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ORIGNAL ARTICLE

Methotrexate-Quercetin loaded solid lipid nanoparticles in the treatment of freund's complete adjuvant induced rheumatoid arthritis

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Abstract

The objective of the current study was to prepare and characterize the methotrexate-Quercetin loaded solid lipid nanoparticles and to determine their anti-inflammatory effect on induced arthritis rat model. Rheumatoid arthritis (RA) is a chronic autoimmune disease defined by inflammation, tissue damage and functional impairment. Methotrexate is most effective choice in rheumatoid arthritis. Quercetin is a flavonoid that is abundant in fruits and vegetables and known for its anti-inflammatory properties. For this study, animals were divided into 5 groups. First group was normal control; second group was kept as arthritic control and was provided with 0.1 ml freund's complete adjuvant (FCA); third group as standard group and was provided with Methotrexate-Quercetin plane (MTX-QU Plane) (T1); fourth group as test group 1 and provided with Methotrexate- Quercetin loaded solid nanoparticles low doses (MTX-QU SLNs LD) (T2) and fifth group as test group 2 and was provided with Methotrexate-Quercetin loaded solid nanoparticles high doses (MTX-QU SLNs HD) (T3). The physicochemical properties of nanoformulations were carried out by their zeta size, zeta potential and drug loading efficiency. The results of the study showed that Methotrexate- Quercetin loaded solid nanoparticles decreases the arthritis signs and symptoms as compared to the Methotrexate-Quercetin plane drugs.

Keywords

Rheumatoid Arthritis
Methotrexate
Quercetin
Solid lipid nanoparticles
Freund's Complete Adjuvant
Nano-medicine.

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Introduction

Nanotechnology is a developing field which involves the development of nano-particles for various applications (Nanda and Saravanan, 2009). Generally, the nano-particles size ranges between 10-1000 nm (Ulbrich and Lamprecht 2010). Most importantly, due to their minimum size and dimensions, these nano-particles acts as fundamental tool that can be utilized in complicated biological processes even at molecular and cellular levels (Ihsan et al., 2015). The exact cause of RA is unclear but there is involvement of complex interaction between environmental and various genetic factors (Majithia and Geraci, 2007). Major symptoms linked with RA include hyperplasia, bone erosion and cartilage destruction (Wang et al., 2018). Rheumatoid arthritis is mediated by certain proinflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) that leads to erosion of bone, joint abnormalities and cartilage destruction (Bhalekar et al., 2016). Pathological change linked with RA is the formation of synovial pannus. Pannus consists of proliferated cells, inflammatory cells, new capillaries and celluloses with similar features of tissue tumors (Yamada, 1998).

The therapeutic strategies for rheumatoid arthritis include disease-modifying antirheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), immune response mediator inhibitors and glucocorticoids (Escandell et al., 2007). Inflammatory

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cytokines current therapies such as IL-1, IL-6 and TNF-α are productive in the treatment of RA (Choudhary et al., 2018). NSAIDs use is limited in RA due to gastrointestinal complications (Yuan at al., 2012). Although glucocorticoids are used as an effective anti-inflammatory agent but their long-term exposure may cause skin atrophy, muscle atrophy and glaucoma (Russell et al., 2001). Methotrexate (MTX) is the most effective choice in the treatment of RA due to low cost, rapid onset, long-term safety and better response (Chen et al., 2013). Methotrexate was first time introduced in 1940s as folic acid antagonist. Methotrexate has grown into a standard treatment for rheumatoid arthritis. MTX drug combination strategy is helpful for clients for whom the calculated correctly dose of MTX do not give enough control of disorder (Kremer et al., 2003). The interest is increasing in flavonoids due to their therapeutic approaches to treat the inflammatory diseases (Ross and Kasum, 2002).

Flavonoids are basically grouped as flavanones, flavones, flavonols, anthocyanidins and catechins (Kaswar et al., 2016). Among these subclasses, the polyphenols are highly attracted because of their efficient applications. Many bio-chemicals and epidemiological studies of these polyphenols suggest their wide range of spectrum including pharmacological, biological and anticancer activities (Kumar and Pandey, 2013). Flavonoids inhibit tremendous enzyme count. These are also antithrombotic due to their potential to scavenge anions from superoxides. These anions are powerful active antagonists of prostacycline. Deletion of polyphenols from superoxide anions facilitates the formation of antiaggregatory prostaglandin. The antiaggregatory consequence of flavanols can be due to the limitation of isoprostanes development (Robak and Gryglewski, 1996). Among flavonoids, Quercetin is familiar which is found in vegetables, fruits and famous due to its anti-inflammatory and antioxidant potentials. Quercetin lowers the TNF-α production, which is one of the most important cytokine present in RA (Hasany et al., 2013)

Freund's complete adjuvant contains water and oil suspension with heat inactivated mycobacterium (Wiedemann et al., 1991). Now the research paying attention on many new approaches using lipid-based nanocarriers such as solid lipid nano-particles (SLN) (Kapoor et al., 2014). Solid lipid nano-particles (SLNs) provide advantages of control release, easy penetration, and physical stability and secure against labile degeneration of drug (Wissing et al., 2004). These particles are developed to be extremely selective for the cells, permit slow release of anti-inflammatory drugs, decreases systemic toxicity and improvement of drug distribution in body (Serra and Santamaria, 2015). Recently, nanoformulations including nanoemulsion, lipid nanocarriers, polymeric particles and liposomes are used. These are highly interesting because they have better adhesion to biological membrane while delivery of active drugs in controlled fashion (Tsai et al., 2015). More research was necessary to check the anti-inflammatory effect of drugs combination using lipid-based nano-carriers. Therefore, the current study was performed to prepare and characterize the methotrexate-Quercetin loaded solid lipid nanoparticles and to determine their anti-inflammatory effect on induced arthritis rat model.

Materials and Methods

Materials: This study was performed at Institute of Physiology and Pharmacology, University of Agriculture Faisalabad by using following materials; FCA (In vivo-Gen France), Methotrexate (United Biotech), Quercetin (kandel), Lauric Acid (Sigma Aldrich), Stearic Acid (Sigma Aldrich), COX-1 Kit (China ELISA Shanghai) and TNF-α (China ELISA Shanghai).

Animals: Total thirty (30) healthy male albino rats of 8 weeks old were selected in this study. All these animals were kept in animal house of Institute of Physiology and Pharmacology, University of Agriculture Faisalabad. The trial was conducted on animals with the permission of Bioethical committee of University of Agriculture Faisalabad.

Experimental Protocol: All rats were randomly divided into 5 groups. The groups detail and treatments are given in Table 1.

Synthesis of low and high-dose Methotrexate-Quercetin loaded solid lipid nano-particles: For low dose Methotrexate-Quercetin loaded solid lipid nano-particles, Stearic acid (170 mg), lauric acid (170 mg), quercetin (126mg) and methotrexate (10mg) were

Table 1: Experimental Protocol

Group No.	Group Name	No. of Animals	Treatment
1	Normal Control	6	Routine diet for 21 days
2	Arthritic Control	6	Routine diet for 21 days with FCA
3	Standard Group	6	MTX-QU per oral for 14-day post FCA induced arthritis in rat (MTX-QU Plane)
4	Test Group 1	6	low doses of MTX-QU loaded solid lipid nano-particles, per oral for 14 days post FCA induced arthritis in rat (MTX-QU SLNs LD)
5	Test Group 2	6	high doses of MTX-QU loaded solid lipid nano-particles, per oral for 14 days post FCA induced arthritis in rat (MTX-QU SLNs HD)

weighed on weighing balance. For high dose Methotrexate-Quercetin loaded solid lipid nano-particles, Stearic acid (340 mg), lauric acid (340 mg), quercetin (252mg) and methotrexate (20 mg) were weighed on weighing balance. Acids to drugs ratio were (5:2). Hot melt encapsulation method was used for preparation of nanoparticles. Both acids were added in the beaker, magnetic stirrer was put in beaker, and then beaker was placed over hot plate at 60 degree Celsius and 120 rpm. After homogenous mixture of both acids, 500 µl and 1000 µl of ethanol was added followed by both drugs in low and high dose nanoparticles respectively. Then 3% solution of Tween 80, 20 ml and 40 ml was added in both low and high dose respectively. Temperature was gradually reduced up to 26 degree Celsius for 30 minutes. After 30 minutes, hot plate knob was switched off and sample was left for cooling at room temperature. For the purification, the sample was placed in appendorf tubes and centrifuged at 14800 rpm for 15 minutes. Supernatant was discarded and the pellets were dissolved in distil water to remove the surfactant (Mehnert and Mader, 2012).

Characterization of low and high-dose Methotrexate-Quercetin loaded solid lipid nano-particles:

Nanoparticles were characterized for their zeta size and potential and encapsulation efficiency.

Zeta size: Zeta size was measured with the help of zeta sizer (Malvern).

Zeta Potential: Zeta potential was measured with help of Dynamic light scattering analyzer.

Encapsulation Efficiency: The encapsulation efficiency for Methotrexate-Quercetin was determined at 302 nm through following mentioned formula;

% Encapsulation efficiency = (Methotrexate-Quercetin weight in nano-particles)/initial weight of Methotrexate-Quercetin) * 100 (Bagad and Khan, 2015).

Statistical Analysis: The final data was analyzed by one- and two-way analysis of variance (ANNOVA). The difference between the mean values was subjected to Tuckey test. The P value was set at 95 % confidence interval (P<0.05).

Results

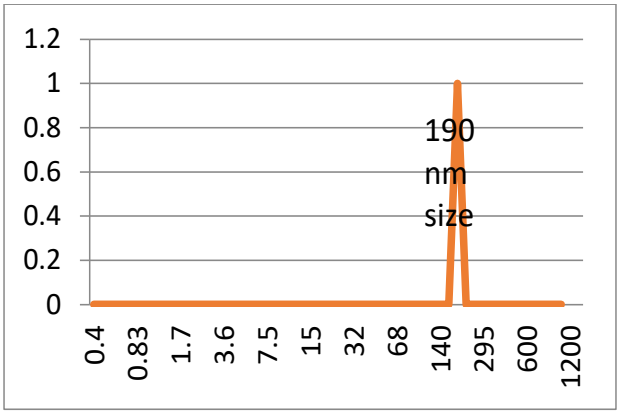
Zeta size for Low and high dose nanoparticles: Zeta size for low and high dose MTX-QU SLNs was 160 nm and 190 nm as given in Fig: 1A and Fig: 1B respectively.

Zeta Potential for Low and high dose nanoparticles: Zeta potential for low and high dose MTX-QU SLNs was -18.2 mV and -15.5 mV as given in (Fig: 2A) and (Fig: 2B) respectively.

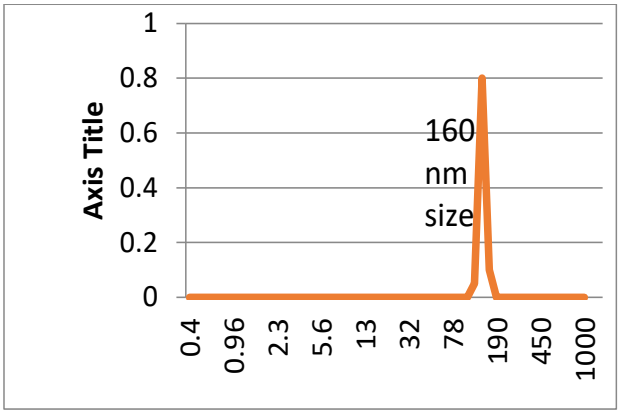
Entrapment Efficiency: The entrapment efficiency of low dose MTX-QU SLNs was 92.6% and high dose MTX-QU SLNs was 91.7% respectively (Table 2).

Table: 2: Encapsulation Efficiency

Name of Preparations	% Encapsulation
MTX-QU SLNs Low Dose (LD)	92.6%
MTX-QU SLNs High Dose (HD)	91.7%



(Fig: 1A)



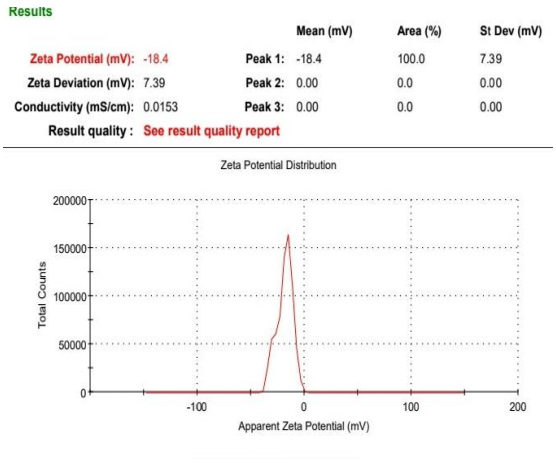
(Fig: 1B)

Fig. 1: Zeta size of methotrexate-quercetin loaded solid lipid nanoparticles low dose(A) and high dose (B)

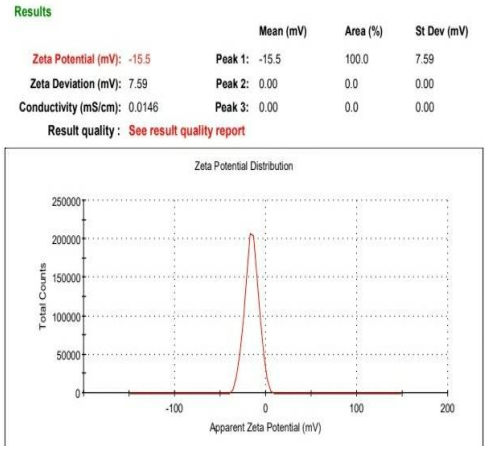
Inflammatory Mediators parameters: Graphical comparison of TNF-α level (Fig: 3A) and COX-1 (Fig: 3B) among different treatment groups are showing significant results among different treatment groups as P< 0.05.

Hematological Parameters: Graphical comparison of Hematological parameters i.e. WBCs (Fig: 4A), HB (Fig: 4B), MCV (4C), MCH (4D), HCT (4E), RBCs (4F), MCHC (4G) and Platelets (4H) among different treatment groups is showing significant results among different treatment groups of rats (P< 0.05).

Determination of Oxidative Stress Parameters: Graphical comparison of Catalase (CAT) (Fig: 5A) and Super oxide dismutase (SOD) (Fig: 5B) among different treatment groups is showing significant results among different treatment groups at P< 0.05.

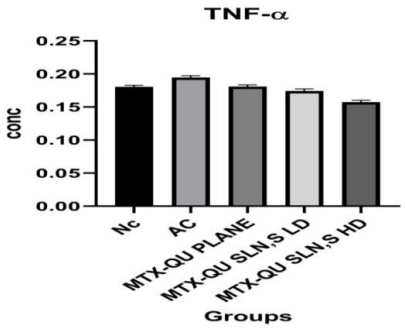


(Fig: 2A)

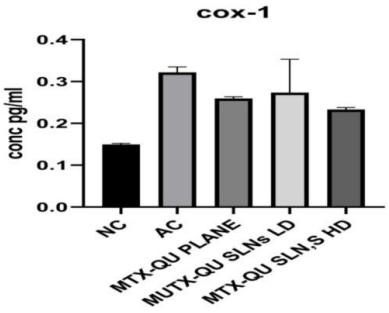


(Fig: 2B)

Fig. 2: Zeta potential of methotrexate-quercetin loaded solid lipid nanoparticles low dose(A) and high dose (B)



(Fig: 3A)



(Fig: 3B)

Fig. 3: TNF-α and COX-1 levels in rats treated with different doses of drugs.

Determination of Paw volume and body weight:
Graphical comparison of Paw volume (Fig: 6A) and body weight (Fig: 6B) among different treatment groups is showing significant results among different treatment groups as $P< 0.05$.

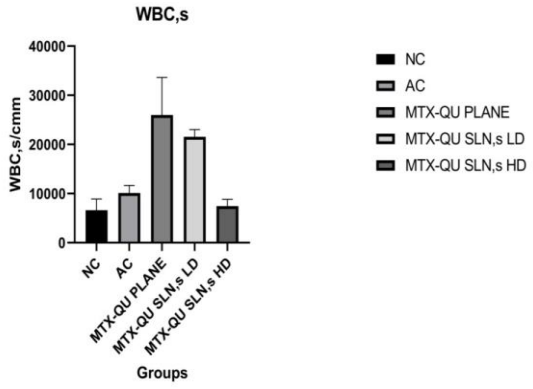
X-ray Analysis

X-ray analysis of normal joint in normal control group (Fig: 7A) showing no inflammation, Arthritic control group (Fig: 7B) showing inflammation, (Fig: 7C) showing joint treated with MTX-QU Plane, (Fig: 7D) showing joint treated with MTX-QU SLNs LD and (Fig 7E) showing joint treated with MTX-QU SLNs HD. Less inflammatory response is observed in low and high dose treatments as compared to control.

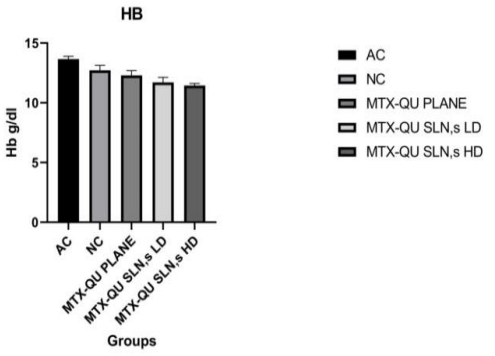
Histopathological Examination: In histopathology, it can be seen that in normal control group, joint is protected and no inflammation observed as shown in Fig. (8A). But in arthritic control group, it can be seen that there is severe inflammation of joint. Multinucleated cells can be seen. There is large number of inflammatory cells as shown in figure (8B). In MTX-QU Plane treated group, it can be seen that there is much inflammation on joint. Large number of inflammatory cells can be observed as shown in figure (8C). In MTX-QU SLN, s LD treated group, it can be observed that joints are still inflamed. Infiltration of inflammatory cytokines can be observed as shown in figure (8D). In MTX-QU SLN, s HD treated group, it can be observed that there is still disruption of joint cells. Inflammation comparatively decreased as compared to rest of all treatments as shown in Fig. (8E).

Discussion

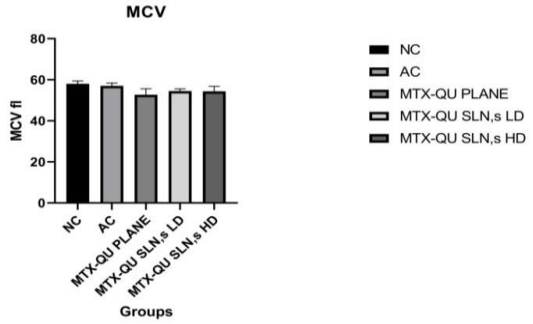
Quercetin (QU) is a flavonoid that is abundant in fruits and vegetables. It is still important to explain the exact mechanism of the anti-inflammatory effects of QU in arthritis. We clearly demonstrate, in addition to ex vivo results, that quercetin reduces the development of TNF alpha, major inflammatory, pro-arthritic macrophage mediators and cycloxygenase (Shen et al., 2002).



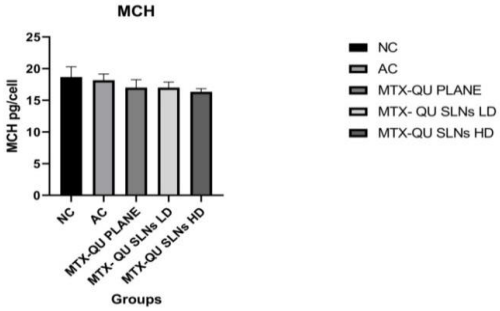
(Fig: 4A)



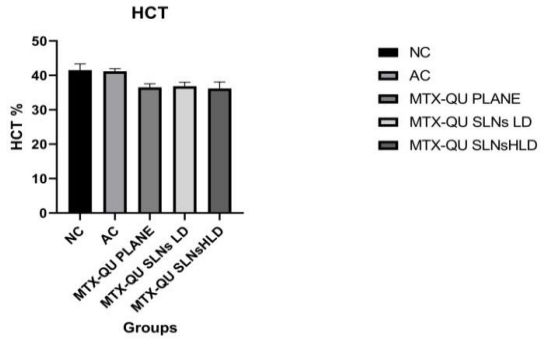
(Fig: 4B)



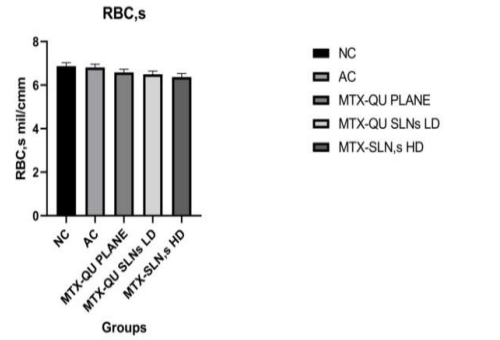
(Fig: 4C)



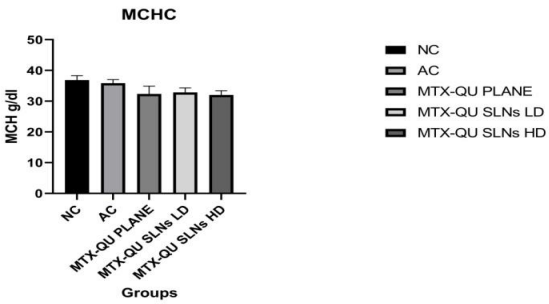
(Fig: 4D)



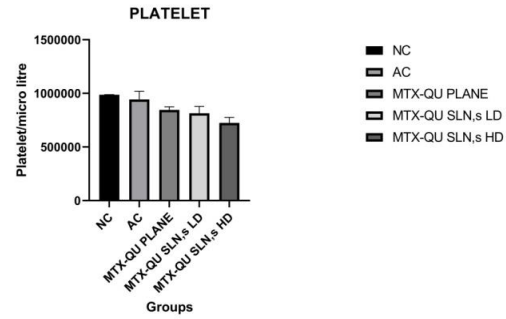
(Fig: 4E)



(Fig: 4F)

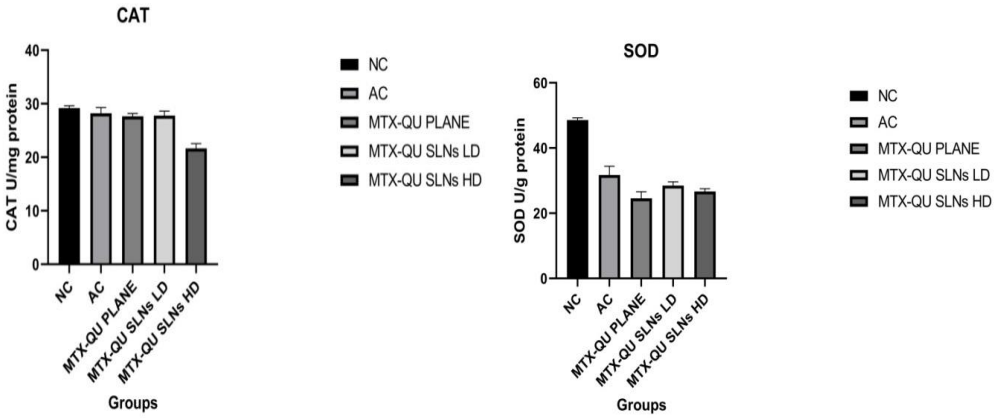


(Fig: 4G)



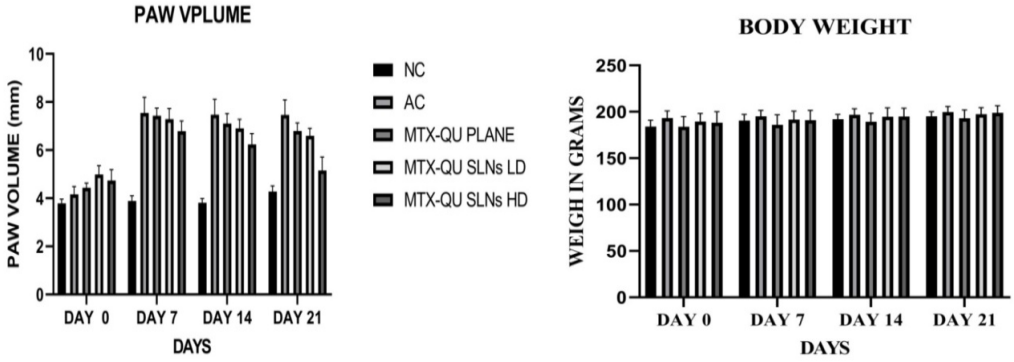
(Fig: 4H)

Fig. 4: Hematological parameters in rats given different treatments.



(Fig: 5A) **(Fig: 5B)**

Fig. 5: Anti-oxidant parameters catalase (CAT) and super oxide dismutase (SOD) in different treatment groups of rats



(Fig: 6A) **(Fig: 6B)**

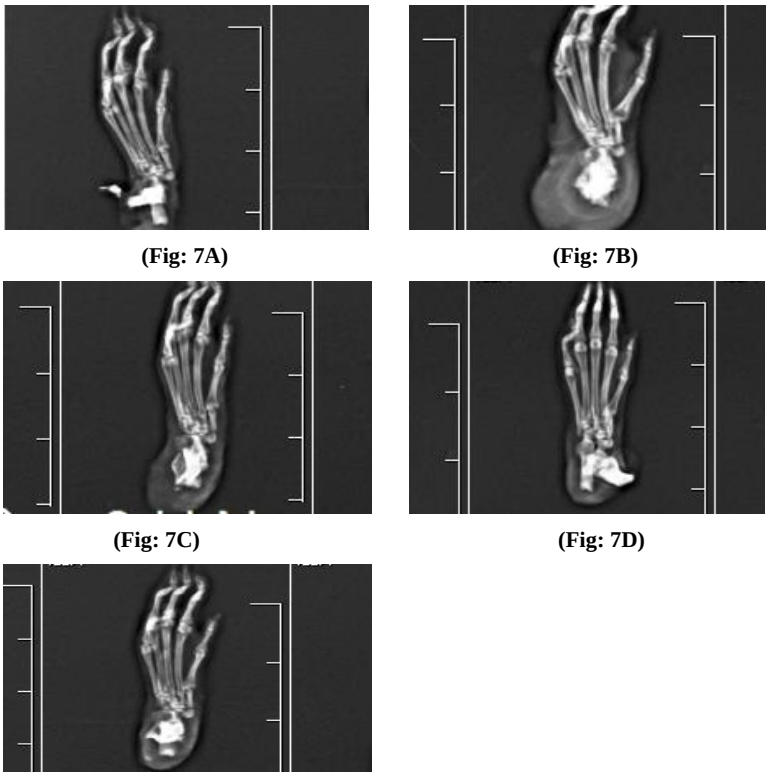
Fig. 6: Graphs of paw swelling and body weight of rats in different treatment groups.

Since anti-carcinogenic therapies are increasingly being used as adjuvant treatment for high-risk patients. The complications of chronic diseases are a major concern. Methotrexate that is commonly used in Anti-metabolite treatment for cancer or different types of arthritis due to oxidative reactions, it is considered to have toxic effects that happen in liver during metabolism. Due to the absence specificity of MTX for malignant cells can cause multiple tissues toxicity (Ayromlou et al., 2011).

Zeta size is an important tool in the characterization of nano-formulations. The created nano-formulations showed zeta size of 160 nm (Fig: 1A) and 190 nm (Fig: 1B) respectively for low and high dose nano-formulations which is present in 10-1000 nm range. This thing confirms the synthesis of nano-formulation. Nano-formulations carrying size of less than 200 nm are found to be more effective for the delivery of the therapeutic agents (Rizvi and Saleh, 2017).

Zeta potential of any formulation tells about the stability of any colloidal suspension (Moore et al., 2015). For the measurement of zeta potential, the nano-formulations were first diluted in the distilled water and were analyzed under the D.L.S analyzer (Bhattacharjee, 2016). Both of the nano-formulations exhibited zeta potential of -18.2 mv (Fig: 2A) and -15.5 mv (Fig: 2B) for low and high dose respectively. The negative zeta potential with solid lipids showed the emerging stability of particles.

To determine the encapsulation efficiency of the nano-formulations, indirect procedure was used. First of all the sample was centrifuged and its supernatant was separated to calculate the free drug concentration. Then total concentration of drug present in the nano-formulation was calculated. MTX-QU SLNs showed maximum absorbance at 302 nm wavelength. The encapsulation efficiency of the nano-formulations was 92.6 % for low dose and 91.7% for high dose nano-particles as given in Table 2.



(Fig: 7A) **(Fig: 7B)**

(Fig: 7C) **(Fig: 7D)**

(Fig: 7E)

Fig. 7: X-ray analysis of joints of different treatment groups of rats.

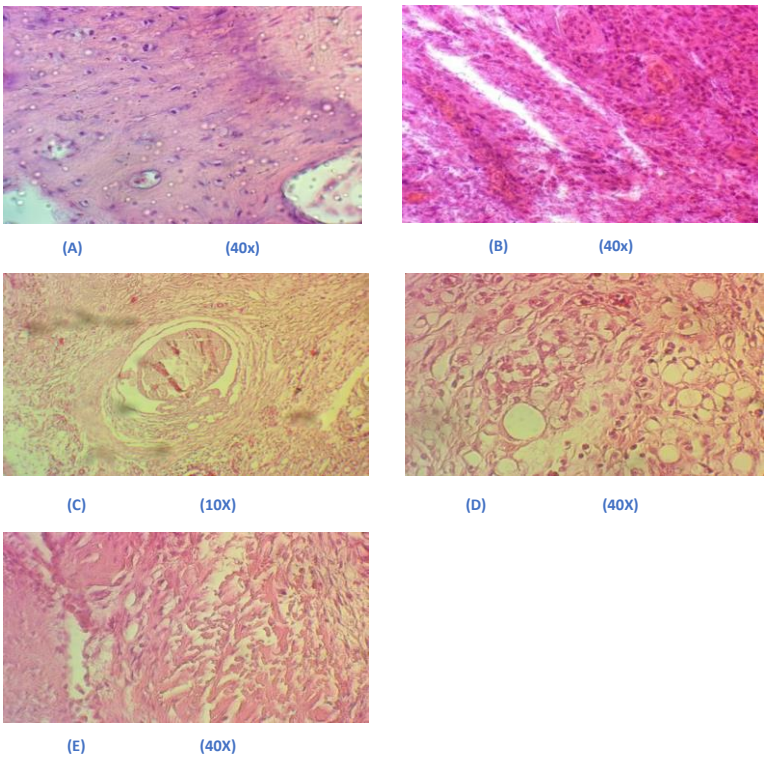


Fig. 8: Histological examination of joints of different treatment groups of rats.

In this trial, the arthritis was induced by injecting FCA 0.1 ml in the paw of rat. FCA produced the inflammation because it activates the immune system. The anti-rheumatic effect of MTX-QU plan was compared with the MTX-QU SLN, s Low doses and High doses. The arthritic group showed significant increase in the paw volume as compared to the control group.

Oxidative stress was evaluated by monitoring the level of SOD and CAT. MTX-QU SLN, s decreased the level of SOD (Fig: 5B) and CAT (Fig: 5A) as compared to the control groups. The latest studies showed that oxygen radicals and hydrogen have been shown to the undesired adverse effects of peroxides are correlated with the undesired effects of multiple anti-tumors drugs. Though, the generation of Reactive oxygen species (ROS) and Cellular damage can result from the development of hydroxyl radicals. The probable ROS mechanism is due to high levels of Purine-catabolizing enzyme activities (Çetin et al., 2008).

Hematological examination was conducted in order to check the blood parameters including WBCs, (Fig: 4A), Hb (Fig: 4B), RBC s, (Fig: 4F), Hematocrit (Fig: 4E), MCV (Fig: 4C), MCH (Fig: 4D), MCHC (Fig: 4G) and platelet aggregation (Fig: 4H). Due to presence of the methotrexate, there was reduction in these parameters in treatment groups similar to the observations of Akacha et al. (2010).

Inflammatory mediators including TNF-α, cyclooxygenase-1 were checked by using ELISA kits by performing different dilutions. Then the standard curve was generated to check their efficiency. There was significance decreased in TNF-α (Fig: 3A) and cyclooxygenase-1 (Fig: 3B) in the treated groups as compared to arthritic control group (Morikawa et al., 2003). The development of TNF-alpha is still one of the most powerful agents in progression of arthritis. Disease modifying antirheumatic drugs such as Methotrexate is used for the treatment of RA that works via the release of adenosine. Although the adenosine receptor involved in this action remains controversial (Prabhakar et al., 1995).

Histopathological examination was conducted to check the effect of MTX-QU SLN, s with high and low doses. The arthritic control group showed significance destruction of knee joint with infiltration of inflammatory cytokines. Results showed that there was minimum reduction in the destruction of joint MTX-QU SLN, s treated group as compared to the MTX-QU plane and arthritic control (Kim and Kang, 2015).

Conclusion: It can be suggested that Methotrexate-Quercetin loaded solid lipids using nanotechnology-based approaches have better potential to compete the inflammation as compared to Methotrexate-Quercetin plane. Combination of the Stearic and lauric acids exhibited beneficial effects with the consistency in the reduction of arthritis. Furthermore, Methotrexate-Quercetin loaded solid lipids also lowers the oxidative stress that will ultimately protect the nervous cells and decreases the inflammatory mediators (COX-1 and TNF-α) in arthritis.

Conflicts of interest: We declare that there are no conflicts of interest for this study.

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