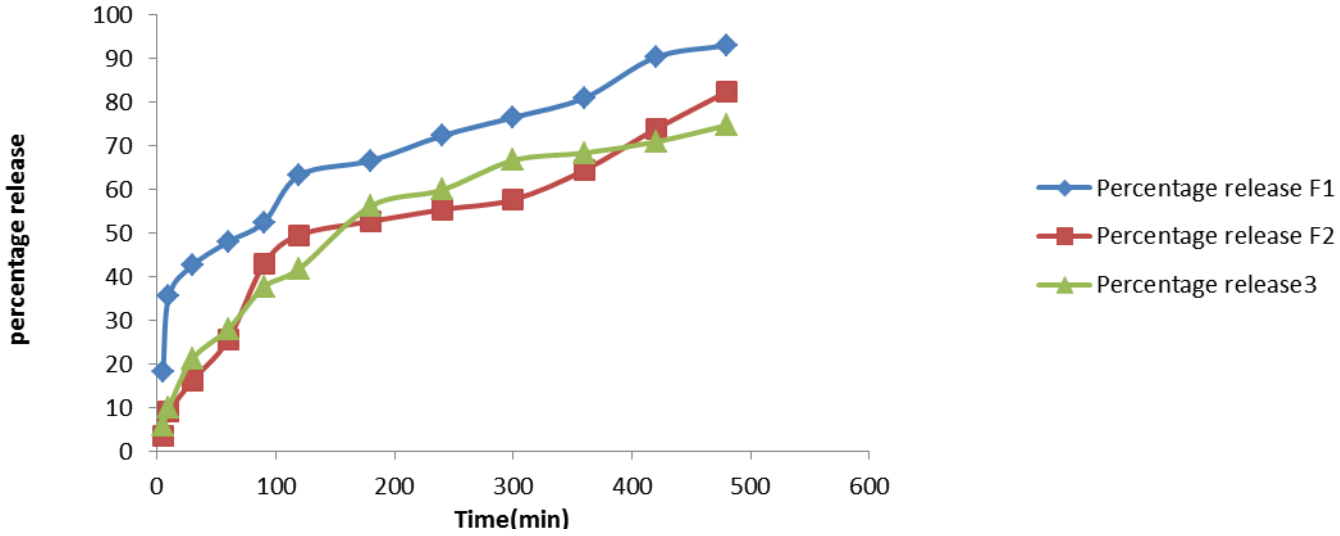

Figure 14. Comparison of dissolution study of formulations F1, F2 and F3.

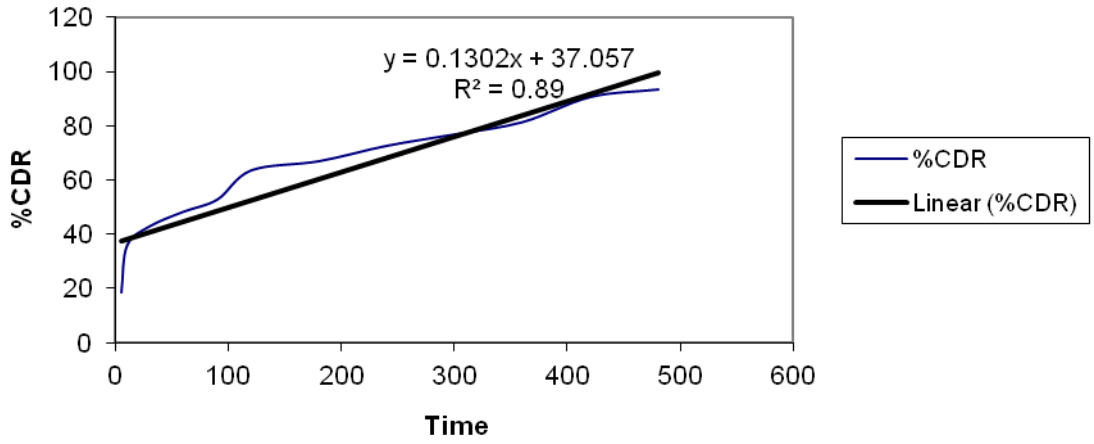

Figure 15. Zero order release kinetics of magnetic microspheres.

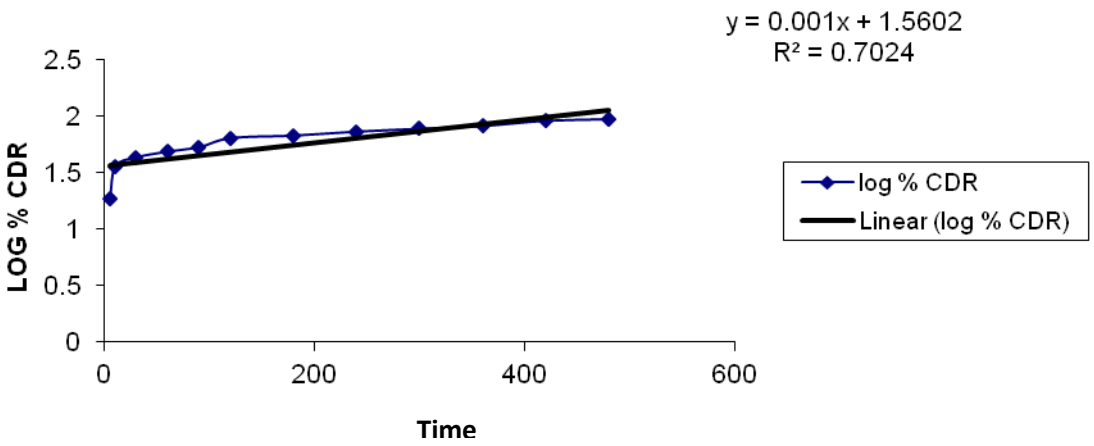

Figure 16. First order release kinetics magnetic microspheres.

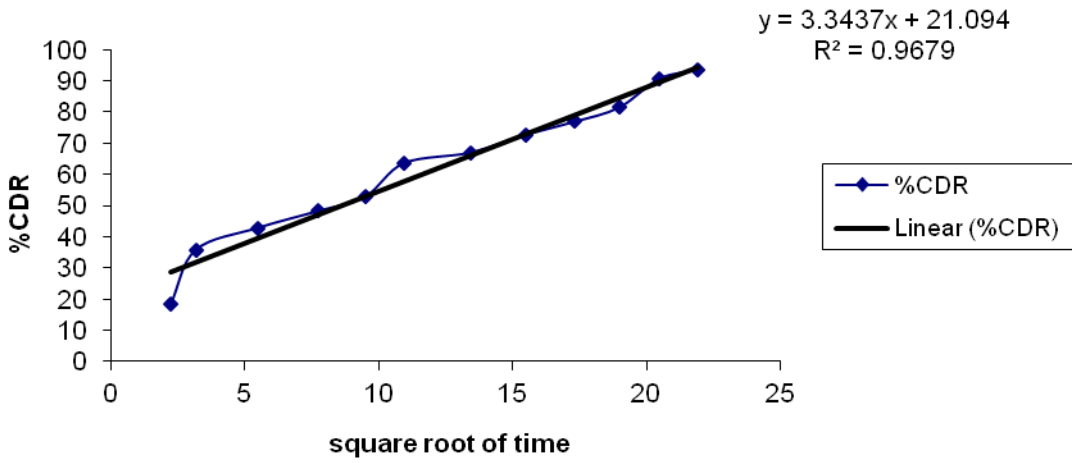

Figure 17. Higuchi model release kinetics of magnetic microspheres.

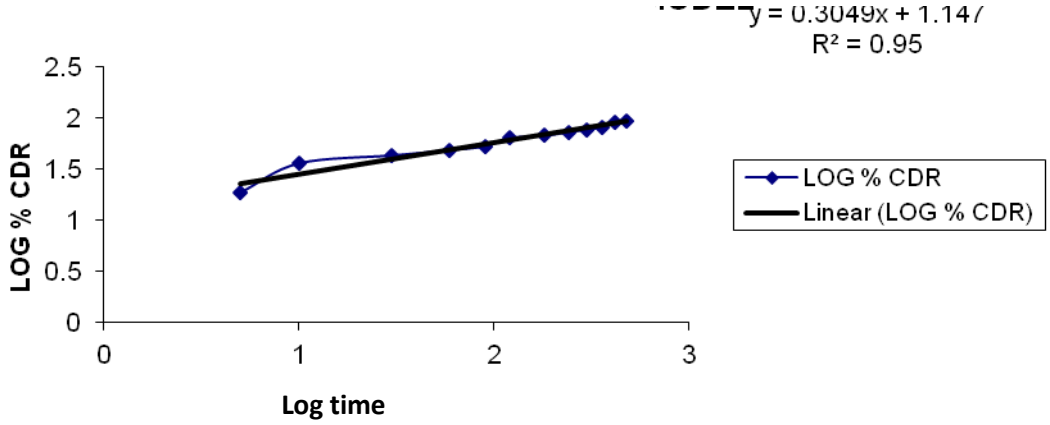

Figure 18. Korsemeyer Peppas model release kinetics of magnetic microspheres.

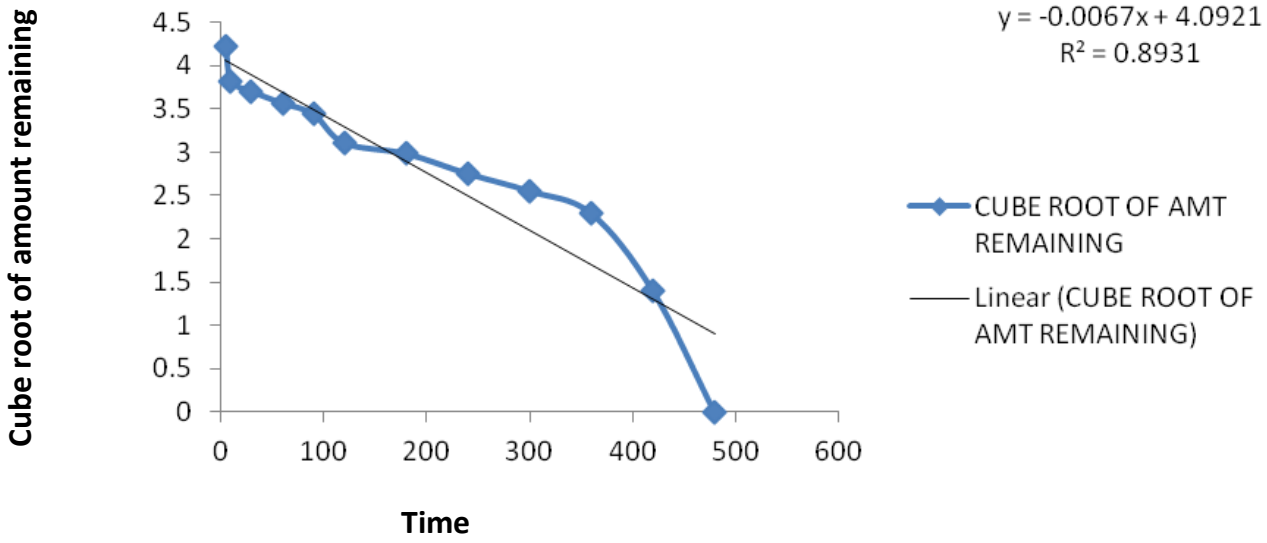

Figure 19. Hixon Crowell model release kinetics of magnetic microspheres.

Table 3. Flow characteristics of magnetic microspheres.

Code	Carr's index (%)	Hausner's ratio	Angle of repose	Flow character
F1	9.89	1.17	27.5	Excellent
F2	16.47	1.19	30	Good
F3	9.56	1.0	27.5	Excellent
F4	26.9	1.43	40.99	Passable
F5	27.3	1.23	42.28	Passable
F6	30.1	1.22	44.36	Poor
F7	10.6	1.2	27.6	Excellent
F8	19.8	1.4	25.5	Fair
F9	22.8	1.3	29.5	Fair

models was found to be average. It was found that formulation F-3 (chitosan 1:3) obeys Higuchi model.

10. On the basis of drug content, magnetic responsiveness, particle size morphology, *in vitro* release, release kinetics formulation F-3 (chitosan 1:3) was found to be most optimized.

Table 4. Drug content and entrapment efficiency of magnetic microspheres.

Formulation code	Drug content (%)	Entrapment efficiency (%)
F1	48	86.0
F2	31.3	89.1
F3	26.3	92.4
F4	43	70
F5	29.66	82.27
F6	26.33	84.6
F7	44.6	70.188
F8	31.3	88.3
F9	26.3	90.56

Table 5. Particle size of the formulated microspheres.

Code	Particle size (µm)
F1	10
F2	20
F3	100
F4	10
F5	20
F6	30
F7	10
F8	20
F9	100

Table 6. Percentage magnetite entrapped in magnetic microspheres.

Formulations code	Percentage magnetite entrapped
F1	51
F2	59
F3	73
F4	23
F5	37
F6	51
F7	45
F8	56.5
F9	70.605

Table 7. Percentage release of the formulated magnetic microspheres.

Formulation code	Percentage release
F1	95.00
F2	84.60
F3	76.10
F4	82.89
F5	76.98
F6	74.90
F7	86.67
F8	80.90
F9	76.90

DISCUSSION

As drug: polymer ratio increases the release of drug decreases due to formation of a rigid polymer matrix. Also, with increase in drug: polymer ratio particle size increases, thus surface area is decreased and release of drug is retarded. This proposed a method for targeted drug delivery by applying high magnetic field gradients within the body to an injected super paramagnetic fluid carrying the drug with the help of modest uniform magnetic field. 5-ASA was used as a model drug. In the present study, an attempt was made to formulate 5-ASA magnetic microspheres in order to study targeting efficiency, enhance bioavailability, reduce dose, thereby improving patient compliance.

Conflict of Interest

No competing interests were disclosed.

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African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Toxicological evaluations of ethanolic crude seed extract of *Corchorus olitorius*

Maxwell Osaronowen Egua[1]*, Emmanuel Udo Etuk[1], Shaibu Oricha Bello[1] and Sanusi Wara Hassan[2]

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The toxicological effect of the ethanolic seed extract of *Corchorus olitorius* was evaluated in white albino rats as a prelude to further pharmacology assessment of the plant seed extract. The acute, sub acute and chronic effects of the extract following oral administration in the animal were studied. The lethal medium dose (LD_{50}) of the extract was estimated to be higher than 5000 mg/kg. Oral administration of 250, 500 and 1250 mg/mg doses daily for 28 days did not produce any death among the rats. But there was significant ($P < 0.05$) and dose dependent increase in the renal biochemical parameters (sodium, potassium and urea level) and the liver biochemical parameters [the alkaline phosphatase (AP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), conjugated bilirubin and total bilirubin] supported by histological evidences (tubular necrosis in the kidney and from hepatic fatty change to multifocal hepatocyte necrosis in the liver). In the chronic 90 days study, where even higher doses at 1250, 2500 and 3750 mg/kg were administered orally, in which there were mortalities recorded. Renal biochemical parameters showed a significant ($P < 0.001$) decrease in sodium, increase in potassium and increase in urea while the AST, ALT and albumin were all significantly ($P < 0.05$) increased in the liver. The derangements were all supported by histological evidences, mild medulla lyphiod aggregation, multifocal area of renal lyphiod aggregations, portal vein congestion and multifocal hepatic necrosis. The heamatological system showed an increase in the lymphocyte count ($P < 0.05$) and ($P < 0.001$) in both the 28 and 90 days studies, respectively.

Key words: *Corchorus olitorius* (CO), lethal medium dose (LD_{50}), alkaline phosphatase, alanine aminotransferase.

INTRODUCTION

Herbal medicine or medicinal practice is of great importance to man and his health (Franstisek, 1998). In Africa, hundreds of plants are used traditionally for the management of various diseases. To date, however, only a few of these African medicinal plants have received scientific scrutiny, despite the fact that the World Health Organization (WHO) has recommended that medical and scientific examinations of such plants should be undertaken (WHO, 1980). *Corchorus olitorius* (CO) is an annual, much-branched herb, 90 to 120 cm tall, with

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glabrous stems, leaves 6 to 10 cm long and 3.5 to 5 cm broad, with pale yellow flowers and black trigonous seeds (Kirtikar and Basu, 1975). The leaves of *C. olitorius* was reported to have hypoglycaemic effect (Abo et al., 2008) and high antibacterial activity (Adegoke and Adebayo, 2009).

The seed protein enriched diet was found to increase rats' body weight (Laskar et al., 1986). There was failure to produce adverse effects in young chicken, with levels of seeds (*C. olitorius*) up to 5% of the diet (Johnson and Toleman, 1984). The seeds were found to contain reasonable percentage of biologically active cardiac principals (Sharaf and Negm, 1969). The plant stem is a source of jute fibre, and folkloric uses includes, seeds for purgative, leaves for dysentery, fever, gonorrhea and demulcent (Watt and Breyer-Brandwijk, 1962). The part of the plant targeted in this study is the seed believed to have a greater hypoglycaemic effect (ethanolic extract) as claimed by a traditional medicinal practitioner. Also claimed is its lethality. The safety of the crude seed ethanolic extract of *C. olitorius* was evaluated in white albino rats as a prelude to the assessment of its hypoglycemic effect. The null hypothesis was adopted, that is, no toxicity effect exists, both in short or long term use.

MATERIALS AND METHODS

Plant

The plant seed was purchased in the market, identified by Mshelia H.E in the Department of Pharmacognosy and Ethnopharmacy, Pharmacy School, Usmanu Danfodiyo University, and a herbarium Voucher specimen number (PCG/UDUS/TIL/0002) was obtained.

Preparation of plant extract

The *C. olitorius* seeds were pulverized to powder using a blending machine. The soxhlet extractor was used for extraction of the dried powdered seed using ethanol (99.9%). 10 g of the powdered seed extract was placed inside the thimble made from thick filter paper, which was loaded into the main chamber of the soxhlet extractor. The soxhlet extractor was placed onto a flask containing the extraction solvent (ethanol). The soxhlet was equipped with a condenser. The solvent was heated to reflux and the solvent vapour travelled up the distillation arm and flooded the chamber housing the thimble of solid and around the condenser. The condenser ensured that the solvent vapours cooled and dripped down into the chamber housing the solid material. The chamber containing the solid material slowly filled with warm solvents with some of the desired compound dissolved in the warm solvent. When the soxhlet chamber was almost full, the chamber was emptied by a siphon side arm. This cycle was allowed to repeat many times, over hours and days. During each cycle, a portion of the non-volatile compound (powdered extract) dissolved in the solvent. After many cycles, the desired compound was concentrated in the distillation flask. The advantage of this system was that instead of many portions of warm solvent passed through the sample; just one batch of solvent was recycled. The non-soluble portion of the extract remained in the thimble and was discarded. The extract isolated was kept at −20°C until tested.

Laboratory animals

Albino rats of both sexes from the Biological Sciences Department of Usmanu Danfodiyo University (UDUS) were used for the study. The rats were housed in metal cages in the laboratory at temperature between 35 to 37°C; 12 h/12 h light/dark cycle and maintained with free access to standard rat feeds and water for 7 days before experimentation. 12 h before experimentation, food was withdrawn but water was available *ad libitum*.

Acute oral toxicity studies

The Organisation for Economic Co-operation and Development (OECD) 420 guideline for testing of chemicals (2001) (acute oral toxicity-fixed dose procedure) was used. The seed extract of *C. olitorius* (5000 mg/kg body weight) were administered to five (5) female rats (one after the other at an observation period of 24 h) in a single oral dose using a feeding tube. The dose was chosen following a sighting study conducted using the following doses 5, 50, 300, and 2000 mg/kg. Observations for toxic symptoms was made and recorded systematically, 30 min, 1, 2, 3, 4, 5 and 6 h after administration. Finally the number of survival was noted after 72 h and 14 days for each animal. The toxicological effect was assessed on the basis of mortality, which was expressed as LD_{50}.

Sub acute toxicity

Thirty two (32) rats was randomly selected and divided into four groups of animals labeled A to D, and each group with 8 rats of equal sexes. The initial and weekly weights of the rats were recorded. The animals in group D was left without extract administration to serve as control while those in Groups A, B and C received low (250 mg/kg), medium (500 mg/kg) and high (1250 mg/kg) doses of the herbal extract, respectively daily. Administration of the extract was oral by feeding tube for 28 days. Male rats were separated from female within the groups. The fluid and water consumption of the animals was observed daily. Their various body weights were recorded weekly throughout the period of the study. On the last day of the experiment (29th day), blood samples were collected through cardiac puncture following chloroform anaesthesia. Blood samples for biochemical analysis were collected in non-heparinized bottles and that for hematological studies in ethylenediaminetetraacetic acid (EDTA) bottle. The liver, kidney, pancreas and heart were collected and stored in 10% formalin for histological study. Any rats that died during the test period were analyzed for pathological lesions.

Chronic toxicity

Thirty two (32) rats was randomly selected and divided into four groups of animals labeled A to D. Very high doses were chosen for this study to simulate its local use and because of its safety profile on LD_{50} estimation as well as the knowledge of the results obtained from the sub-acute toxicity not reflecting the lethality warned of by the traditional users. The initial and weekly weights of the rats were recorded. The animals in group D was left without extract administration to serve as control, while those in Groups A, B and C received 1250 mg/kg (0.25 of LD_{50}), 2500 mg/kg (0.5 of LD_{50}) and 3750 mg/kg (0.75 of LD_{50}) doses of the herbal extract, respectively daily. Administration of the extract was oral by feeding tube for 90 days (Loomis, 1996). Male rats were separated from female within the groups. The fluid and water consumption of the animals was observed daily. Their various body weights were recorded weekly throughout the period of the study. On the last day of the experiment (91st day), blood samples were collected through

cardiac puncture following chloroform anaesthesia. Blood samples for biochemical analysis were collected in non-heparinized bottles and that for hematological studies in EDTA bottle. The liver, kidney, pancreas and heart were collected and stored in 10% formalin for histological study. Any rat that died during the test period was tested pathologically.

Haematological studies

The blood sample was collected and inserted in EDTA bottle. Several parameters were determined, the packed cell volume (PCV), red blood cell count (RBC) and white blood cell count (WBC) by a computerized method using the Swelab Alfa Auto.

Biochemical studies

The blood samples collected was centrifuged, using centrifuge model 8000D for 5 min and the sera was collected in plain test tubes and stored in the deep freezer at -17°C until required for the following analysis.

The Randox kit procedure of Richmond (1973) was used for Cholesterol determination.

The Jendanssik and Gof (1997) colorimetric method was used (Randox kit) for bilirubin determination.

The Randox kit method of Doumac et al. (1997) was used for albumin determination.

The Randox kit colorimetric method of Sood (1999) was used for alkaline phosphate determination.

The Randox kit, Reitman et al. (1957) method was used for alanine amino transferase (ALT).

The Randox kit, Reitman et al. (1957) method was used for aspartate amino transferase (AST) determination

Randox kit, Rec (1972) Urease-Bertholot colorimetric method was used for Urea determination while Teco diagnostics kit (1996) method was used for urea determination.

Slyke titration method, Van slyke (1992) was used for bicarbonate determination.

Sodium and potassium concentrations of electrolyte were determined using the flame photometry method; flame photometer model – 6400A manufactured by Bran Science and Instrument Co. Eng. was used. A dilution of 1:100 with distilled water was done by pipetting 0.1 ml of serum into 10 ml of distilled water. The 6400A flame photometer was used only after the liquid of standard concentration was made. To have the comparison determination, absolute value of the concentration of electrolyte sorted (Na or K) were read off the machine following sample introduction and switch to either electrolyte (Na or K) being tested was effected.

Histopathological studies

The animals were sacrificed after general anesthesia, with chloroform, a day after the last dose of the drug administration. At autopsy, the liver, heart, kidney and pancreas of each rat were removed and weighed. The tissue samples were then fixed in formalin for histopathological examination. The tissues were fixed in formalin-acetic acid fixative, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin for histological examination.

Statistical analysis

Results were expressed as Mean ± standard deviation (SD). The analysis of variance (ANOVA) was used to compare means using Tukey Kramer test. Statistical significances was considered at $p \leq 0.05$.

RESULTS

Soxhlet extraction yield of ethanolic extract of *C. olitorius*

10 yields were calculated and the mean yield calculated from these.

$$\text{Yield 1} = \frac{\text{Weight loss by thimble } (v_1 - v_2)}{\text{Weight of sample } (v_0)} \times 100$$

v_1 = weight of residue = 49.39 g
v_2 = weight of empty filter paper = 1.56 g
v_0 = weight of sample = 50 g
= 95.66% = % weight loss by thimble
Therefore % yield = 100% - 95.66
Yield 1 = 4.34%

$$\text{Mean yield} = \frac{4.34 + 4.7 + 4.46 + 5.12 + 4.74 + 4.7 + 4.56 + 4.9 + 4.82 + 4.68}{10} = 4.70\%$$

Acute oral toxicity studies

No death was recorded following the single dose administration in either the control or treated groups given 5 g/kg of ethanolic seed extract of *C. olitorius* orally. The animals were however noticed to be hypoactive (slower) in the first 24 h on comparing with the control. The animals did not show any other changes in general behavior or other physiological activities. The LD_{50} of the extract was estimated to be greater than 5000 mg/kg.

Sub acute toxicity

There were no obvious physical changes and no death recorded over the 28 days study in either the control or treated groups. The animals did not show any changes in general behavior or other physiological activities. There were no differences in water and feeds intake in both the control and the treatment groups. There were no significant changes in the weight of the rats in both the control and treated groups (Table 1). There were also no

Table 1. The effect of extract on body weight of rats treated for 28 days.

Week	Control A	250 mg/kg (B)	500 mg/kg (C)	1250 mg/kg (D)	Stat. (one-way ANOVA)
0	126.75±22.52	140.40±14.31	140.0±9.93	137.10±31.32	P=0.544 ($F_{3,28}$=0.7278)
1	140.73±28.0	173.28±17.02	161.0±16.68	156.43±34.39	P=0.1003 ($F_{3,28}$=2.288)
2	149.47±25.05	171.26±29.80	168.64±17.60	160.51±31.51	P=0.3675 ($F_{3,28}$=1.095)
3	153.48±23.47	179.73±31.67	176.48±18.63	167.01±32.42	P=0.2355 ($F_{3,28}$=1.502)
4	160.78±24.19	179.43±29.32	175.36±15.10	170.50±33.45	P=0.5510 $F_{3,28}$=0.7156
Mean wt.	146.29±24.19	168.03±23.56	164.65±14.61	158.29±32.11	P=0.3172 ($F_{3,28}$=1.230)
Mean wt. gain	34.03±10.10	38.98±24.18	35.35±15.20	33.4±12.62	P=0.9163 ($F_{3,28}$=0.1692)

Values are mean ± SD (n=8). *significant difference p<0.05

Table 2. The effect of extract on organ weight of rats treated for 28 days.

Organ	Control A	250 mg/kg (B)	500 mg/kg (C)	1250 mg/kg (D)	Stat. (one-way ANOVA)
Liver	4.98±0.41	5.08±0.77	5.47±0.51	4.68±0.78	P=0.1248 ($F_{3,28}$=2.085)
Pancreas	0.46±0.08	0.57±0.11	0.57±0.11	0.54±0.10	P=0.1067 ($F_{3,28}$=2.230)
Heart	0.51±0.07	0.54±0.07	0.54±0.08	0.53±0.08	P=0.7942 ($F_{3,28}$=0.3434)
Left kidney	0.47±0.07	0.51±0.05	0.54±0.1	0.51±0.09	P=0.5334 ($F_{3,28}$=0.7467)
Right kidney	0.47±0.06	0.51±0.07	0.55±0.09	0.51±0.61	P=0.2035 ($F_{3,28}$=1.636)

Values are mean ± SD (n=8). *significant difference p<0.05.

Table 3. The effect of extract on organ weight relative to body weight of rats treated for 28 days.

Organ	Control (A)	250 mg/kg (B)	500 mg/kg (C)	1250 mg/kg (D)	Stat. (one-way ANOVA)
Liver	3.13E2±2.48E3	2.85E2±3.21E3	3.14E2±3.79E3	2.76E2±2.39E3	P=0.0321* ($F_{3,28}$=3.377)
Pancreas	2.65E3±1.09E3	3.19E3±4.26E4	3.26E3±6.13E4	3.21E3±5.87E4	P=0.3002 ($F_{3,28}$=1.279)
Heart	3.16E3±3.63E4	3.07E3±5.39E4	3.82E3±2.15E3	3.16E3±3.08E4	P=0.5356 ($F_{3,28}$=0.7428)
Left kidney	2.97E3±1.42E4	2.90E3±3.25E4	3.04E3±4.45E4	2.99E3±1.78E4	P=0.8252 (0.2999)
Right kidney	3.77E3±2.38E3	2.84E3±1.93E4	3.11E3±3.55E4	3.08E3±3.60E4	P=0.4820 ($F_{3,28}$=0.8428)

Values are mean ± SD (n=8). *significant difference p<0.05 E=exponential. *In stat column indicate column means is significantly greater than expected by chance.

changes noticed in the organ weights of rats as well as organ weight relative to body weight (Tables 2 and 3). The organs of both control and treated groups were unremarkable and comparable to each sex.

Sub acute toxicity hematological and biochemical observations

The hematological analysis (Table 4) showed no significant differences in some of the parameters examined in either the control or treated groups. But the white blood cell count (WBC) and the lymphocyte count had a dose dependent rise from the control, significantly ($p < 0.05$). There was a rise in the red blood cell count (RBC) and packed cell volume (PCV), though insignificantly in the extract treated groups. There was a significant difference of the treatment groups from the control in all the liver biochemical parameter except for total protein and albumin that showed none (Table 5). Alkaline phosphatase showed a highly significantly dose dependant rise with $p < 0.001$ in the 1250 mg/kg group. The ALT in all the treatment groups showed a highly sig-nificant dose dependant rise with $p < 0.001$ on comparing with control. The AST showed a highly significant dose dependent rise with $p < 0.001$ in the 500 and 1250 mg/kg groups.

Total bilirubin also had a highly significantly dose dependent rise with $p < 0.001$ in all the treatment groups. But the conjugated bilirubin had a significant difference from the control in both the 500 and the 1250 mg/kg groups. The cholesterol also had a significant ($p < 0.001$) dose dependent rise between the treatment groups and the control (Table 5). The electrolyte urea and creatinine results all showed a significant rise, dose dependently from the control (Table 6). Sodium had a rise that was highly significant ($p < 0.001$) in the 500 and 1250 mg/kg groups. Potassium was highly significant ($p < 0.001$) in the 1250 mg/kg group and only significant ($p < 0.05$) in

Table 4. Heamatological parameters of rats treated with extract for 28 days.

Parameter	Control (A)	250 mg/kg (B)	500 mg/kg (C)	1250 mg/kg (D)	Stat. (one-way ANOVA)
RBC×10^{12}/L	5.95±0.57	5.92±1.83	6.78±0.65	7.04±0.43	P=0.2499 ($F_{3,21}$=1.476)
RDW%	21.38±6.68	20.69±4.44	22.33±3.76	19.78±2.06	P=0.8004 ($F_{3,22}$=0.3347)
MCVFl	65.98±8.30	60.21±9.24	60.63±4.64	57.7±9.83	P=0.3904 ($F_{3,22}$=1.050)
PCV%	39.80±2.76	34.41±8.98	41.21±5.66	40.58±7.12	P=0.2207 ($F_{3,22}$=1.888)
HGB	12.40±0.56	11.10±2.83	12.43±1.33	13.5±1.12	P=0.1732 ($F_{3,21}$= 1.827)
WBC×10^{9}/L	2.80±1.63	3.46±2.00	6.51±1.79*	6.66±2.61*	P=0.0029* ($F_{3,22}$=6.351)
Lym×10^{9}/L	2.57±1.44	3.20±1.94	6.00±1.71*	5.82±2.31*	P=0.0046* ($F_{3,22}$=5.765)
Gran×10^{9}/L	0.10±0.2	0.10±0.14	0.24±0.18	0.28±0.13	P=0.1417 ($F_{3,28}$=2.012)
PLT×10^{9}/L	308.33±112.6	325.50±68.48	346.43±99.59	288.00±96.37	P=0.7399 ($F_{3,28}$=0.4208)

Values are mean ± SD (n=8). *significant difference(p<0.05) **significant difference (p<0.01) and ** *significant difference(p<0.001). *In stat column indicate column means is significantly greater than expected by chance.

Table 5. Liver function test of rats treated with extract for 28 days

Parameter	Control (A)	250 mg/kg (B)	500 mg/kg (C)	1250 mg/kg (D)	Stat. (one-way ANOVA)
Alkaline phosphatase	140.76±6.73	144.00±7.42	150.96±6.41	208.05±46.69***	P=0.0001* ($F_{3,28}$=13.847)
SGPT u/L (ALT)	14.22±1.39	18.36±1.90***	20.10±1.39***	21.08±0.87***	P=0.0001* ($F_{3,28}$=35.646)
SGOT u/L (AST)	123.10±5.19	138.46±4.05	306.2±72.48***	295.98±147.76***	P=0.0001* ($F_{3,28}$=16.24)
Conjugated bilirubin (µmol/L)	0.12±0.01	0.149±0.031	0.154±0.018*	0.154±0.019*	P=0.0150* ($F_{3,28}$=4.14)
Total bilirubin (µmol/L)	0.29±0.06	0.44±0.05***	0.46±0.09***	0.52±0.03***	P=0.0001* ($F_{3,28}$=21.215)
Total protein (g/l)	5.19±0.47	5.31±0.36	5.16±0.38	5.30±0.30	P=0.8100 ($F_{3,28}$=0.3211)
Albumin (µmol/L)	2.31±0.29	2.33±0.20	2.41±0.29	2.46±0.18	P=0.5662 ($F_{3,28}$=0.6894)
Cholesterol (mmol/L)	120.50±2.67	127.25±3.92**	129.88±4.39***	131.88±1.73***	P=0.0001* ($F_{3,28}$=17.587)

Values are mean ± SD (n=8). *significant difference (p<0.05) and ***significant difference (p<0.001). *In stat column indicate column means is significantly greater than expected

Table 6. Renal biochemical parameters of rats treated with extract for 28 days.

Parameter	Control (A)	250 mg/kg (B)	500 mg/kg (C)	1250 mg/kg (D)	Stat. (one-way ANOVA)
Sodium (Mmol)	133.25±1.83	138.00±2.27	146.37±5.48***	143.88±±5.41***	P=0.0001* ($F_{3,28}$=16.477)
Potassium (Mmol)	3.56±0.34	4.19±0.48	4.24±0.35*	4.64±0.68***	P=0.00014* ($F_{3,28}$=6.813)
Bicarbonate (Mmol)	20.63±0.52	20.25±0.71	20.00±0.76	19.25±1.04**	P=0.0109* (4.477)
Urea (mg/dl)	5.96±0.61	7.78±0.75	10.81±1.75***	17.44±2.78***	P=0.0001* ($F_{3,28}$=69.270)
Creatinine (mg/dl)	1.10±0.09	1.26±0.11	1.50±0.13*	2.50±0.50***	P=0.0001* ($F_{3,28}$=46.512)

Values are mean ± SD (n=8). *significant difference(p<0.05) **significant difference (p<0.01) and ***significant difference(p<0.001).

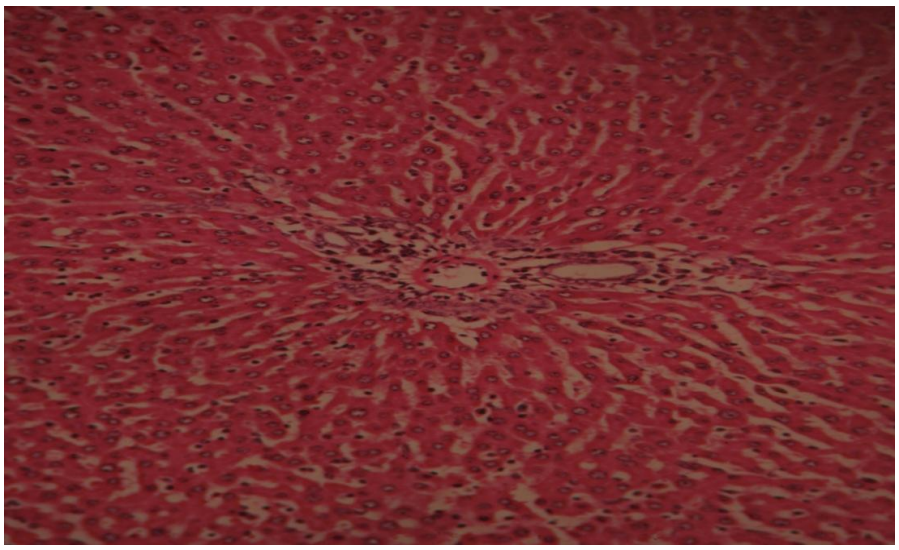

Figure 1. Photomicrograph of liver with portal tract(portal vein, hepatic artery and bile duct. H&E ×20.

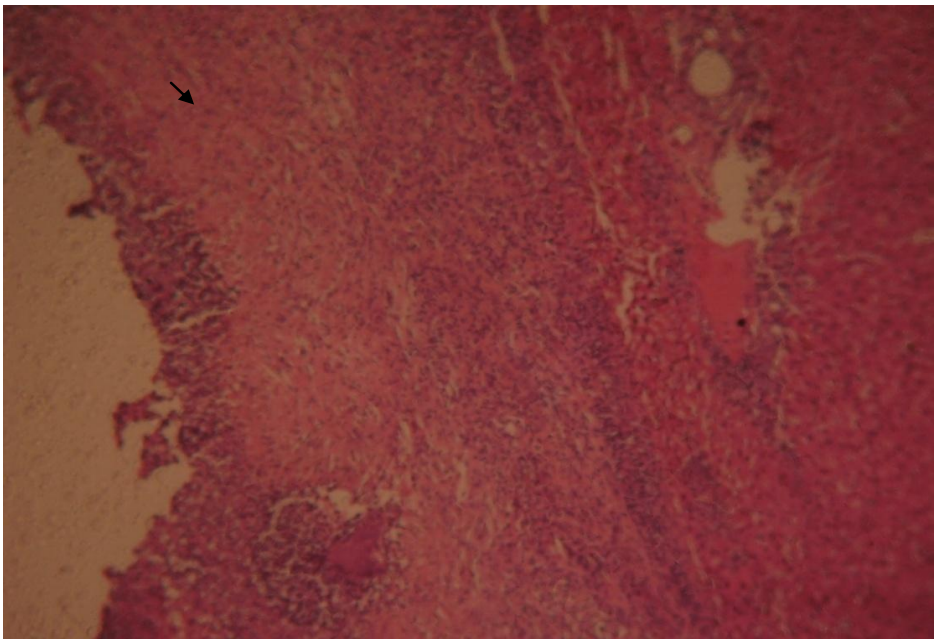

Figure 2. Photomicrograph of liver showing cyst with a mass of actinomycosis. H&E ×20.

the 500 mg/kg group. Bicarbonates had a fall in its level that was very significant ($p < 0.01$) in the 1250 mg/kg group. Urea level showed a highly significant ($p < 0.001$) rise in its 500 and 1250 mg groups. Creatinine was highly significant ($p < 0.001$) in the 1250 mg group and only significant ($p < 0.05$) in the 500 mg group.

Histopathological tissue analysis for the sub acute study

In the liver, the control group had a normal hepatic architecture maintained (Figure 1). The 250 mg/kg dose group was normal as in control. The 500 mg/kg dose group showed a cyst (actinomycosis) and others with mild hepatic necrosis (Figures 2 and 3). The 1250 mg/kg dose group showed hepatic fatty change (Figure 4) and vacuolar degeneration (Figure 5). The kidney of the rats treated with 250 and 500 mg/kg groups both had no pathological changes in the renal tissues (Figure 6). But the 1250 mg/kg group showed acute tubular necrosis (tubulorrhexis) (Figure 7). There were no obvious histopathological changes seen in both the heart and the pancreas of the rats (Figures 8 and 9), respectively.

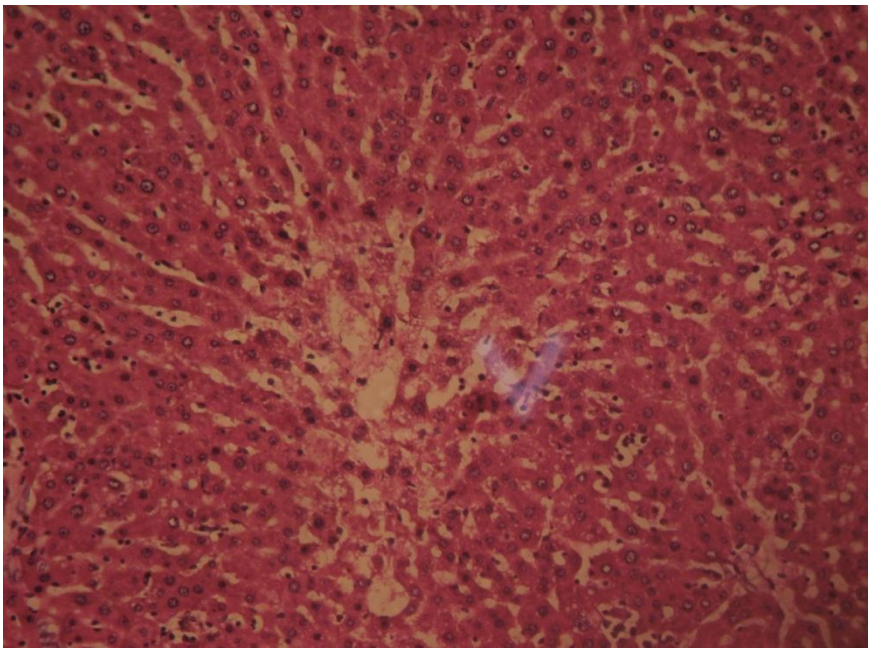

Figure 3. Photomicrograph of liver showing mild hepatocyte necrosis. H&E ×20.

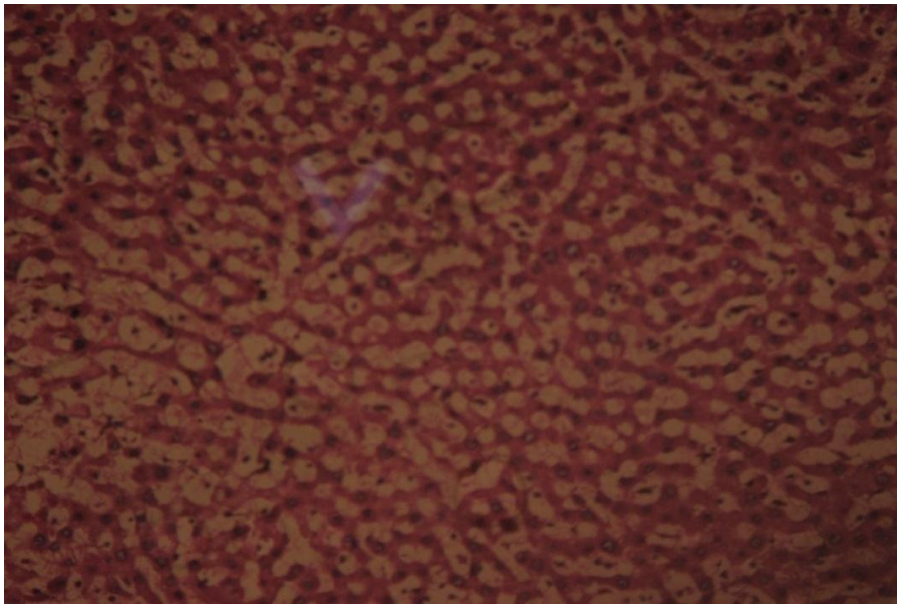

Figure 4. Photomicrograph of liver showing fatty change. H&E ×20.

Chronic toxicity study

There were changes and deaths in the treatment groups recorded over the 90 days study. The rats in the highest dose (3750 mg/kg) group had diarrhea/soft stool as from the 6th week of treatment and they were less active than the other groups. The rats that died in the low dose (1250 mg/kg) group did not show any sign of illness, and the deaths occured from the 20th day of study while those that died in the highest dose group (3750 mg/kg) were inactive for several days before dying (death occurring from the 34th day of study). There was no death recorded in the 2500 mg/kg dose and control groups. The weekly weight of the rats did not show any significant difference but the mean weight gain of rats over the period of study showed that there was a significant

Figure 5. Photomicrograph of liver showing vacuolar degeneration. H&E ×40.

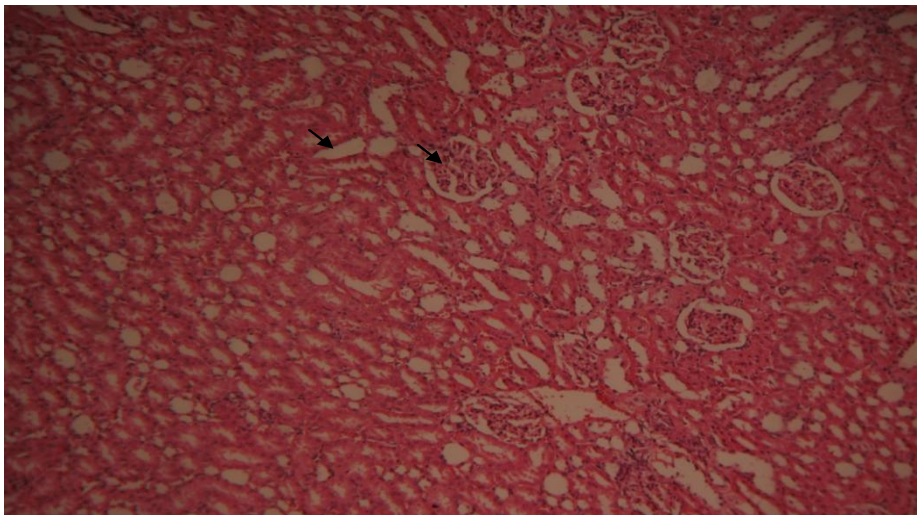

Figure 6. Photomicrograph of kidney showing normal tubules and glomeruli. H&E ×20.

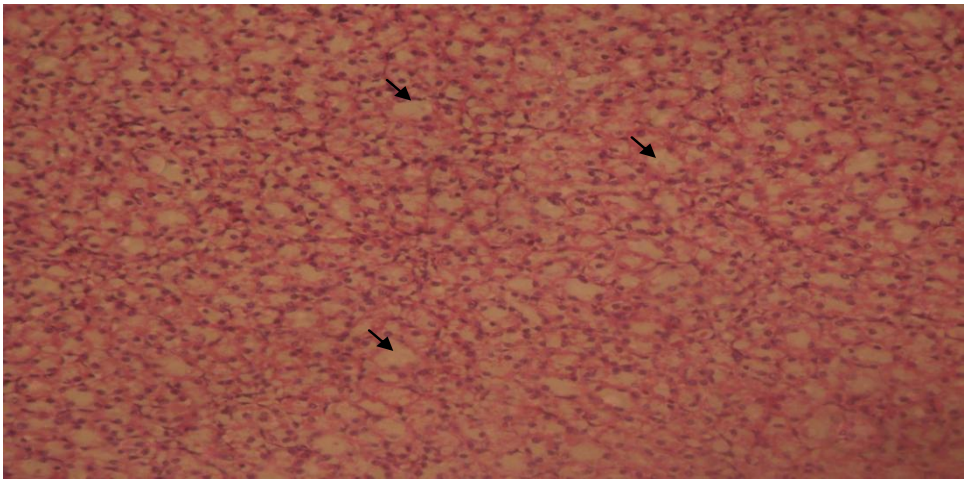

Figure 7. Photomicrograph of kidney showing tubular necrosis (tubulorrhexis). H&E ×20.

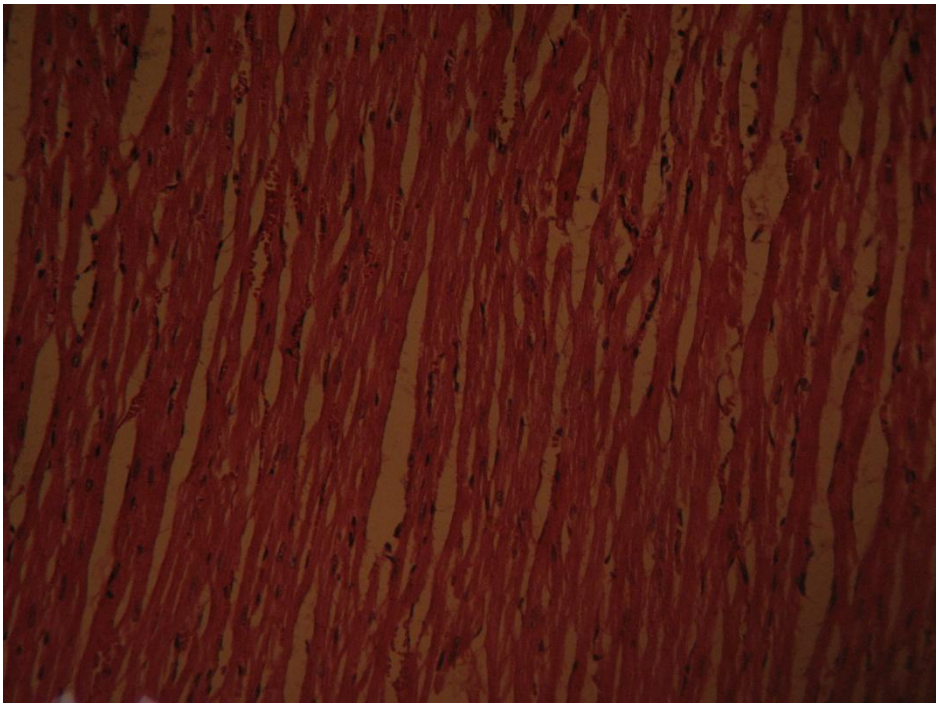

Figure 8. Normal cardiac muscle. H&E ×20.

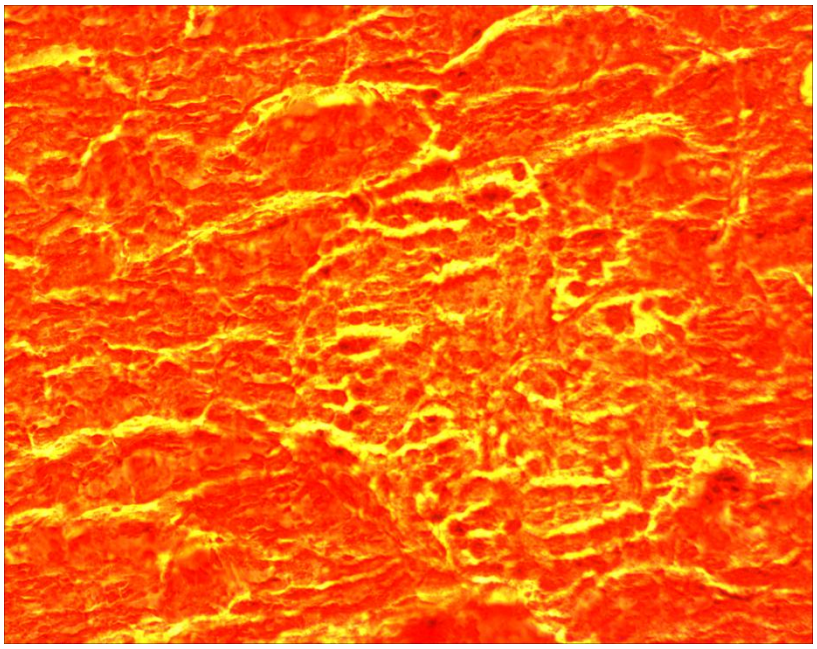

Figure 9. Normal pancreas. H&E ×20.

difference within the groups (Table 7). The rats in the medium dose group had the lowest weight gain that was of statistical significance. The control group had the highest weight gain. The heart of the rats was noticed to have reduced in weight significantly dose dependently (Table 8); the smallest being the 3750 mg/kg group and next being the 2500 mg/kg group significantly. The control group's heart was noticed to be larger than that of the treated groups. Other organ weights did not have any significant change within the groups. The trend was the same in the organ weight relative to body weight. The heart being of significance in organ weight relative to body weight in both the 1250 and 2500 mg/kg groups dose dependently (Table 9).

Table 7. The Effect of extract on the body weight (g) of rats treated for 90 days.

Week	Control A	Low dose (1250 mg) B	Medium dose (2500 mg) C	High dose (3750 mg) D	Stat. (one-way ANOVA)
0	168.41±38.41	180.35±35.54	198.88±43.02	179.15±28.99	P=0.4333 ($F_{3,28}$=0.9426)
1	183.11±35.65	182.41±42.54	194.78±47.88	173.20±27.81	P=0.7492 ($F_{3,28}$=0.4069)
2	197.40±34.33	193.88±43.39	198.46±50.60	176.74±31.89	P=0.6900 ($F_{3,28}$=0.4930)
3	199.01±35.17	205.22±52.41	202.96±53.42	177.89±35.24	P=0.6073 ($F_{3,28}$=0.6211)
4	214.33±38.90	221.81±40.90	207.55±57.02	172.71±35.48	P=0.1540 ($F_{3,28}$=1.897)
5	218.06±34.38	219.91±37.04	210.31±55.51	181.43±29.69	P=0.2779 ($F_{3,26}$=1.357)
6	232.84±30.03	225.07±36.71	217.84±64.88	182.24±28.64	P=0.1505 ($F_{3,26}$=1.924)
7	232.23±26.85	228.77±41.08	211.83±59.17	189.24±29.822	P=0.2096 ($F_{3,26}$=1.617)
8	228.94±31.85	234.73±41.19	216.91±61.59	188.9±36.87	P=0.2443 ($F_{3,26}$=1.476)
9	234.05±31.01	244.34±49.41	224.59±73.08	190.87±41.23	P=0.2563 ($F_{3,26}$=1.431)
10	236.11±30.77	250.54±52.44	229.58±71.57	205.12±42.49	P=0.4811 ($F_{3,25}$=0.8472)
11	239.38±29.71	257.43±56.53	228.35±71.94	207.58±43.44	P=0.4376 ($F_{3,24}$=0.9382)
12	241.75±31.73	258.88±58.03	226.35±70.74	212.05±44.54	P=0.4670 ($F_{3,24}$=0.8766)
13	248.37±33.04	258.52±54.47	228.53±67.42	215.80±49.03	P=0.4796 ($F_{3,24}$=0.8514)
Mean weight	219.69±31.28	228.40±45.92	214.03±59.60	193.72±35.47	P=0.5884 ($F_{3,24}$=0.6538)
Mean weight gain	83.00±26.68	74.65±34.55	29.57±39.98*	32.85±34.28	P=0.0096* ($F_{3,24}$=4.759)

Values are mean ± SD (n=8). *significant difference (p<0.05).

Table 8. Effect of extract on organ weight (g) of rats treated for 90 days.

Organ	Control A	Low dose (1250 mg) B	Medium dose (2500 mg) C	High dose (3750 mg) D	Stat. (one-way ANOVA)
Pancreas	0.56±0.09	0.64±0.16	0.61±0.13	0.67±0.08	P=0.3586 ($F_{3,24}$=1.125)
Right kidney	0.78±0.07	0.83±0.17	0.76±0.19	0.67±013	P=0.3252 ($F_{3,24}$=1.217)
Left kidney	0.79±0.05	0.80±0.17	0.72±0.20	0.64±0.13	P=0.2601 ($F_{3,24}$=1.425)
Liver	7.09±0.94	7.55±1.76	6.89±1.80	7.08±1.61	P=0.8815 ($F_{3,24}$=0.2200)
Heart	0.92±0.09	0.81±0.19	0.71±0.15*	0.68±0.08*	P=0.0086* ($F_{3,24}$=4.883)

Values are mean ± SD (n=8). *significant difference(p<0.05)* In stat column indicate column means is significantly greater than expected by chance.

Table 9. Effect of extract on organ weight relative to body weight of rats treated for 90 days.

Organ	Control A	Low dose (1250 mg) B	Medium dose (2500 mg) C	High dose (3750 mg) D	Stat. (one-way ANOVA)
Pancreas	2.33 3±7.36E4	2.48 E 3±2.8E4	2.78 E3±7.52E4	3.18 E3±5.25E4	P=0.09 ($F_{3,24}$=2.372)
Right kidney	3.06 E±5.15E4	3.22E3±3.40E4	3.36 E3±2.15E4	3.11 E3±1.85E4	P=0.3803 ($F_{3,24}$=1.07)
Left kidney	3.22E3±4.37E4	3.10E3±3.02E4	3.18 E3±3.05E4	2.96 E3±2.23E4	P=0.5194 ($F_{3,24}$=0.7750)
Liver	0.0286±0.0025	0.0292±0.0036	0.0286±0.0040	0.0337±0.0100	P=0.2964 ($F_{3,24}$=1.303)
Heart	3.75E3±4.79E4	3.11E3±2.91E4*	3.15 E3±3.25*	3.24 E3±5.39E4	P=0.0237* ($F_{3,24}$=3.778)

Values are mean ± SD (n=8). *significant difference(p<0.05). *In stat column indicate column means is significantly greater than expected by chance.

The WBC showed a highly significant rise in its level in the 3750 mg/kg dose group. All other haematological parameters were of no significant difference (Table 10). The liver, SGPT (ALT), showed a dose dependent rise in its level that was significant in the 3750 mg/kg dose group (Table 11). So did SGOT (AST) have a dose dependent rise in its levels that was significant in the 2500 and 3750 mg/kg groups. Albumin was severely raised raised significantly in the 3750 mg/kg group (Table 11). Cholesterol rather had a dose dependent fall in its level that was highly significant in the low dose (1250 mg/kg) group (Table 11). Renal biochemical parameter showed that sodium, potassium and urea level all had a significant change in their levels. The sodium had a drop while potassium and urea had rather a rise in their levels (Table 12).

Table 10. Heamatological parameters of rats treated with extract for 90 days.

Parameter	Control (A)	Low dose B	Medium dose C	High dose D	Stat. (one-way ANOVA)
RBC×10^{12}/L	7.17±0.64	6.65±0.28	6.73±0.40	6.99±0.92	P=0.3855 ($F_{3,21}$=1.064)
RDW%	16.71±0.77	15.34±0.28	16.43±0.98	17.60±1.80	P=0.0219* ($F_{3,21}$=3.966)
MCVFl	49.03±1.13	47.22±1.33	49.65±1.41	47.42±4.82	P=0.2478 ($F_{3,21}$=1.484)
PCV%	35.11±2.85	31.56±2.03	33.43±2.20	33.00±3.87	P=0.1844 ($F_{3,21}$=1.766)
HGB	12.91±1.00	11.68±0.84	12.33±0.80	12.26±1.53	P=0.2539 ($F_{3,21}$=1.460)
WBC×10^{9}/L	7.43±2.40	7.04±0.67	7.31±3.41	19.00±8.46***	P=0.0003* ($F_{3,21}$=9.650)
Lym×10^{9}/L	5.31±2.38	5.40±0.85	5.07±2.05	10.63±7.07	P=0.0521 ($F_{3,21}$=3.03)
Gran×10^{9}/L	1.29±0.69	0.90±0.37	1.43±0.37	3.74±4.45	P=0.1453 ($F_{3,21}$=1.997)
PLT×10^{9}/L	462.88±54.61	431.00±39.45	402.00± 57.70	475.60± 43.25	P=0.0727 ($F_{3,21}$=2.686)

Values are mean ± SD (n=8). *significant difference(p<0.05). ***significant difference(p<0.001). *In stat column indicate column means is significantly greater than expected by chance.

Table 11. Liver function test of rats treated for 90 days with extract.

Parameter	Control A	Low dose (1250 mg) B	Medium dose (2500 mg) C	High dose (3750 mg) D	Stat (one-way ANOVA)
Alkaline phosphatase (u/L)	48.63±13.04	25.20±25.94	42.60±16.16	54.17± 25.06	P=0.1453 ($F_{3,24}$=1.987)
SGPT u/L (ALT)	15.00±5.90	28.00±12.59	22.00±11.99	39.83± 24.78*	P=0.0328* ($F_{3,24}$=3.462)
SGOT u/L (AST)	32.50± 9.70	64.80± 28.39	73.63± 30.13*	73.83±36.02*	P=0.0192* ($F_{3,24}$=4.034)
Conjugated bilirubin (µmol/L)	0.68±0.25	0.14±0.00	0.39±0.53	0.38±0.28	P=0.1149 ($F_{3,24}$=2.215)
Total bilirubin (µmol/L)	1.27±0.31	0.72±0.42	1.44±0.41	3.32±4.41	P=0.1884 ($F_{3,23}$=1.732)
Total protein (g/L)	6.78±0.93	6.48±1.27	6.62±0.27	6.17±1.11	P=0.6651 ($F_{3,24}$=0.5314)
Albumin (µmol/L)	2.42±0.30	2.36±0.63	2.67±0.51	4.17±1.32**	P=0.0006* ($F_{3,22}$=8.293)
Cholesterol (mmol/L)	43.75±10.61	107.5±15.00***	56.25± 10.61	40.00±10.95	P=0.0001* ($F_{3,22}$=34.321)

Values are mean ± SD (n=8). *significant difference (p<0.05). **significant difference (p<0.01).

Table 12. Renal biochemical parameters of rats treated with extract for 90 days.

Parameter	Control A	Low dose B	Medium dose C	High dose D	Stat. (one-way ANOVA)
Sodium (Mmol)	148.50± 7.93	136.80±5.40**	131.38± 1.51***	136.00± 2.45***	P=0.0001* ($F_{3,23}$=16.004)
Potassium (Mmol)	4.35±1.00	6.66± 2.35	5.03±1.25	7.50±2.29*	P=0.009* ($F_{3,23}$=4.886)
Biocarbonate (Mmol)	21.00±1.77	21.17±2.99	22.00±2.88	22.17±1.72	P=0.7460 ($F_{3,24}$=0.4119)
Urea (mg/dl)	79.11±29.26	88.30±42.63	69.56±29.77	152.33±68.10*	P=0.0086* ($F_{3,24}$=4.887)
Creatinine (mg/dl)	0.84±0.42	0.75±0.29	0.70±0.26	0.55±0.05	P=0.3695 ($F_{3,24}$=0.3695)

Values are mean ± SD (n=8). *significant difference (p<0.05) **significant difference (p<0.01) and ***significant difference (p<0.001).

Histopathological tissue analysis for chronic toxicity

In the liver, the control group had a normal maintained hepatic architecture (Figure 10). The treatment groups all showed congestion of portal vein (Figure 11), portal triaditis (Figure 12) and multifocal hepatocyte necrosis (Figure 13). In the kidney, the control group showed a normal cortex and medulla with normal glomeruli and tubules (Figure 14). The 1250 and 2500 mg/kg groups showed mild focal lyphoid aggregation within the medulla (Figure 15). The 3750 mg/kg group showed multifocal multifocal areas of lyphoid aggregations (Figure 16)

The heart was normal histologically

In the pancreas, the control group displayed a normal endocrine and exocrine pancreatic architecture (Figure 17) rich with large islet cell clusters. The 1250 mg/kg group pancreatic tissue was as in the control. The 2500 mg/kg group showed a normal architecture but with fewer islet cell clusters noticed (Figure 18). These islet cells clusters were far much fewer in the 3750 mg/kg group

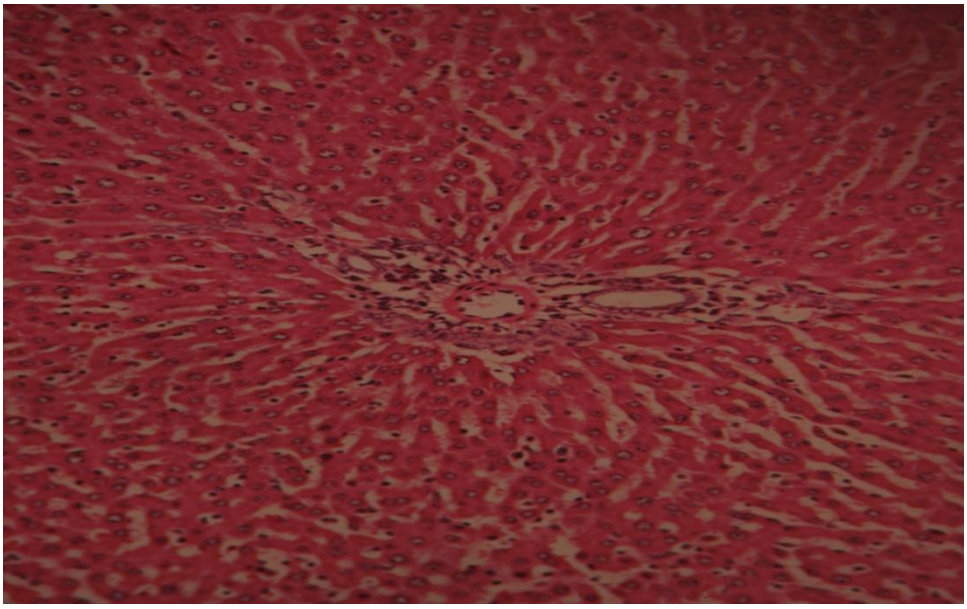

Figure 10. Normal hepatic tissue. H&E ×20.

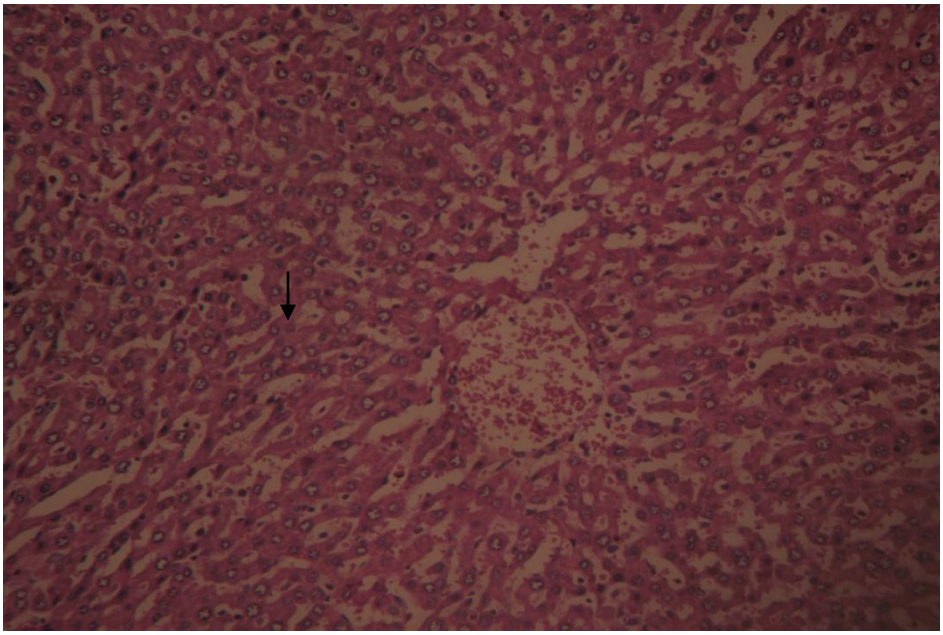

Figure 11. Photomicrograph of liver showing mild congestion of portal vein and background sinusoidal congestion. H&E ×20.

(Figures 19 and 20).

DISCUSSION

Previous toxicity studies gave much reference to the adverse effect of *C. olitorius* seeds on the heart. In this study, there were no deaths recorded following a single oral administration of 5 g/kg of ethanolic seed extract of *C. olitorius*. The LD_{50} of the extract was therefore estimated to be greater than 5000 mg/kg in albino rats. In earlier studies, the LD_{50} of a 10% alcoholic extract of the seeds in water were 0.75 g/kg BW in mice and 6 g/kg BW in toads (Sharaf and Negm, 1969). The test limit of 5000

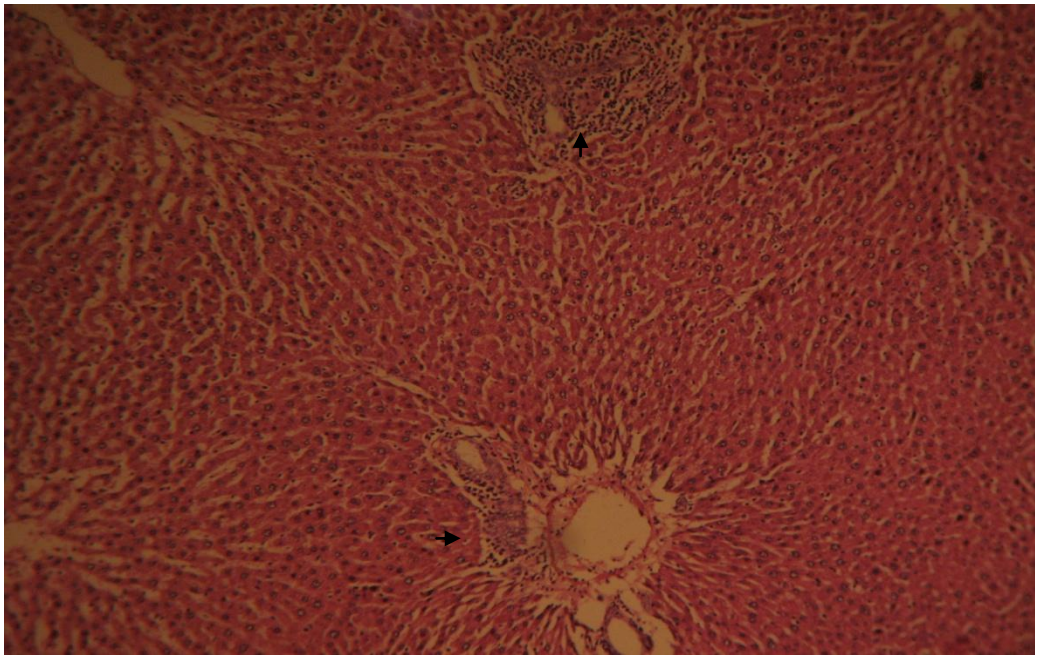

Figure 12. Photomicrograph of liver showing portal triaditis. H&E ×20.

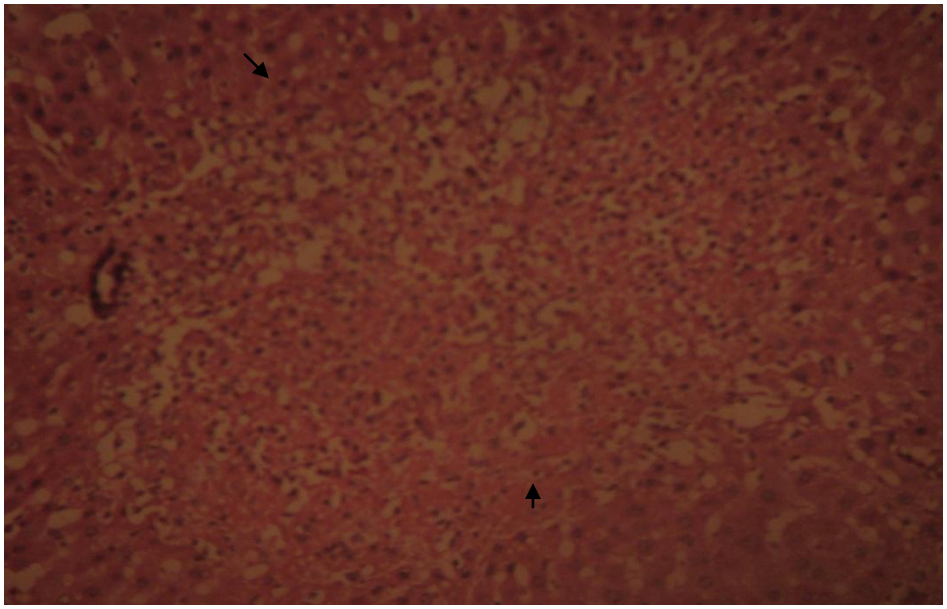

Figure 13. Photomichrograph of liver showing hepatocyte necrosis. H&E ×20.

mg/kg for acute oral toxicity is generally considered to be the point, at which it can be concluded that a test substance is practically non-toxic or non-lethal after an acute exposure (OECD, 2001).

In the sub acute toxicity study, in which rats were treated orally with 250, 500 and 1250 mg/kg of ethanolic seed extract of *C. olitorius* for 28 days, there were no deaths recorded also, indicating further the safety profile at these doses. But there were deaths recorded in the chronic toxicity study where even higher doses were employed (in the bid to exclude toxicity) over 90 days, at 1250, 2500 and 3750 mg/kg levels. The rats that died in the low dose (1250 mg/kg) group were believed to have died from fighting, since they were apparently healthy the previous day. While those that died in the highest dose group (3750 mg/kg) obviously resulted from the effect of

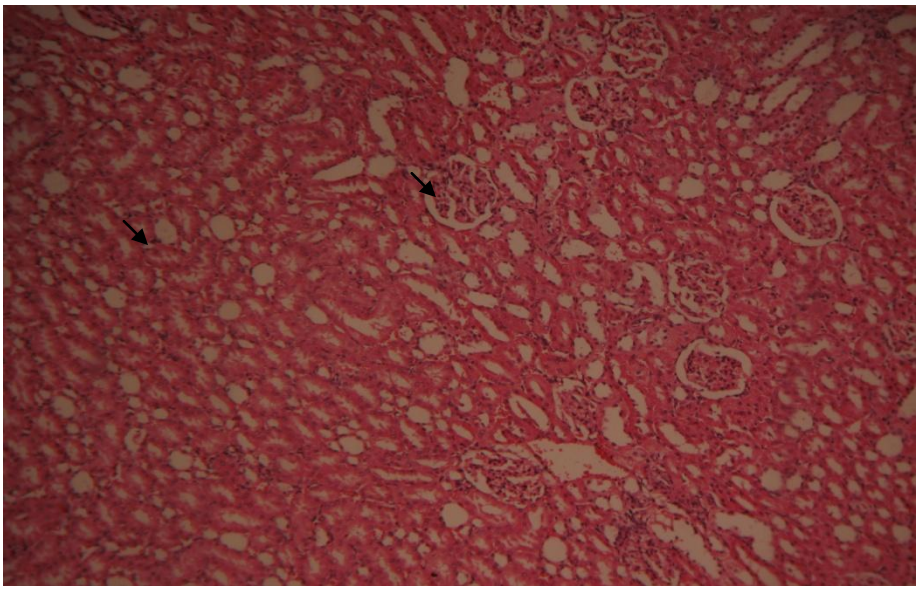

Figure 14. Photomicrograph of kidney showing normal glomeruli and tubules. H&E ×20.

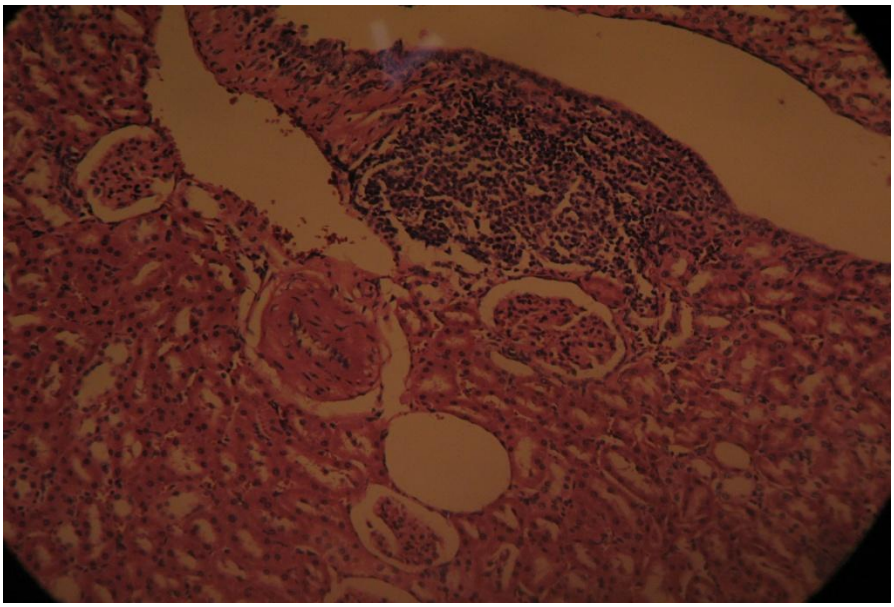

Figure 15. Photomicrograph showing lymphocytic infiltration of the kidney parenchyma. H&E ×20.

the seed extract of *C. olitorius* being inactive for several days before dying. The probable cause of deaths could be from the cardiotoxic effect of *C. olitorius* seed (Sharaf and Negm, 1969). The extract seems to produce a reduction in weight gained by the animals as it was noticed in the 90 days study that rats in the highest dose (3750 mg) and medium dose (2500 mg) group had a lower weight gain than the low dose (1250 mg) group. The control group had the highest weight gain.

A healthy weight loss is known to improve health in many ways (reduce blood pressure, lower cholesterol level and reduce risk of developing type 2 diabetes) (Stephen, 2006). Generally there were no obvious changes in the organ weight and organ weight relative to body weight of the rats in the 28 days study. In the chronic 90 days toxicity, only the heart was noticed to have reduced in weight significantly and dose dependently. The heart weight relative to body weight in both the 1250 and the 2500 mg/kg

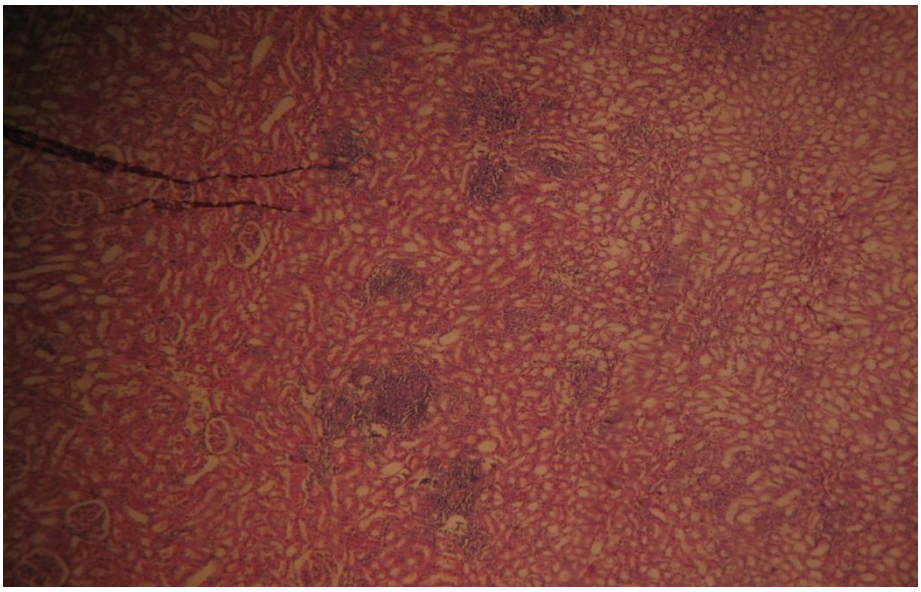

Figure 16. Photomicrograph showing multifocal lymphocytic infiltration of the kidney parenchyma. H&E ×20.

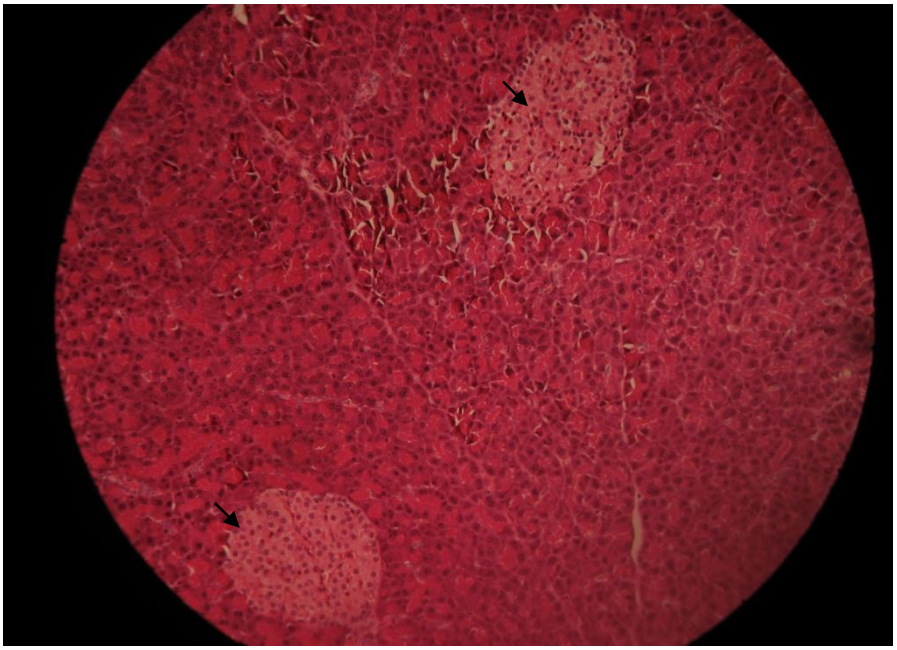

Figure 17. Photomicrograph of pancreas showing endocrine(2 cluster or islet cells) and exocrine pancreas. H&E ×20.

groups were of significance, dose dependently. Toxic activity of *C. olitorius* have been attributed to olitorisides and corchoroside which are found to possess a strophanthin-like action on the heart (Sharaf and Negm, 1969). The histological report showed the heart as being normal in both the 28 and 90 days study. The cholesterol in the 28 days toxicity study had a significant dose dependent rise between the treatment groups and the control. But in the 90 days study, the cholesterol rather had a dose dependent fall in its level that was highly significant in the low dose (1250 mg/kg) group. These cholesterol results were difficult to explain.

The white blood cell count (WBC) and the lymphocyte count had a dose dependent rise from the control significantly, in the 28 days sub acute toxicity study, indicating an effect of the ethanolic seed extract of *C. olitorius*; this

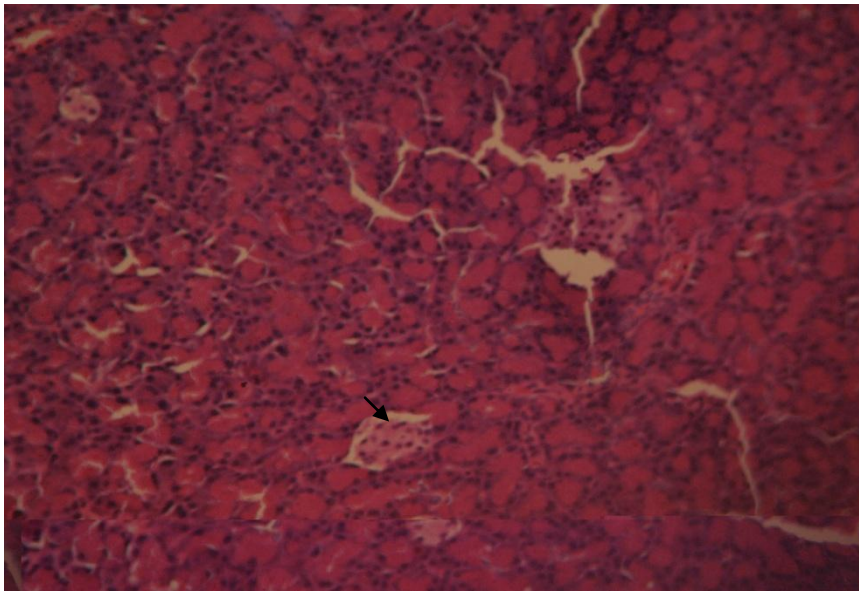

Figure 18. Photomicrograph of pancreas showing smaller cluster of islet cells. H&E ×20.

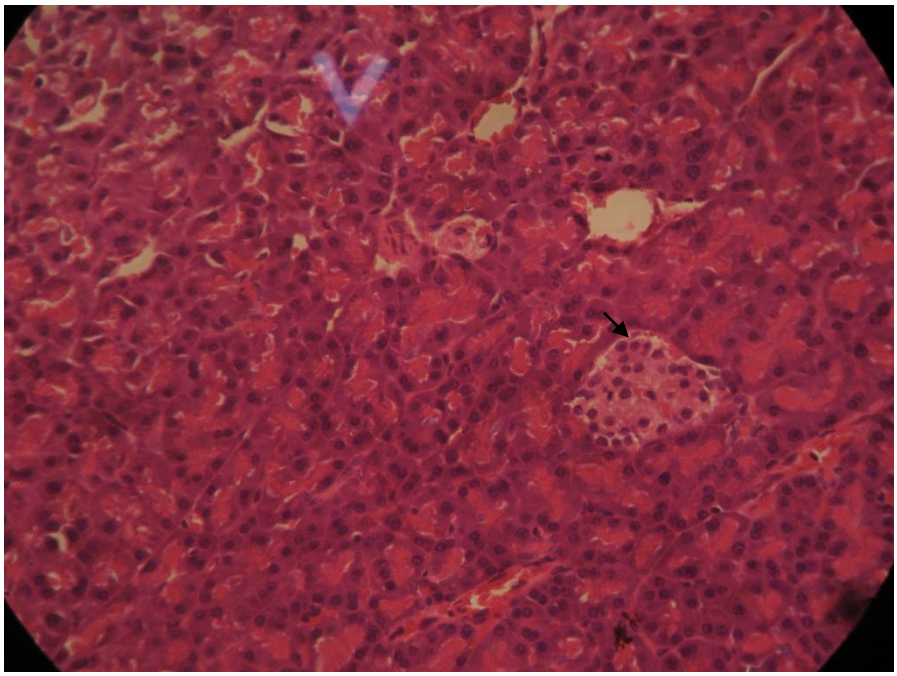

Figure 19. Photomicrograph of pancreas showing fewer clusters of islet cells. H&E ×20.

was supported by the fact that the WBC showed a highly significant rise in its level in the 3750 mg/kg dose group, in the 90 days toxicity study. There was a rise in the red blood cell count (RBC) and packed cell volume (PCV), though insignificantly. It would be in place to conclude that the extract has boosted haemopoitic cells production and may alleviate aneamic condition and raised immune response. This inference may support report of folklore use of *C. olitorius* in the treatment of gonorrhea, chronic cystitis, fever and tumours (Zeghichi et al., 2003) and Zakaria et al. (2006) report of *C. olitorius* anti inflammatory and anti pyretic activity in rats. The increase WBC and RBC may be due to the high content of iron and folate in *C. olitorius*, useful for prevention of aneamia

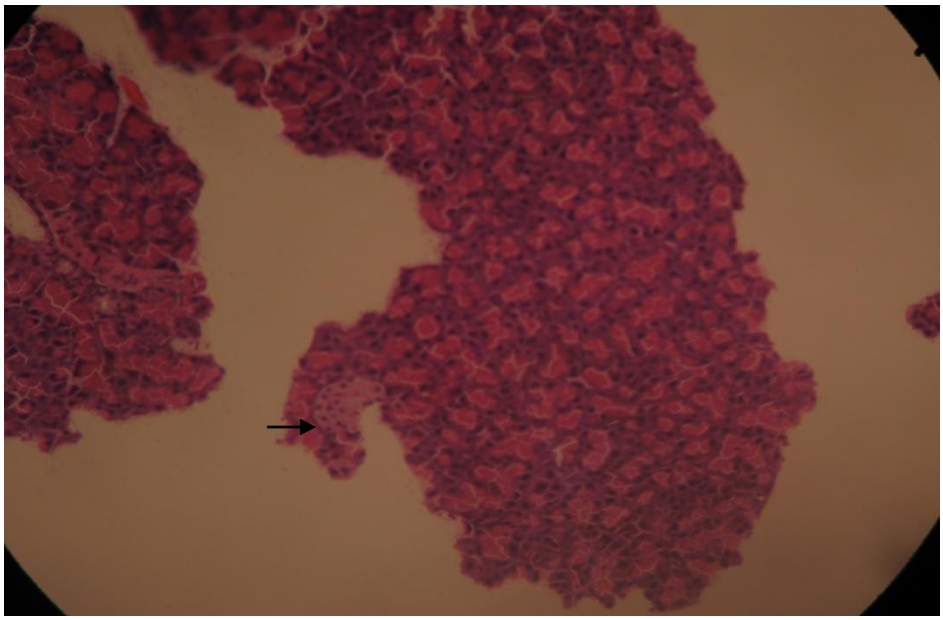

Figure 20. Photomicrograph of pancreas showing fewer clusters of islet cells. H&E ×20.

(Oyedele et al., 2006).

In the 28 days toxicity, there was a significant difference of the treatment groups from the control in all the liver biochemical parameter except for total protein and albumin that showed none. This was also the case in the 90 days study. These findings all indicates a dose dependent compromised hepatic milieu that was supported by the histological reports. Likewise, when *C. olitorius* seed protein enriched diet were fed to albino rats, slight fatty infiltration in the liver of test animals was seen, also observed was that AST, ALT and total lipid of liver increased significantly (Laskar et al., 1986).

The renal biochemical parameter in both the 28 days and the 90 days studies showed that sodium, potassium and urea level all had a significant change in their levels. The imbalance in the electrolyte may be due to the mineral elements content of the extract (Fe, Mg, Cu, Zn, Ca Na and K) found to be present in the plant leaf (Amanabo, 2012) and raised urea is evidence of renal affectation of the ethanolic seed extract of *C. olitorius* which was supported by the histological findings already mentioned.

The evaluation has shown that the ethanolic crude extract of *C. olitorius* seed may be safe following oral administration in albino rats especially at low doses (less than 1000 mg/kg). The evidences of compromised renal and hepatic milieu, supported by histological results at repeated high dose usage suffices for caution and selection of a proper dose in its use locally. The extract improved both the white and red cell count in rats, an indication that it may possess the potential to boost immunity and treat aneamia.

ABBREVIATIONS

AP, Alkaline phosphatase; **ALT,** alanine amino transferase; **AST,** aspartate amino transferase; **H&E,** heamatoxylin and eosin stain.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Development and *in-vitro* characterization of lornoxicam loaded ethyl cellulose microspheres prepared by emulsion solvent evaporation method

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Advancement in drug delivery could come from innovating improvement to the existing drug delivery system. Lornoxicam (Lxm) loaded ethyl cellulose microspheres were prepared by emulsion solvent evaporation technique and also to investigate the effect of variations in drug concentration, polymer concentration, internal phase volume, continuous phase volume and emulsifier concentration on the particle size, shape, % yield, percent entrapment efficiency and *in vitro* drug release behavior. The scanning electron microscopy (SEM) revealed that microspheres had good spherical geometry with smooth surface. The result showed that the maximum yield of the microspheres was found to be 64.23 ± 0.25%, with particle size in the range of 64.24 ± 1.82 to 81.83 ± 3.43 µm and encapsulation efficiency was found to be in a range of 60.34 ± 1.63 to 71.61 ± 1.20%. The average particle size and entrapment efficiency of microspheres were enhanced with increasing polymer concentration but reduced with increasing internal phase volume, external phase volume and emulsifier concentration. *In vitro* release profile of microspheres was in the range of 75.65 ± 2.3 to 87.78 ± 2.3% at the end of 12 h. It was concluded that Lxm loaded ethyl cellulose microspheres formulation showed sustained effect over a period of 12 h.

Key words: Ethyl cellulose, lornoxicam, microspheres, solvent evaporation, sustained release.

INTRODUCTION

In the last few decades, advancement in controlled/ sustained drug delivery systems has led to attainment of more effective therapy, that is delivery of drug over a long period of time, avoiding the large fluctuations and reducing the need of several administrations (Duarte et al., 2007). Over the past few decades, microspheres are one of the microparticulate systems which have shown tremendous potential and are used for sustained or controlled drug delivery and to improve the therapeutic efficacy (Davis and Illum, 1988; Ritschel, 1989). Ethyl cellulose (EC) is a water insoluble, biocompatible and nontoxic cellulose polymer and is studied extensively as encapsulating agent for sustained release of drugs (Chowdary et al., 2004; Wu et al., 2003).

Lornoxicam (Lxm), also known as chlortenoxicam, is a member of the oxicam group widely used for the symptomatic treatment of pain and inflammation in patients with rheumatoid arthritis and osteoarthritis. However, Lornoxicam usefulness is limited due to its short half-life that ranges from 3 to 5 h (Skjodt and Davies, 1998). Hence

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Table 1. Formulation variables of Lxm loaded ethyl cellulose microspheres (LECM).

Formulation code*	Lxm (mg)	EC (mg)	Drug:polymer ratio	DCM (ml)	Distilled water (ml)	Emulsifier concentration (%)
LECM-D_1	200	600	1:3	10	100	1.0
LECM-D_2	400	600	2:3	10	100	1.0
LECM-D_3	600	600	3:3	10	100	1.0
LECM-P_1	200	200	1:1	10	100	1.0
LECM-P_2	200	400	1:2	10	100	1.0
LECM-P_3	200	600	1:3	10	100	1.0
LECM-P_4	200	800	1:4	10	100	1.0
LECM-I_1	200	600	1:3	5	100	1.0
LECM-I_2	200	600	1:3	10	100	1.0
LECM-I_3	200	600	1:3	15	100	1.0
LECM-C_1	200	600	1:3	10	50	1.0
LECM-C_2	200	600	1:3	10	100	1.0
LECM-C_3	200	600	1:3	10	150	1.0
LECM-E_1	200	600	1:3	10	100	0.5
LECM-E_2	200	600	1:3	10	100	1.0
LECM-E_3	200	600	1:3	10	100	1.5

*Effect of drug concentration (D_1-D_3), polymer concentration (P_1-P_4), internal phase volume (I_1-I_3), external (continuous) phase volume (C_1-C_3), emulsifier concentration (E_1-E_3).

it requires repeated dosing which lead to local irritation and ulceration, and hence is the cause of the patient's non-compliance. To reduce the frequency of dosing and improve the patient compliance, controlled/sustained release formulation is desirable.

In the present investigation, we made an attempt to prepare various ethyl cellulose microspheres of Lxm by emulsion-solvent evaporation technique with varying various formulation variables like drug concentration, polymer concentration, volume of internal phase, volume of external phase and emulsifier concentration (Tween 80). The effect of the mentioned formulation variables on particle size, shape, % yield, drug entrapment efficiency and drug release behavior were investigated for the development of Lxm loaded ethyl cellulose microspheres to provide oral sustained drug delivery for a longer period of time.

MATERIALS AND METHODS

Chemicals

The drug, Lornoxicam was obtained as a gift sample from Zydus Cadila Healthcare Limited, India. EC was purchased from Central Drug House (P) Ltd., New Delhi, India. Dichloromethane (DCM) and Tween 80 procured from Central Drug House Pvt. Ltd., New Delhi, India. All other chemicals used were of analytical grade.

Preparation of Lxm loaded ethyl cellulose microspheres

The Lxm loaded ethyl cellulose microspheres were prepared by emulsion solvent evaporation method with some modification (Atyabi et al., 2005; Duarte et al., 2006). Weighed amount of polymer and Lxm were dissolved in dichloromethane as the internal phase. The prepared organic phase was then added drop wise to the water, containing Tween-80 (surfactant), which acts as external (continuous) phase. The mixture was stirred with mechanical stirrer at controlled stirring speed of 1000 rpm. The formed oil-in-water (o/w) emulsion was stirred continuously at room temperature until complete evaporation of dichloromethane and formation of solid microspheres. The prepared microspheres were filtered, washed with excess of distilled water and dried in a desiccator under vacuum at room temperature.

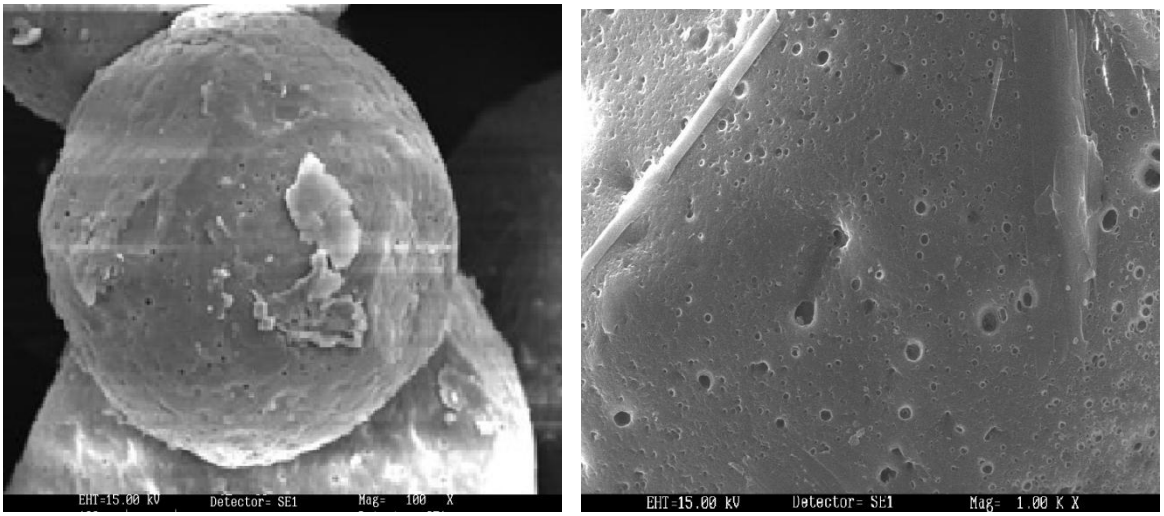
Optimization of Lxm loaded ethyl cellulose microspheres

Various formulation variables such as: drug concentration, polymer concentration, volume of internal phase, volume of external phase, emulsifier concentration, which could affect the preparation and properties such as particle size, shape, % yield, drug entrapment efficiency and *in vitro* drug release of the microspheres were studied for the development of Lxm loaded ethyl cellulose microspheres. The compositions and formulation code of the microspheres are given in Table 1.

Characterization of Lxm loaded ethyl cellulose microspheres

Shape and surface morphology

Shape and surface morphology of the formulations were studied by scanning electron microscopy (SEM) (JEOL JSM-1600, Tokyo, Japan) using a gold sputter technique. The samples for SEM were prepared by lightly sprinkling the microspheres on a double adhesive tape, which was stuck on an aluminum stub and coated with gold to a thickness of about 300 Å under an argon atmosphere in a high-vacuum evaporator. The coated samples were then randomly scanned and photomicrographs were taken.



(a) (b)

Figure 1. SEM images of Lxm loaded ethyl cellulose microspheres. (a): at 100× magnification, (b): at 1000× magnification.

Particle size analysis

Microspheres were studied microscopically for their size using a calibrated ocular eyepiece. In this method, the samples were mounted on a slide and placed on a mechanical stage. The calibrated ocular eyepiece was fitted with a micrometer by which the particle size of the sample was determined (Martin et al., 2005).

Determination of percent yield and entrapment efficiency

The dried microspheres were weighed and percentage yield of the prepared microspheres was calculated by the following Equation 1 (Garud and Garud, 2012), where Wm is the weight of the microspheres and Wdp is the expected total weight of drug and polymer used for the preparation.

$$\text{Yield (\%)} = (Wm / Wdp) \times 100 \quad (1)$$

For the determination of entrapment efficiency, microspheres (100 mg) were accurately weighed and crushed and were suspended in 50 ml of phosphate buffer solution (PBS) (pH 6.8) and the resulting mixture was kept for shaking on mechanical shaker. At the end of 2 h, it was filtered, diluted appropriately and analyzed for drug content spectrophotometrically (n = 3) at 376 nm (Shimadzu 1700, Japan). Entrapment efficiency was calculated using Equation 2 (Garud and Garud, 2012), where A is actual drug concentration and T is the theoretical drug concentration.

$$\text{Entrapment efficiency (\%)} = (A / T) \times 100 \quad (2)$$

***In-vitro* drug release studies**

The drug dissolution test of microspheres was carried out by the paddle method specified in United States Pharmacopeial (USP) XXIII. The *in vitro* drug release of various microspheres formulations were studied in simulated gastrointestinal pH conditions: simulated gastric fluid (0.1 N HCl, pH 1.2) for the first 2 h, followed by simulating intestinal fluid (phosphate buffer solution, PBS, pH 6.8) up to 12 h, at 37 ± 0.5°C. Samples (1 ml) were withdrawn at regular time intervals and replaced with the same volume of test volume of test medium to maintain sink conditions. The withdrawn samples were suitably diluted, filtered through a 0.45 µ membrane filter and analyzed spectrophotometrically. All the tests were carried out in triplicate.

Differential scanning calorimetry study

The differential scanning calorimetry (DSC) of pure drug (Lxm), ethyl cellulose (EC), physical mixture of Lxm and EC, blank EC microsphere and Lxm loaded EC loaded microsphere was performed using a Pyris Diamond DSC-4 (Perkins Elmer, Wellesley, MA) in order to assess the drug excipient compatibility study. Thermograms were obtained at a scanning rate of 10°C min^{-1} conducted over a temperature range of 25 to 350°C in a liquid nitrogen environment.

RESULTS AND DISCUSSION

Ethyl cellulose microspheres of Lxm were successfully prepared by emulsion solvent evaporation method. SEM image of Lxm loaded ethyl cellulose microspheres showed that all microspheres were spherical and uniform with presence of some of the drug adhered to the surface of microspheres in its native crystalline form (Figure 1a and b).

Effect of drug concentration on particle size, percent yield and entrapment efficiency

The amount of drug was varied with respect to constant polymer concentration with drug to polymer ratio of 1:3 (LECM-D_1), 2:3 (LECM-D_2) and 3:3 (LECM-D_3) in order to investigate the effect of drug concentration, the resulting average particle size of microspheres were found to be 75.11 ± 1.12, 78.77 ± 2.92 and 81.83 ± 3.43 µm, respectively

Table 2. Effects of formulation variables on particle shape, size, % yield and drug entrapment efficiency of Lxm loaded ethyl cellulose microspheres.

Formulation code	Shape	Average particle size[$] (µm)	Yield[$] (%)	Drug entrapment efficiency[$] (%)
LECM-D_1	Spherical	75.11±1.12	64.23±0.25	71.61±1.20
LECM-D_2	Spherical	78.77±2.92	60.32±0.72	67.88±2.37
LECM-D_3	Spherical	81.83±3.43	58.27±0.45	61.47±1.43
LECM-P_1	Irregular shape	64.24±1.82	61.73±0.34	60.34±1.63
LECM-P_2	Spherical	67.38±2.02	62.32±0.08	65.15±1.06
LECM-P_3	Spherical	75.11±1.12	64.23±0.25	71.61±1.20
LECM-P_4	Spherical	76.25±1.36	65.02±0.58	70.19±2.06
LECM-I_1	Spherical	79.10±1.98	59.75±0.04	71.37±2.18
LECM-I_2	Spherical	75.11±1.12	64.23±0.25	71.61±1.20
LECM-I_3	Spherical	73.60±2.35	65.40±0.20	67.10±1.31
LECM-C_1	Spherical	79.32±2.06	67.04±0.45	70.97±2.34
LECM-C_2	Spherical	75.11±1.12	64.23±0.25	71.61±1.20
LECM-C_3	Spherical	71.45±1.68	59.09±0.48	65.64±2.21
LECM-E_1	Irregular shape	77.41±1.45	65.68±0.18	72.48±2.11
LECM-E_2	Spherical	73.11±1.12	64.23±0.25	71.61±1.20
LECM-E_3	Rough surface	72.29±1.79	61.26±0.37	68.24±1.60

[$]All data were expressed as mean ± SD. n = 3.

(Table 2). The average particle size of microspheres increased with increasing Lxm concentration may be due to increased content of the internal phase (drug and polymer) leading to bigger emulsion droplets resulting in a comparatively increase in size of microspheres. The percentage yield of microspheres gradually decreased with increasing the drug ratio, which might be due to increase in viscosity of internal phase and hence rapid solvent evaporation before formation of a continuous emulsion leading to and hence reduced percent yield. The drug entrapment efficiency was found to decrease progressively with increasing the drug ratio for preparation (Table 2), which might be attributed due to greater payload on polymer matrix which resulted in an increase in drug leaching into the continuous phase before the solidification could occur, moreover reduced % yield may be attributed to a reduction in drug entrapment efficiency. The highest entrapment efficiency was found with LECM-D_1 (1:3) that is, 71.61 ± 1.20 (Table 2), therefore this formulation was selected as optimum.

Effect of polymer concentration on particle size, percent yield and entrapment efficiency

The effect of increasing polymer concentration (drug to polymer ratio) from LECP-P_1 (1:1), LECP-P_2 (1:2), LECP-P_3 (1:3) and LECP-P_4 (1:4) on microspheres characteristics were shown in Table 2. The average particle size and percent yield of Lxm loaded ethyl cellulose microspheres were found to increase from 64.24 ± 1.82 to 76.25 ± 1.36 µm and 61.73 ± 0.34 to 65.02 ± 0.58% on varying drug: polymer ratio from 1:1 to 1:4, respectively. The average particle size was found to increase significantly with increasing the polymer concentration. With increasing the polymer concentration the internal phase viscosity increased which produced larger droplets upon emulsification, particle size were increased (Vasir et al., 2003). Percent yield was slightly increased on increasing polymer concentration. However, this increase was not significant. The entrapment efficiency was found to be in the range of 60.34 ± 1.63 to 71.61 ± 1.20% (Table 2).

The entrapment efficiency increased progressively with increasing polymer concentration because the increased polymer content provided more binding site for the drug molecules and more particles of Lxm were coated leading to higher encapsulation efficiency (Khan et al., 2010). EC microspheres (LECM-P_3) with drug: polymer ratio (1:3) shows highest entrapment efficiency that is, 71.61 ± 1.20%. However, further increase in the concentration of EC resulted in a decrease in the entrapment efficiency which may be due to aggregation of polymer matrix as a consequence of higher viscosity of internal phase in which drug did not uniformly dispersed in smaller droplets upon the induced shear for the preparation.

Effect of internal phase volume on particle size, percent yield and entrapment efficiency

In the preparation of Lxm loaded EC microspheres, when the volume of internal phase was increased from 5 ml to 15 ml (Table 2), percent yield of microspheres increase

may due to reduction in viscosity of internal phase as a consequence of a uniform dispersion, resulting in homogeneous emulsion and consequently increased yield upon its solidification. Before the formation of stable emulsion at given stress the emulsion droplets are not divided into smaller droplets resulting in lumps and hence reduced yield of microspheres. The average particle size of microspheres was decreased and found to be 79.10 ± 1.98, 75.11 ± 1.12 and 73.60 ± 2.35 on increasing internal phase volume of 5 ml (LECM-I_1), 10 ml (LECM-I_2) and 15 ml (LECM-I_3), respectively. Increase in internal phase volume from 5 to 15 ml also seemed to have decreased the entrapment efficiency from 71.61 ± 1.20 to 67.10 ± 1.31. This, which was due to the leaching of drug particle from internal phase to continuous phase was increased because of decreased viscosity of the drug-polymer solution. The internal phase volume of LECM-I_2 (10 ml) was found to be optimum for EC microspheres preparation, as the drug entrapment efficiency was highest (Table 2).

Effect of continuous (external) phase volume on particle size, percent yield and entrapment efficiency

Variations in continuous phase volume were also studied and results suggested that as the volume of the processing medium was increased from 50, 100 and 150 ml, the % yield and particle size decreased gradually and was found to be 67.04 ± 0.45, 64.23 ± 0.25, 59.09 ± 0.48% and 79.32 ± 2.06, 75.11 ± 1.12, 71.45 ± 1.68 µm, respectively (Table 2). This can be attributed to the fact that larger volumes of continuous phase resulted in less collisions between emulsion droplets and fine dispersement of emulsion, thereby yielding small and uniform microspheres (Saravanan et al., 2003). When the continuous phase volume was increased, entrapment efficiency of the drug was found to decrease. This may be due to increase of the partitioning of the drug into the increased volume of the continuous phase. Continuous phase volume of 100 ml (LECM-C_2) was found to be optimum for the preparation of EC microspheres (Table 2).

Effect of emulsifier concentration on particle size, percent yield and entrapment efficiency

The effect of emulsifier concentration on the formation of microspheres were examined, the average particle size and percent yield of microspheres was found to vary from 79.32 ± 2.06 to 71.45 ± 1.68 µm and 67.04 ± 0.45 to 59.09 ± 0.48% on varying emulsifier concentration (Tween 80) from 0.5 to 1.5%, respectively. Increased surfactant concentration led to the formation of globules with a lower average size and stabilization of the emulsion droplets avoiding their coalescence, resulting in smaller microspheres (Maia et al., 2004). An optimum concentration is required to produce finest stable dispersion. Below this concentration, the dispersed globules/droplets are fused to produce larger globules that require lower emulsifier concentration for stabilization (according to their reduced surface area). Above the optimum concentration, no significant decrease in particle size and microsphere having rough surface was observed. The drug entrapment efficiency varied from 72.48 ± 2.11 to 68.24 ± 1.60% during preparation of microspheres (Table 2), for the preparation of spherical shape and high entrapment efficiency. Required emulsifier concentration was 1.0% for Lxm loaded EC microspheres.

***In-vitro* drug release studies**

The effect of the drug to polymer ratio (LECM-D1 to LECM-D3) on the Lxm release from microspheres is shown in Figure 2, drug release from microspheres is notably affected by when the ratio of drug to the polymer is increased. It was found to increase with increasing drug content ratio. By increasing the amount of drug content, a point is reached when the solid drug particles upon dissolution begin to form continuous pores or channels within the matrix, ultimately leading to the diffusion of dissolution medium within polymer matrix and increasing the drug/dissolution medium interaction and hence increasing dissolution rate were observed (Song et al., 1981).

The results of effect of polymer concentration showed that the rate of Lxm release from LECM-P1, LECM-P2, LECM-P3 and LECM-P4 formulations was found to be 36.47 ± 0.98, 30.83 ± 1.44, 23.29 ± 1.46 and 20.59 ± 1.38% during the first 2 h in SGF, was significantly reduced and thereafter only LECM-P3 and LECM-P4 formulations followed the drug release pattern extending up to 12 h in dissolution medium SIF (pH 6.8). Lxm release from EC microspheres followed the order LECM-P1 > LECM-P2 > LECM-P3 > LECM-P4 (Figure 3). The study showed that the rate of Lxm release from EC microsphere was progressively decreased from 87.48 ± 2.68 to 75.65 ± 2.3% by increasing the polymer concentration, suggesting that the release from microspheres could be controlled by polymer concentration. This may be due to the increased density and thickness of polymer matrix at higher polymer concentrations that acted as a barrier for penetration medium, thereby retarding the diffusion of the drug, resulting in decreasing overall drug release from the polymer matrix (Al-Kassaa et al., 2007; Ramachandran et al., 2010).

The effect of the volume of internal phase and continuous phase on drug release (%) was shown in Figure 4 and 5, respectively. Internal phase volume does not have any significant effect on drug release. On the other hand, significant increase in the drug release rate was observed

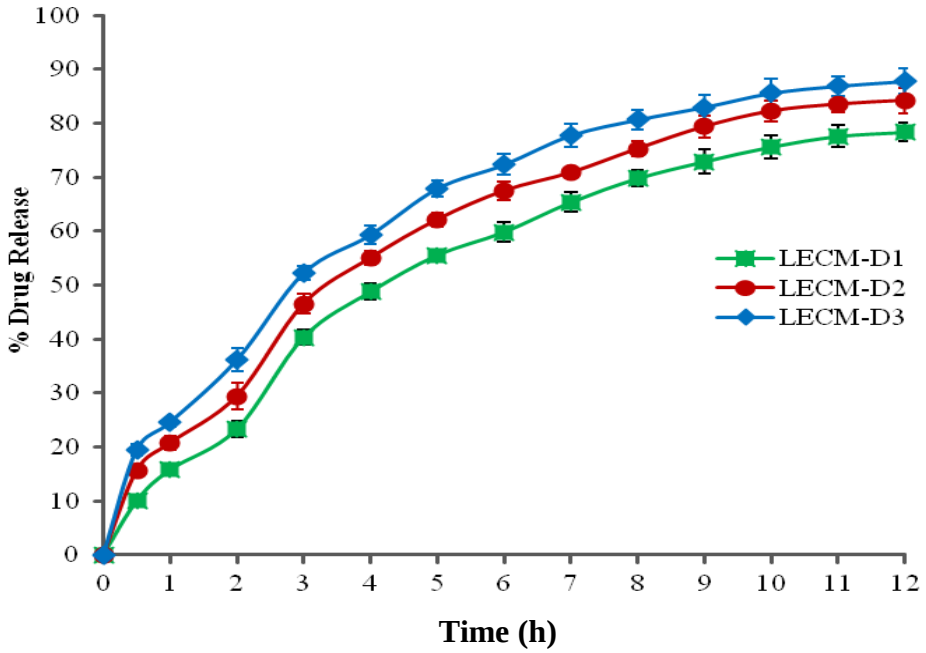


Figure 2. Effect of drug concentration on Lxm release from ethyl cellulose microspheres.

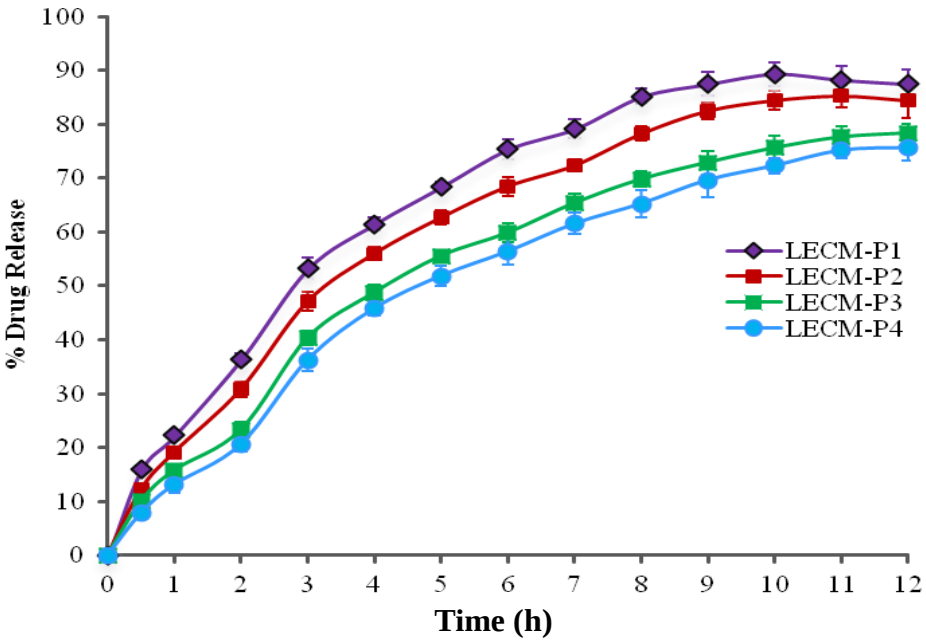


Figure 3. Effect of polymer concentration on Lxm release from ethyl cellulose microspheres.

with increased volume of continuous (external) phase. It may be due to a decreased size of the microspheres and hence a larger surface area exposed to dissolution (Dahiya and Gupta, 2011). The release of drug from EC microspheres also depends on the concentration of emulsifier (Tween 80), which was used at the time of preparation preparation as a stabilizer. As the concentration of Tween 80 increased from 0.5% (LECM-E1), 1.0% (LECM-E2) and 1.5% (LECM-E3), the release rate was increased (Figure 6). It may also be due to a decreased size of the microspheres and hence a larger surface area exposed to dissolution medium.

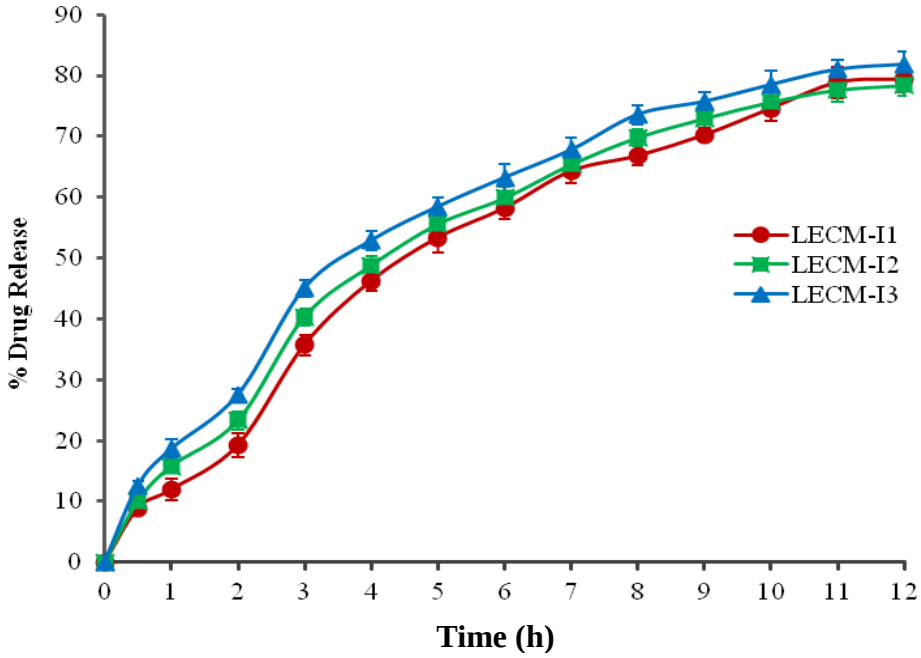


Figure 4. Effect of internal phase volume on Lxm release from ethyl cellulose microspheres.

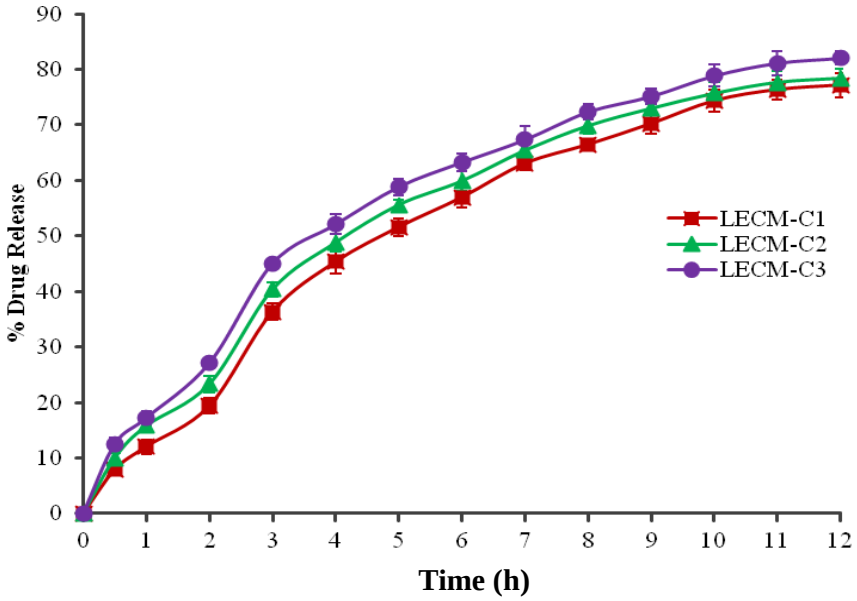


Figure 5. Effect of external (continuous) phase volume on Lxm release from ethyl cellulose microspheres.

Differential scanning calorimetry study

The DSC curves of pure Lxm (A), EC (B), physical mixture of Lxm and EC (C), blank EC microspheres (D) and Lxm-loaded EC microsphere (E) are shown in Figure 7. It was evident from the DSC profile that pure Lxm was typical of a crystalline substance, exhibiting a sharp exothermic peak at 232°C, corresponding to its melting. Thermogram of EC also displays a large exothermic peak around 180°C. The thermograms of the physical mixtures of Lxm with EC showed the existence of the drug exothermic peak, which could indicate the absence of interaction between Lxm and EC. The examination of EC empty microspheres revealed an exotherm peak due to the melting point of EC. The DSC profile of the drug appeared at the temperature corresponding to its melting

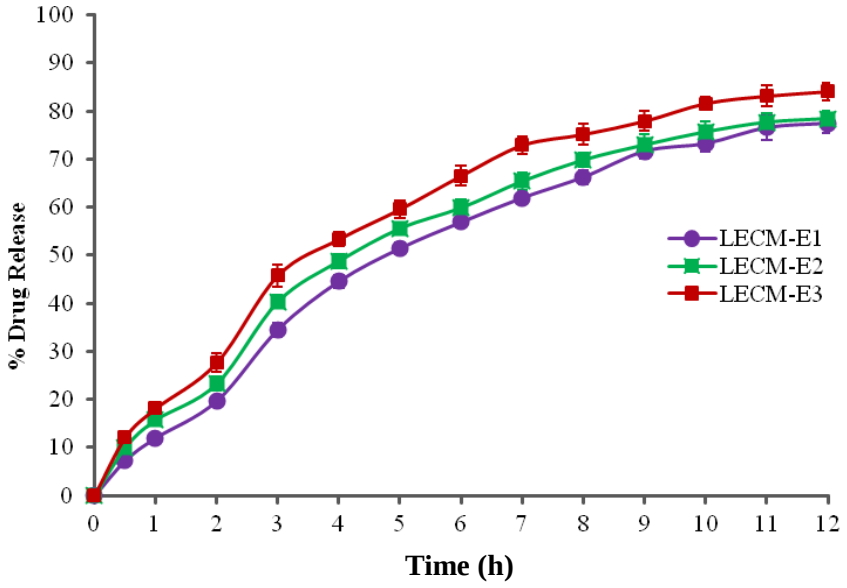


Figure 6. Effect of emulsifier concentration on Lxm release from ethyl cellulose microspheres.

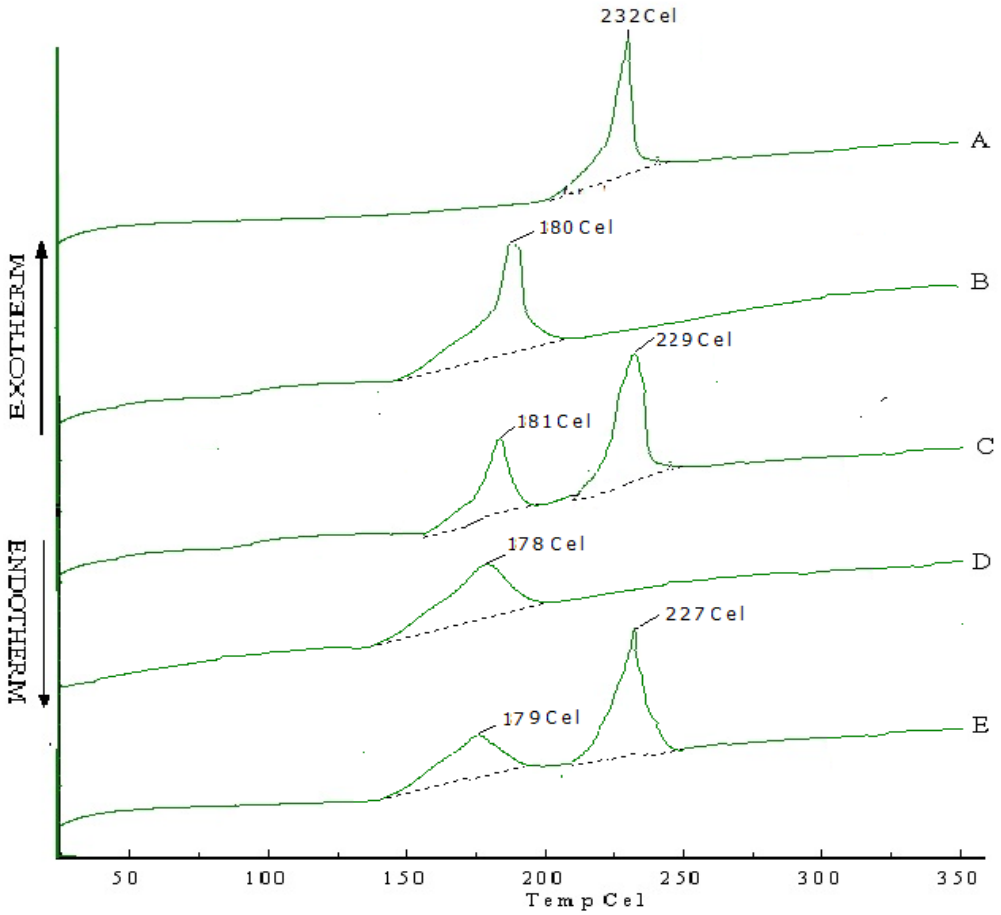


Figure 7. DSC thermo grams of pure Lxm (A), EC (B), physical mixture of Lxm and EC (C), blank EC microspheres (D) and Lxm- loaded EC microsphere (E).

point in the Lxm-loaded EC microspheres but with the loss of its sharp appearance. It appears that there is a significant reduction of drug crystallinity in the microspheres. The DSC study apparently revealed that the drug was compatible with the polymer and neither drug decomposition nor drug-polymer interactions occurred in the freshly prepared microspheres.

Conclusion

It can be concluded that the emulsification solvent evaporation method is a simple, reproducible method and the prepared microspheres had good spherical geometry. This study also concluded that ethyl cellulose microspheres formulation containing drug: polymer ratio (1:3), 10 ml internal phase volume, 100 ml continuous phase volume and 1.0% emulsifier concentration was optimized on the basis sphericity, highest entrapment efficiency and drug release study. The *in vitro* drug release studies showed that Lxm loaded ethyl cellulose microspheres formulation showed better sustained effect over a longer period of time. However, ability of ethyl cellulose microsphere to incorporate the drug and provide the sustained release for oral administration can be considered as one of the promising formulation technique for preparing a multiparticulate drug delivery system (polymer microsphere) of Lornoxicam, and the use of ethyl cellulose for the microsphere preparation has provided delays in drug release at a longer period which is suitable for oral drug delivery systems.

Conflict of interest

No competing interests were disclosed.

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