



Evaluation of novel methotrexate nanoparticles for anticancer action

Amaresha S

Department of Pharmacy, JJT University, Jhunjhunu, Rajasthan, India

Corresponding Author: Amaresha S

ABSTRACT

Methotrexate-chitosan nanoparticles (MTX-NP) were prepared by method of Tripolyphosphate (TPP) ionic cross-linking. Influence of preparation conditions on physicochemical properties were analyzed by DLS, SEM & FTIR. *In vitro* drug release properties were investigated by UV-VIS spectroscopy. *In vitro* anti-cancerous activities (MTT assay, apoptosis assay, cell cycle arrest & ROS indices) of MTX/MTX-NP were evaluated on HT-1080 & MCF-7 cells. Comparative (MTX/MTX-NP) *in vivo* pharmacokinetic & Acute/sub acute toxicity studies carried out on Wistar rats. In conclusion, prepared MTX-NP has played significant role in increasing *in vitro*-anti cancer efficacy, *in vivo* pharmacokinetic profiles & as well helps in reducing toxic effect.

Keywords: *Invitro*, *Invivo* anticancer activities, Methotrexate nanoparticles and toxicity studies

INTRODUCTION

General clinical recognitions were made in any event once dependably, ideally in between time (s) constantly and considering apex time of anticipated effects in wake of dosing. Accomplishment state of creatures was recorded. No not as much as twice reliably, all creatures were searched for loathsomeness and mortality. Creatures in satellite party made preoccupation plans for follow-up attestations were kept for impel 14 days without treatment to see surrendered event, or confirmation of, or recuperation from unsafe impacts.

Haemato-biochemical examinations

Haemato-biochemical parameters were outlined utilizing blood tests amassed from every single one of

creatures on day 0, 14 and 28 by retro-orbital plexus cut structure utilizing microhematocrit tight tubes under ketamine (Aniket®, Neon Labs, India, 40 mg/kg, i.p) and xylazine (Xylazine®, Indian Immunologicals, India, 10 mg/kg, i.m) anesthesia. Calcium Disodium EDTA (Merck, India) was utilized as anticoagulant at 1.5 mg/mL of entire blood for estimation of hematological parameters [1-7]. Running with hematological parameters were examined by utilizing completely changed platelet counter (Erma®).

- Total erythrocyte check (TEC) (106 cells/mm³)
- Total leucocyte check (TLC) (10³ cells/mm³)
- Packed cell volume (PCV) (%)
- Haemoglobin fixation (Hb) (g/dL).

MATERIALS AND METHODS

Study design

Table 1: Details of doses administered to male & female rats

Group	Dose (mg/kg)	No. of male rats	No. of female rats
Group I (Normal Control)	Normal saline (2 mL/kg)	6	6
Group II MTX	150	6	6
Group III Low dose MTX-NP	150	6	6
Group	Dose (mg/kg)	No. of male rats	No. of female rats
Group IV Medium dose MTX-NP	300	6	6
Group V High dose MTX-NP	600	6	6
Group VI Satellite MTX-NP	600	6	6

Observations

General clinical recognitions were made in any event once dependably, ideally in between time (s) constantly and considering apex time of anticipated effects in wake of dosing. Accomplishment state of creatures was recorded. No not as much as twice reliably, all creatures were searched for loathsomeness and mortality. Creatures in satellite party made preoccupation plans for follow-up attestations were kept for impel 14 days without treatment to see surrendered event, or confirmation of, or recuperation from unsafe impacts.

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- Total erythrocyte check (TEC) (106 cells/mm³)
- Total leucocyte check (TLC) (103 cells/mm³)
- Packed cell volume (PCV) (%)

- Haemoglobin fixation (Hb) (g/dL).

Serum normal science

Serum was isolated from anticoagulant free blood tests and refrigerated at - 40°C until empower biochemical examination. Running with biochemical estimations were done by utilizing serum fundamental science self-loader analyzer (Erba-Chem no under 5) with individual Erba business reagent packs.

Alkaline phosphatase (ALP) (IU/L)

The protein is found over incalculable animals, prokaryotes and eukaryotes alike, with tantamount universally useful of suppression at any rate in various fundamental structures true to earth they work in. It expect noteworthy part in support inside liver and progression inside skeleton. In light of its boundless noteworthiness in these regions, its fixation in circulatory structure is utilized by diagnosticians as biomarker in picking end, for example, hepatitis or osteomalacia.

Serum transaminase (AST and ALT) (IU/L)

Transaminases or aminotransferases are annoys that catalyze transamination reaction between amino ruinous and α -keto harming. They are indispensable in mix of amino acids, which shape proteins [8-17]. Transaminase mixes are major in time of various

amino acids, and surveying groupings of various transaminases in blood is basic in diagnosing and following indisputable desolations.

For example, closeness of lifted transaminases can be pointer of liver and cardiovascular damage. Two urgent transaminase mixes are Aspartate transaminase (AST), by and wide called serum glutamic oxaloacetic transaminase (SGOT); and Alanine transaminase (ALT), comparatively called Alanine aminotransferase (ALAT) or serum glutamate-pyruvate transaminase (SGPT).

Creatinine (mg/dL)

A serum Creatinine test measures level of Creatinine in blood and gives check of how well kidneys channel (glomerular filtration rate).

Blood Urea Nitrogen (BUN) (mg/dL).

Blood urea nitrogen (BUN) is mending test that assessments extent of urea nitrogen found in blood. Liver produces urea in urea cycle as waste outcome of assimilation of protein. Fundamental driver of increment in BUN are: high protein eat up less calories, lessen in glomerular filtration rate (GFR) (suggestive of renal dissatisfaction) and in blood volume (hypovolemia), congestive heart disillumination, gastrointestinal discharge, fever, and enlarged catabolism. Hypothyroidism can cause both decreased GFR and hypovolemia, in any case BUN-to-Creatinine degree has been acknowledged to be brought down in hypothyroidism and brought up in hyperthyroidism.

Pathology

Around total of examination medium-term fasted rodents were yielded under ether anesthesia and exposed to disconnected necropsy including examination of outside surface of body, all openings, cranial, thoracic and stomach devastations and their substance.

Get-together of organs for histopathological consider

After medium-term fasting, rodents were gauged and yielded intelligently on day 29 and necropsy was driven on each body to watch any gross miserable individual changes. organs viz., liver, kidney, stomach, stomach related tract and testis were confined from running with tissues utilizing saline and gathered by setting on sullyng paper and painstakingly walloped to expel abundance of saline.

They were accumulated in Neutral Buffered Formalin (NBF) for histopathological inspect and they were overseen for histopathology by routine paraffin embeddings procedure. Bits of five microns thickness were cut and recolored with Haematoxylin and Eosin (Luna, 1968).

Quantifiable examination

The varieties between get-togethers and periods were endeavored by two way ANOVA, utilizing Bonferroni show test surrendering to frameworks of Steel et al., 1996. Examinations among gatherings and between periods were endeavored by Bonferroni post-test. P see <0.001 was considered as goliath. Mean attributes and standard botch up of mean were managed and every single one of characteristics were displayed as Mean \pm SEM (GraphPad Prism 5 Software, 2007).

Toxicity studies

Destructiveness is essential stress for anticancer prescriptions. These blends demonstrate thin therapeutic record, with little complexity between measurements required for antitumor effect & that accountable for unacceptable threat. Their endorsed estimations are settled by peril endpoint. Moreover, toxic quality is observed sooner than therapeutic effect, hence, deadly effects address critical endpoint for pharmacodynamic examinations of cytotoxic prescriptions. Learning of destructiveness illustrations & guideline factors of toxic nature of anticancer medicines is required before showing data of these examinations. Hematological toxicities address essential toxic nature of cytotoxic. In any case, non-hematological toxicities have ended up being more basic than hematological toxicities as pharmacodynamic endpoints in couple of conditions, for instance, high-estimations chemotherapy related with bone marrow transplantation. This paper will depict essential lethality of cytotoxic drugs, & its factors of both between & intra-tireless variability. Learning of harmful quality case of drug constitutes pre-essential before showing its pharmacodynamics.

Harmful quality can be evaluated by effect substance has on living thing, tissue or cell. We understand that individuals will respond differently to comparable measurement of substance because of different factors including their sexual introduction, age & body weight. Thusly people level measure of harmful quality is routinely used.

Exceptional oral peril contemplate

Exceptional hurtfulness portrays opposing effects of substance that result either from single presentation or from different exposures in short time span (typically under 24 hours). To be depicted as exceptional risk, antagonistic effects should occur inside 14 significant lots of association of substance. Serious peril is perceived from interminable hurtfulness, which portrays hostile prosperity impacts from repeated exposures, every now & again at cut down levels, to substance over more drawn out time (months or years).

LD50 cut-off regard

Extreme oral harmful quality examination of MTX & MTX-NP was started with measurements of 300 mg/kg b. wt. In preliminary length (14 days) there was no watched mortality in case of MTX-NP treated assembling; where as in case of MTX treatment, mortality of one animal was recorded. In this way with another social affair of animals examination was repeated with same estimations of drug to avow mortality/survivability. Watched comes to fruition were same. So as showed by principles next level of higher measurement (2,000 mg/kg b. wt.) was picked & resultant animal mortality was seen to be three & two independently if there ought to be event of MTX & MTX-NP social occasions. Thusly, already specified medications lethality in Globally Harmonized Classification System (GHS) requested to Category 4 (>300-2000 mg/kg b. wt); where individual LD50 cut-off estimation of MTX & MTX-NP was seen to be 500 & 1000 mg/kg b. wt. Along these lines comparative lethal measurement of MTX-NP was seen to be impressively higher than MTX.

Clinical signs & Body weight

The treated social affairs (MTX & MTX-NP) were kept for observation for period of 14 days. rats were dull, demoralized, slow & anorectic in condition. animals had clinical signs of keeps running with lessened body weight, unforgiving hair coat & couple of animals were found to have swollen face. At terminal stage animals showed gasping & passing.

Repeated estimations 28-day oral noxious quality examination

Wistar pale cleaned singular rodents including 6 male and 6 female rodents in every get-together were gavaged with MTX (200 mg/kg b. wt) and MTX-NP

(400, 200 and 100 mg/kg b. wt) as high, medium and low estimations, reliably for 28 days and results were recorded. typical control assemble creatures were gavaged with kept running of factory saline (2mL/kg b. wt) and satellite get-together of MTX-NP were gavaged with high estimations of 400 mg/kg b. wt and after fundamental length were kept up for additional 14 days without relationship of MTX-NP.

Clinical signs

The creatures gave clinical recommendations, for example, trouble, deficiency, anorexia and separation of inner parts [18-20]. Level of give of clinical hints was less in low and medium estimations of MTX-NP separated from MTX and MTX-NP treatment get-togethers. Relating to bodyweight, there was fundamental decrease in all various treatment social affairs; where most stunning reducing in body weight was seen in MTX treatment.

Alanine aminotransferase (ALT)

The mean qualities got for ALT (U/L) on day 0, 14 and 28 of primer time range for Normal control (Group I), MTX control gathering (Group II), MTX-NP treated amassing (Group III, IV, V and VI) for both male and female rodents are given.

Alanine aminotransferase (ALT) in male rodents

The mean serum ALT fixation (U/L) in low (Group III), medium (Group IV), high (Group V) and satellite (Group VI) estimation of male rodents composed with MTX-NP on day 14 and 28 were 49.41 ± 0.26 , 56.74 ± 0.27 , 68.67 ± 0.26 , 70.03 ± 0.61 and 68.30 ± 0.21 , 75.76 ± 0.71 , 88.29 ± 0.21 , 88.22 ± 0.67 , solely. These attributes were on extremely essential level low ($P < 0.001$) showed up diversely in connection to MTX control (83.11 ± 1.08 and 100.48 ± 0.29 freely) and out and out high ($P < 0.001$) emerged from standard control (33.55 ± 0.30 and 33.33 ± 0.20 , autonomously) (Table. 29 and Fig. 26).

The mean serum ALT fixation (U/L) of all treatment get-togethers (Group II to bunch V) were for the most part fascinating ($P < 0.001$) to one another on day 14 and 28 of examination.

Alanine aminotransferase (ALT) in female rodents

The mean serum ALT focus (U/L) in low (Group III), medium (Group IV), high (Group V) and satellite

(Group VI) estimations of female rodents facilitated with MTX-NP on day 14 and 28 were 46.036 ± 0.65 , 54.04 ± 0.30 , 68.53 ± 0.39 , 69.73 ± 0.35 and 65.83 ± 0.81 , 74.87 ± 0.37 , 86.34 ± 0.54 , 87.20 ± 0.30 , only. These qualities were on extremely fundamental level low ($P < 0.001$) wandered from MTX control (76.14 ± 0.47 and 106.81 ± 0.20 solely) and inside and out high ($P < 0.001$) emerged from normal control (32.30 ± 0.44 and 32.05 ± 0.51 , freely)

The mean serum ALT fixation (U/L) of all treatment get-togethers (Group II to add up to V) were on extremely fundamental level remarkable ($P < 0.001$) to one another on day 14 and 28 of examination.

Aspartate aminotransferase (AST)

The mean attributes got for AST (U/L) on day 0, 14 and 28 of exploratory period for Normal control (Group I), MTX control gathering (Group II), MTX-NP treated collecting (Group III, IV, V and VI) for both male and female rodents are given in Table 30 and Fig. 28 and 29.

Aspartate aminotransferase (AST) in male rodents

The mean serum AST fixation (U/L) in low (Group III), medium (Group IV), high (Group V) and satellite (Group VI) estimation of male rodents composed with MTX-NP on day 14 and 28 were 84.37 ± 0.24 , 97.94 ± 0.32 , 101.42 ± 0.40 , 100.98 ± 0.24 and 105.64 ± 0.30 , 113.39 ± 1.74 , 124.29 ± 0.24 ,

124.62 ± 1.41 , freely. These attributes were when all is said in done detect low ($P < 0.001$) showed up contrastingly in connection to MTX control (120.52 ± 0.35 and 150.13 ± 0.45 , freely) and by and huge high ($P < 0.001$) emerged from keep running of factory control (60.94 ± 0.29 and 60.96 ± 0.30 , independently). The mean serum AST fixation (U/L) of all treatment social events (Group II to store V) were essentially one of kind ($P < 0.001$) to one another on day 14 and 28 of examination.

Aspartate aminotransferase (AST) in female rodents

The mean serum AST fixation (U/L) in low (Group III), medium (Group IV), high (Group V) and satellite (Group VI) estimation of female rodents controlled with MTX-NP on day 14 and 28 were 84.13 ± 0.13 , 91.59 ± 0.16 , 108.87 ± 0.23 , 108.54 ± 0.35 and 99.28 ± 0.67 , 111.77 ± 0.33 , 128.97 ± 0.30 , 128.64 ± 0.49 , freely. These attributes were fundamentally low ($P < 0.001$) showed up diversely in connection to MTX control (121.82 ± 0.30 and 151.85 ± 0.13 freely) and by and large high ($P < 0.001$) emerged from keep running of factory control (59.78 ± 0.54 and 60.05 ± 0.40 , autonomously)

The mean serum AST fixation (U/L) of all treatment get-togethers (Group II to add up to V) were for the most part phenomenal ($P < 0.001$) to one another on day 14 and 28 of examination.

RESULTS AND DISCUSSION

Effect of MTX & MTX-NP on Alanine aminotransferase (U/L) in

Table 2: Male & female rats of sub-acute toxicity study

Male rats ALT (U/L)						
Day	Group I (Normal control)	Group II (MTX Control)	Group III (MTX-NP Low dose)	Group IV (MTX-NP Medium dose)	Group V (MTX-NP High dose)	Group VI (MTX-NP Satellite)
0	33.27 ± 0.24^a	33.67 ± 0.36^a	33.35 ± 0.22^a	33.47 ± 0.31^a	33.43 ± 0.31^a	33.54 ± 0.28^a
14	33.55 ± 0.30^a	83.11 ± 1.08^b	49.41 ± 0.26^c	56.74 ± 0.27^d	68.67 ± 0.26^e	70.03 ± 0.61^e
28	33.33 ± 0.20^a	100.48 ± 0.29^b	68.30 ± 0.21^c	75.76 ± 0.71^d	88.29 ± 0.21^e	88.22 ± 0.67^e
42	0.000	0.000	0.000	0.000	0.000	77.82 ± 0.27

Female rats ALT (U/L)						
Day	Group I (Normal control)	Group II (MTX Control)	Group III (MTX-NP Low dose)	Group IV (MTX-NP Medium dose)	Group V (MTX-NP High dose)	Group VI (MTX-NP Satellite)
0	32.79 ± 0.29^a	31.82 ± 0.31^a	31.66 ± 0.30^a	32.09 ± 0.26^a	31.70 ± 0.25^a	31.94 ± 0.32^a
14	32.30 ± 0.44^a	76.14 ± 0.47^b	46.036 ± 0.65^c	54.04 ± 0.30^d	68.53 ± 0.39^e	69.73 ± 0.35^e

28	32.05±0.51 ^a	106.81±0.20 ^b	65.83±0.81 ^c	74.87±0.37 ^d	86.34±0.54 ^e	87.20±0.30 ^e
42	0.000	0.000	0.000	0.000	0.000	72.38±0.74

Note: Values expressed as Mean ± SEM, n=6, Means bearing different alphabets in row indicate significant difference (p<0.001).

Serum Creatinine in male rats

The mean serum Creatinine (mg/dL) values in low (Group III), medium (Group IV), high (Group V) and satellite (Group VI) estimation of male rodents coordinated with MTX-NP on day 14 and 28 were 0.95±0.01, 1.16±0.01, 1.27±0.00, 1.28±0.00 and 1.03±0.02, 1.12±0.01, 1.36±0.01, 1.33±0.02, solely. These qualities were as a rule sense low (P<0.001) wandered from MTX control (1.36±0.01 and 1.84±0.01, freely) and essentially high (P<0.001) showed up diversely in connection to standard control (0.74±0.01 and 0.75±0.01, autonomously) (Table. 32 and Fig. 32).

The mean serum Creatinine (mg/dL) estimations of all treatment social gatherings (Group II to gather V) were through and through momentous (P<0.001) to one another on day 14 and 28 of examination.

Serum Creatinine in female rodents

The mean serum Creatinine (mg/dL) values in low (Group III), medium (Group IV), high (Group V) and satellite (Group VI) estimations of female rodents controlled with MTX-NP on day 14 and 28 were 0.84±0.01, 0.97±0.00, 1.20±0.01, 1.18±0.00 and 0.89±0.02, 1.05±0.03, 1.29±0.01, 1.29±0.01, freely. These qualities were on exceptionally fundamental level low (P<0.001) emerged from MTX control (1.33±0.02 and 1.64±0.01, autonomously) and essentially high (P<0.001) showed up diversely in connection to basic control (0.73±0.01 and 0.75±0.01, only) (Table. 33 and Fig. 33).

The mean serum Creatinine (mg/dL) estimations of all treatment social events (Group II to bunch V) were out and out intriguing (P<0.001) to one another on day 14 and 28 of examination.

Table 3: Effect of MTX & MTX-NP on serum Creatinine (mg/dL) in male and female rats of sub-acute toxicity study

Male rats Creatinine (mg/dL)						
Day	Group I (Normal Control)	Group II (MTX Control)	Group III (MTX-NP Low dose)	Group IV (MTX-NP Medium dose)	Group V (MTX-NP High dose)	Group VI (MTX-NP Satellite)
0	0.74±0.01 ^a	0.75±0.01 ^a	0.73±0.01 ^a	0.73±0.01 ^a	0.74±0.01 ^a	0.75±0.01 ^a
14	0.74±0.01 ^a	1.36±0.01 ^b	0.95±0.01 ^c	1.16±0.01 ^d	1.27±0.00 ^e	1.28±0.00 ^e
28	0.75±0.01 ^a	1.84±0.01 ^b	1.03±0.02 ^c	1.12±0.01 ^d	1.36±0.01 ^e	1.33±0.02 ^e
42	0.000	0.000	0.000	0.000	0.000	1.16±0.05

Female rats Creatinine (mg/dL)						
Day	Group I (Normal Control)	Group II (MTX Control)	Group III (MTX-NP Low dose)	Group IV (MTX-NP Medium dose)	Group V (MTX-NP High dose)	Group VI (MTX-NP Satellite)
0	0.73±0.01 ^a	0.75±0.01 ^a	0.72±0.01 ^a	0.75±0.01 ^a	0.74±0.01 ^a	0.75±0.02 ^a
14	0.73±0.01 ^a	1.33±0.02 ^b	0.84±0.01 ^c	0.97±0.00 ^d	1.20±0.01 ^e	1.18±0.00 ^e
28	0.75±0.01 ^a	1.64±0.01 ^b	0.89±0.02 ^c	1.05±0.03 ^d	1.29±0.01 ^e	1.29±0.01 ^e
42	0.000	0.000	0.000	0.000	0.000	1.04±0.01

Note: Values expressed as Mean ± SEM, n=6, Means bearing different alphabets in row indicate significant difference (p<0.001).

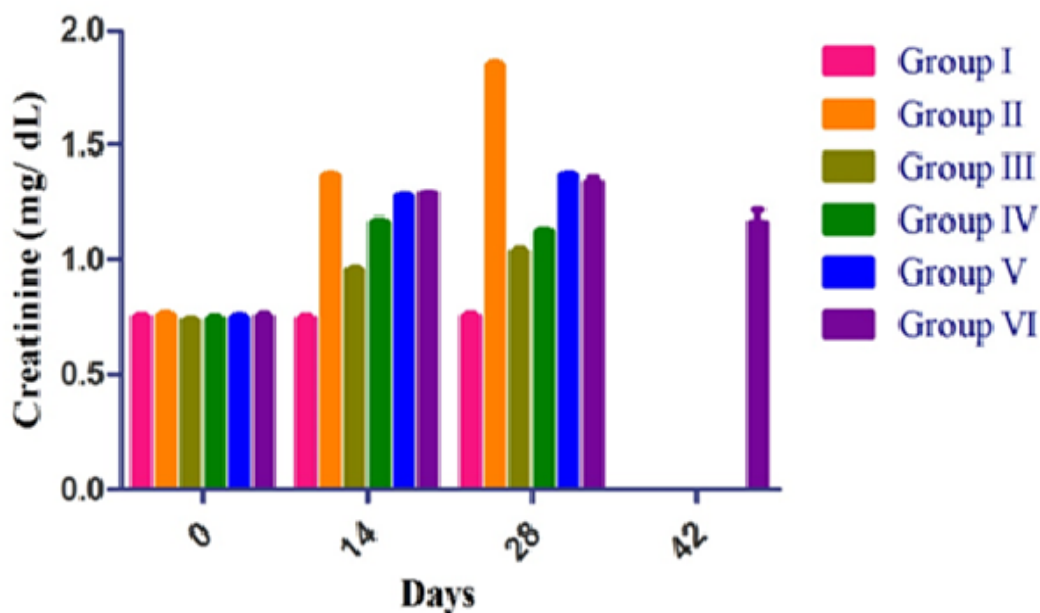


Figure 1: Effect of MTX and MTX-NP on serum creatinine (mg/dl)- sub acute toxicity male rats

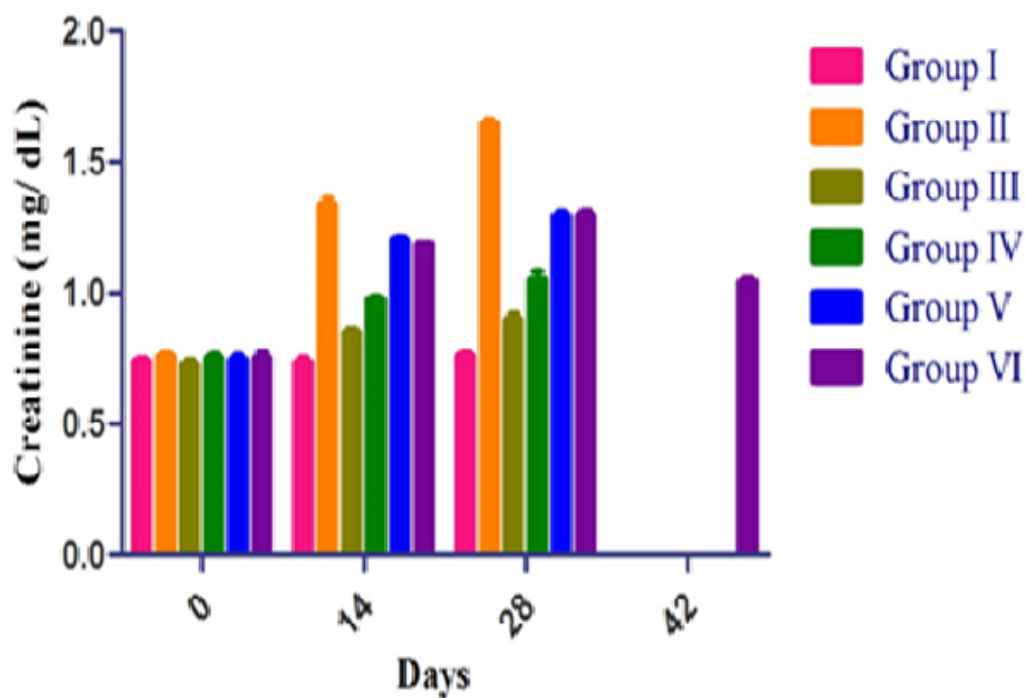


Figure 2: Effect of MTX and MTX-NP on serum creatinine (mg/dl)- sub acute toxicity female rats

Table 4: Effect of MTX & MTX-NP on Total erythrocyte count (10^6 Cells/mm³) in male & female rats of sub-acute toxicity study

Male rats TEC (10^6 Cells/mm ³)						
Day	Group I (Normal Control)	Group II (MTX Control)	Group III (MTX-NP Low dose)	Group IV (MTX-NP Medium dose)	Group V (MTX-NP High dose)	Group VI (MTX-NP Satellite)
0	8.58±0.03 ^a	8.67±0.04 ^a	8.65±0.02 ^a	8.61±0.04 ^a	8.60±0.04 ^a	8.60±0.04 ^a

14	8.50±0.03 ^a	5.98±0.03 ^b	7.58±0.05 ^c	7.22±0.05 ^d	6.90±0.03 ^e	6.81±0.04 ^e
28	8.57±0.03 ^a	3.75±0.05 ^b	6.03±0.01 ^c	5.55±0.06 ^d	4.51±0.04 ^e	4.40±0.05 ^e
42	0.000	0.000	0.000	0.000	0.000	5.28±0.05

Female rats TEC (10 ⁶ Cells/mm ³)						
Day	Group I (Normal Control)	Group II (MTX Control)	Group III (MTX-NP Low dose)	Group IV (MTX-NP Medium dose)	Group V (MTX-NP High dose)	Group VI (MTX-NP Satellite)
0	8.45±0.03 ^a	8.55±0.04 ^a	8.55±0.04 ^a	8.40±0.04 ^a	8.53±0.03 ^a	8.41±0.07 ^a
14	8.50±0.03 ^a	5.96±0.04 ^b	7.52±0.04 ^c	7.20±0.04 ^d	6.86±0.02 ^e	6.83±0.03 ^e
28	8.50±0.01 ^a	3.69±0.04 ^b	6.04±0.01 ^c	5.52±0.07 ^d	4.46±0.03 ^e	4.50±0.03 ^e
42	0.000	0.000	0.000	0.000	0.000	5.33±0.04

Note: Values expressed as Mean ± SEM, n=6, Means bearing different alphabets in the row indicate significant difference (p<0.001).

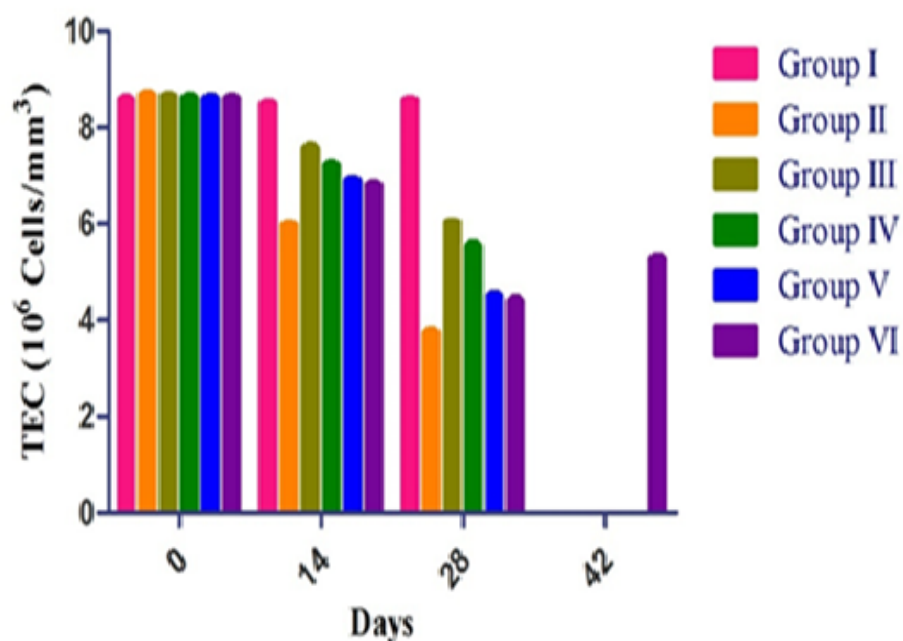


Figure 3: Effect of MTX and MTX-NP on Total Erythrocyte Count- sub acute toxicity male rats

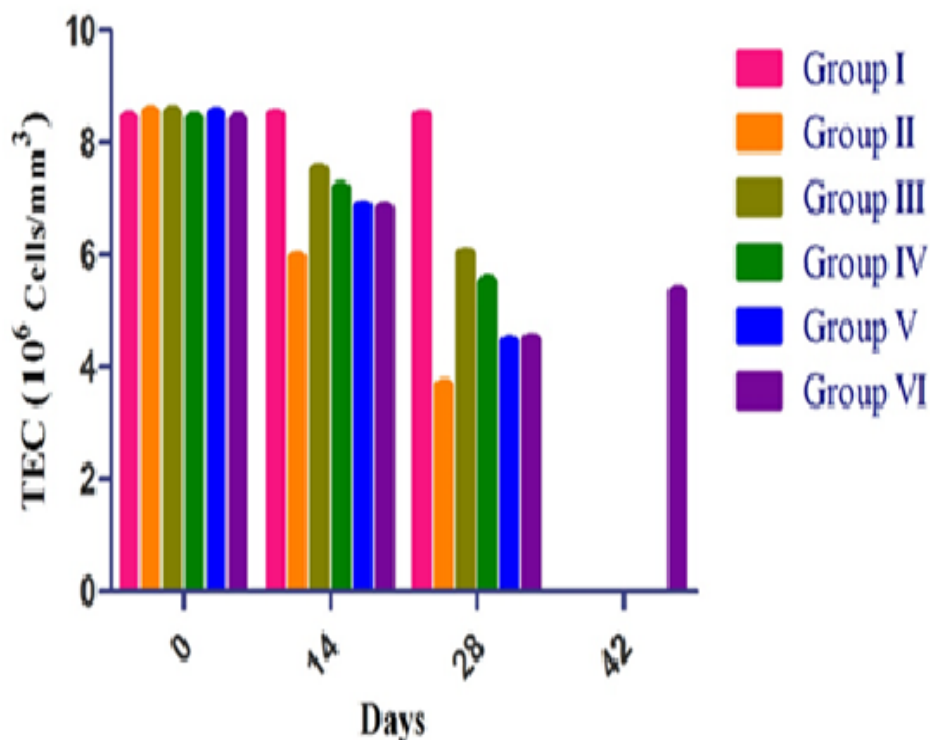


Figure 4: Effect of MTX and MTX-NP on Total Erythrocyte Count- sub acute toxicity female rats

CONCLUSION

It has been vividly observed from the current investigation, the formulations of Methotrexate Nanoparticles definitely have enhanced the intended investigational purpose. However it has been observed an remarkable profound significances in toxicity studies, as it is as safe as the other available

products. Absolutely, considerable and notable improvement in the bioavailability has been seen with the selected nano entity composites. Hence, it is more viable and feasible to fabricate nanoparticles with the Methotrexate, it can be pronounced to be better formulations.

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