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METFORMIN ATTENUATES FREUND'S ADJUVANT INDUCED ARTHRITIS IN MALE RATS

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ABSTRACT

Objective: Metformin is well known anti-diabetic agent. Recently many reports have described the anti-inflammatory effects of metformin on various cell types including human vascular smooth muscle cells and endothelial cells. This study was designed to investigate the anti-arthritic effects of metformin on Freund's complete adjuvant (FCA)-induced arthritis in rats. **Methods**: rats were randomly divided into 4 groups as follows: control group, rheumatoid arthritis (RA) rat model group, indomethacin group and metformin group. The hind paw volume of rats was measured. Oxidative stress and inflammatory cytokines were also analyzed. Western blot analysis was used to evaluate cyclooxygenase-2 (COX-2) protein expression levels. **Results:** The results demonstrated that metformin (200 mg kg^{-1}) was able significantly to reduce hind paw volume. Metformin treatment decreased circulating levels of inflammatory response markers C-reactive protein (CRP), IL-1β, IL-6 and TNF-α. The production of COX-2 and subsequently prostaglandin E2 (PGE2) in FCA rats was reduced significantly by treatment with metformin. In addition, metformin significantly reduced oxidative stress. **Conclusion**: These findings suggest that the inflammatory response and oxidative stress induced by FCA could be attenuated by metformin in RA rat model. Metformin has significant anti-inflammatory effects on FCA rats, which may be associated with the reduction of COX-2 and PGE2 inflammatory mediators.

KEYWORDS: metformin, rheumatoid arthritis, cytokines, adjuvant-induced arthritis.

Abbreviations
COX-2: cyclooxygenase-2
CRP: C-reactive protein
FCA: Freund's complete adjuvant
IL-1β: interleukin-1β
IL-6: interleukin-6
MDA: Malondialdehyde
NO: Nitric oxide
PGE2: prostaglandin E2
RA: rheumatoid arthritis
TNF-α Tumor necrosis factor-α

INTRODUCTION

Rheumatoid arthritis is a chronic inflammatory disorder, characterized by an abundant cellular infiltration consisting of neutrophils, macrophages and lymphocytes, leading to the release of multiple inflammatory cytokines and matrix-degrading enzymes that contribute to the progressive joint destruction (McInnes and Schett, 2007). The pathological characteristics of RA mainly include joint inflammation of the synovial tissue and excessive hyperplasia (Bax et al., 2011). Migration of activated phagocytes and leukocytes into synovial cavity, cartilage erosion and bone degradation are the diagnostic features of RA (Ansari et al., 2015). Tumor necrosis factor-α (TNF- α) and interleukin-1β (IL-1β) are pro-inflammatory cytokines that are pivotal in the pathogenesis of RA (Lu et al., 2014). COX-2 acts as a mediator of angiogenesis, its differential expression determines the production of prostaglandins (Kim et al., 2012).

Metformin, a biguanide, is the first-line oral therapy for type 2 diabetes and the most widely used anti-diabetic drug, alone or in combination with other anti-hyperglycemic agents (Ferrannini, 2014)). Metformin decreases hepatic glucose production through mild inhibition of the mitochondrial respiratory chain complex I (Viollet et al., 2012). Metformin was also shown to possess antioxidant and anti-inflammatory properties (Martin et al., 2013). Recently, metformin was shown to inhibit inflammation and oxidative stress induced by lipopolysaccharide in human middle ear epithelial cell lines (Cho et al., 2016).

However, there are few RA treatment strategies that are effective, reliable and have low toxicity (Doan and Massarotti, 2005). Therefore, the present study was

designed to investigate the anti-inflammatory effects of metformin in RA rat model by investigating its effects on the inhibition of inflammatory response.

MATERIALS AND METHODS

Animals: Male adult albino rats weighing 150-200 grams have been used. Animals were obtained from the animal house, Faculty of Medicine, Assiut University and were housed in animal place with room temperature being maintained at 25±2°C. Animals were fed on a commercial pellet diet and kept under normal light/dark cycle. Animals were given a free access for food and water *ad libitum.*

Induction of arthritis: To induce arthritis, the right hind paw of male albino rats was sterilized with 70% alcohol. Rats were intradermal injected with 0.1 ml of FCA (10 mg ml^{-1}) suspension of heat-killed Mycobacterium tuberculosis (Rajesh et al., 2009 and Yao et al., 2014). Control animals were injected intradermal with saline in equal volume. Chronic inflammation was allowed to progress for 12 days. Rats were divided into 4 groups of eight rats each. Drugs were I.P. administered for 17 days starting on 13^{th} day after arthritis induction and continued till 30 days. Experimental animals were divided as follow:

Group I: Saline treated normal control intradermal injected.

Group II: FCA-induced arthritic control received saline I.P.

Group III: FCA-induced arthritic animals treated with indomethacin 10 mg kg^{-1} I.P.

Group IV: FCA-induced arthritic animals treated with metformin 200 mg kg^{-1}I.P (Ghadge et al., 2016).

On the 31^{th} day, blood samples were collected from the heart and serum was separated by centrifugation and stored at – 80˚C until analysis. The animals were sacrificed by cervical dislocation. The hind paws were separated and immersed in physiological saline to be ready for homogenization.

Hind paw volume measurement. The hind paw volume of all animal groups was measured by water displacement plethysmometer (Coelho et al., 2004) at 0, 13, 20, 24, 27 and 30 day after the injection of FCA emulsion. The inhibited effects of metformin was determined by comparing the changes in volumes of hind paws and expressed in milliliter (ml±SD).

Biochemical assessment

The level of CRP was determined using ELISA kit catalog No. 557825 for the quantitative measurement of rat CRP in serum.

Malondialdehyde, the oxidative stress product of lipid peroxidation, reacts with thiobarbituric acid under acidic conditions at 95°C to form a pink-colored complex with an absorbance at 532 nm (Ohkawa et al., 1979).

Nitric oxide concentration in serum was determined with the Greiss method. The Greiss reagent is made up of a 1% solution of sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihycrochloride in distilled water.

The protein and phenol red of the serum were deleted using zinc sulfate (6 mg/400 µl). Sodium nitrite (0.1 M) was used for the standard curve, and increasing concentrations of sodium nitrite (5, 10, 25, 50, 75, and 100 µM) were prepared. The Greiss solution was added to all microplates, containing sodium nitrite and serum and was read by ELISA reader in 540 nm (Khazaei et al., 2011).

Tumor necrosis factor-α was measured, using a sandwich enzyme immunoassay kit protocol supplied by the manufacturer of the antibodies (Multisciences Biologic Company, Hangzhou, China) and resultant optical density determined, using a microplate reader (Thermo Multiskan MK3) at 450 nm. IL-1β, IL-6 and PGE2 serum levels were determined using ELISA kit (Abcam) according to the manufacturer's instructions.

Western Blotting

Following treatment with metformin, RA tissue samples were removed and homogenized. Total protein was extracted by adding 1ml of Radio-Immuno Precipitation Assay buffer [RIPA (50 mM Tris-HCl pH 8, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 µg ml^{-1} PMSF [phenylmethylsulphonyl fluoride], 1 µg ml^{-1} aprotinin, 1% NP-40)]. Samples were incubated for 30 min. on crushed ice. The tissue lysate was then centrifuged at 12,000×g for 2 min at 4 °C. The supernatant was then transferred to a fresh Eppendorf tube and total protein concentrations were determined using the Bradford protein assay (1976). Denaturation of protein samples was performed by adding loading buffer (0.125 M Tris-HCl, 4% SDS, 20% v/v glycerol, 0.2 M dithiothreitol, 0.02% bromophenol blue, pH 6.8), 1:1 (v/v) at 100 °C for 10 min. followed by centrifugation 3000×g for 1 min. Samples (50 µg of protein) were immediately electrophoresed by SDS-PAGE (BioRad Mini Protein II Electrophoresis gel). Protein bands were transferred to a nitrocellulose membrane (Amersham). The membranes were then incubated in blocking solution [5% non-fat dry milk in TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5)] overnight on a shaker at 4 °C, immuno-stained for COX-2 using rabbit polyclonal primary antibodies (Abcam) and for β-Actin using anti- β-Actin mouse monoclonal primary antibody (Abcam) as a loading control, was conducting according to manufacturer's instructions. Subsequently, the immunoblots were incubated with secondary antibody conjugated with horseradish peroxidase enzyme in 1% BSA in PBS, 0.1% Tween 20. After 1 h incubation at room temperature the immunodetected band visualization was carried out using the chemiluminescent alkaline phosphatase substrate (Immobilom™ Western).

Drugs and chemicals

Metformin was obtained from CID Co. (Cairo, Egypt) dissolved in saline.

Indomethacin: (Nile Co. for pharmaceuticals, Cairo, Egypt) dissolved in saline.

Freund's complete adjuvant (FCA) was purchased from Sigma-Aldrich

Statistical analysis

Statistics was performed using the statistical graph pad prism 5. One way analysis of variables (ANOVA) was used. Significant differences between the groups were determined using a Newman-keuls test. Data were expressed as means ± standard deviation of the mean (SD) and the level of significance between groups were considered significant (*) at $p<0.05$.

RESULTS

Rat hind paw volume. At beginning of the experiment, i.e. day 0, no significant difference was found in rat hind paw volume among all groups. A significant increase in rat hind paw volume was observed for the adjuvant injected group (group-II) on day 13, 20, 24, 27 and 30 day compared to the healthy control rats. Meanwhile, the paw edema volume was significantly reduced in group treated with standard drug of indomethacin and metformin treated group which showed significant difference when compared with the arthritis group (See Table 1).

Table 1. Effect of metformin on rat hind paw swelling in adjuvant-induced arthritis model.

Groups	Dosage mg Kg^{-1}	Rat hind paw volume in ml ± SD					
		Day 0	Day 13	Day 20	Day 24	Day 27	Day 30
Group-I	-	0.850±0.11	1.01±0.10	1.03±0.07	1.05±0.07	1.05±0.33	1.06±0.16
Group-II	-	0.853±0.15	1.86±0.14$^{\#}$	1.85±0.13$^{\#}$	1.85±0.11$^{\#}$	1.88±0.11*	2.02±0.13*
Group-III	10	0.840±0.21	1.79±0.15	1.60±0.11*	1.53±0.14*	1.46±0.05*	1.37±0.05*
Group-IV	200	0.850±0.22	1.81±0.19	1.61±0.12*	1.55±0.08*	1.47±0.06*	1.37±0.06*

Values are expressed as mean ± S.D., n = 8 animals in each group $^{\#}$ Significant result at $p<0.05$ from normal control * Significant result at $p<0.05$ from arthritic control group.

Effect of metformin on the protein expression of COX-2 in the synovial tissues of RA rats

To investigate the potential mechanism underlying the actions of metformin, the protein expression of COX-2 was detected by western blot analysis (Figure 1). Compared with the normal group, a high level of COX-2 protein expression was detected in the RA model group. The metformin-treated group exhibited a significantly decreased COX-2 level in the synovial tissue. Indomethacin also significantly reduced the level of COX-2 in synovial tissue.

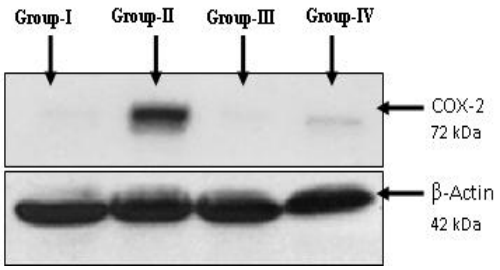

Figure 1. Effect of metformin on COX-2 protein expression in the synovial tissues of FCA rat.

Metformin regulates the concentration of PGE2 in serum

As shown in figure (2), compared with the normal control group, the concentrations of PGE2 significantly increased in serum of RA rats. Treatment with metformin significantly decreased the concentration of PGE2 compared with RA rats. The indomethacin-treated group demonstrated similar results to the group treated with metformin.

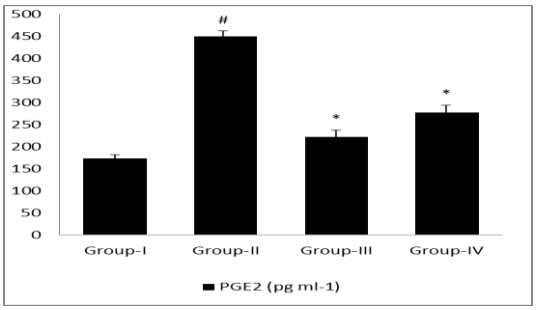

Figure 2. Effect of metformin on concentration of PGE2 in serum of RA model rats

Values are expressed as mean ± S.D., n = 8 animals in each group $^{\#}$ Significant result at $p<0.05$ from normal control * Significant result at $p<0.05$ from arthritic control group.

Effect of metformin on serum levels of CRP, IL-1β, IL-6, MDA, NO and TNF-α.

On day 31 after adjuvant inoculation, levels of CRP, IL-1β, IL-6, MDA, NO and TNF-α in serum were significantly increased in RA model rats (group II) than that of normal control group (group I). After treatment with metformin all of determined parameters in this test were significantly decreased (see Table 2). From the changes of hind paw and the regulation of pro-inflammation markers by metformin, it was showed that they slowed the progression of inflammation.

Table 2. Effect of metformin on CRP, IL-1β, IL-6, MDA, NO and TNF-α in FCA rat.

Groups	CRP (mg L^{-1})	NO (µmol L^{-1})	MDA (nmol ml^{-1})	IL-1β (pg ml^{-1})	IL-6 (pg ml^{-1})	TNFα (pg ml^{-1})
Group-I	0.323±0.019	72.73±6.49	31.47±3.10	34.08±4.08	39.75±5.26	15.67±1.33
Group-II	2.76±0.38#	154.22±9.22#	44.96±1.66#	65.50±8.25#	104.55±16.80#	49.36±4.06#
Group-III	1.61±0.12*	86.52±12.52*	33.74±2.90*	39.64±973*	45.57±5.11*	19.95±1.54*
Group-IV	1.65±0.14*	94.58±8.39*	35.51±1.23*	40.65±7.83*	47.43±6.72*	25.67±1.52*

Values are expressed as mean ± S.D., n = 8 animals in each group # Significant result at p<0.05 from normal control * Significant result at p<0.05 from arthritic control group.

DISCUSSION

FCA-induced arthritis in rats is widely used experimental model for inflammatory arthritis sharing several features of human rheumatoid arthritis (Phadke et al., 1985). Induction of arthritis in adjuvant exposed rats results in generation of inflammatory arthritis by acute periarticular inflammation with synovial mononuclear infiltration followed by synovial hyperplasia and damage to articular bone and cartilage just as in the case of arthritis in human (Kaur and Sultana, 2012). In rheumatoid arthritis, inflammatory mediators stimulate inflammation of the synovial tissues which cause soft tissue swelling along with fluid exudation and cellular influx in the synovium (Jin et al., 2010).

Metformin, a drug widely used to treat type 2 diabetes, inhibits inflammation associated with various disease conditions via blockade of the activity of the transcription factor NF-κB and the secretion of the pro-inflammatory cytokines, TNF-α, IL-6 and IL-1β (Isoda et al.,2006) and the inhibition of COX-2 and inducible nitric oxide synthase (Kalariya et al.,2012). It is generally recognized that RA is an immune-mediated disease with chronic progressive inflammation (Majithia et al., 2007). The results of the present study demonstrated that treatment with metformin decreased paw swelling. Metformin effective in inhibiting the inflammatory response in the present study is comparable to other studies (Pryor and Cabreiro, 2015; Abhimanu and Vijay, 2016).

In various disease conditions, both inflammation and oxidative stress coexist and contribute to morbidity (Biswas, 2016). In view of this, the present study measured the serum level of oxidative stress markers. The serum level of MDA and NO were significantly increased in FCA rat model compared to normal control. Treatment with metformin tend to normalize the levels of these markers like indomethacin. Metformin protects against inflammation by maintaining the redox balance. Inhibiting ROS generation has also been reported as an important mechanism that contributes to its anti-inflammatory actions (Malínskà et al., 2016).

While inflammatory reactions are not the only features of RA, they are the main problem in RA patients. Therefore, controlling inflammatory reactions might be a feasible RA treatment method (Choi et al., 2015). The present study investigated the effect of metformin on the release of pro-inflammatory cytokines and CRP. Pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 stimulate inflammatory responses in arthritic joints and synovial tissues; as such, these cytokines have been reported as potential therapeutic targets for RA (Imboden, 2009; Mariaselvam et al., 2014 and Chaabo et al 2015). The present study shows that daily treatment with metformin suppressed the levels of these pro-inflammatory cytokines as well as CRP in the serum of FCA rats. This is in accordance with other study (Malínskà et al., 2016).

Pro-inflammatory enzyme, including Cox-2, has also been reported to be important for the development of inflammatory responses, and it is over-expressed during the inflammatory process. In addition, this enzyme cause the synthesis and secretion of prostaglandins, including PGE2, which is closely related to pain and inflammatory symptoms and can lead to pain and inflammation in the joints of RA patients (Zheng et al., 2014). PGE2 is a vital inflammatory disease mediator, which is important in inflammatory process (Shindler et al., 2010). A highly selective COX-2 inhibitor is important in the clinical treatment of RA (Lichtenberger et al., 2009). In addition, PGE2 which is excessively expressed in RA, is important in synovial tissue vasodilation, liquid leakage and pain (Gheorghe et al., 2011). The present study found a high expression level of COX-2 protein in synovial tissues and PGE2 in the serum of FCA model rats. Metformin was able to reduce overexpression of the COX-2 protein in synovial tissues and concentrations of PGE2 in the serum. This is in harmony with other studies (Cho et al., 2016; Liu et al., 2016 and Tikoo et al., 2016).

In conclusion metformin played a role in alleviating the inflammatory response. These findings suggest that metformin could suppress the inflammatory response and oxidative stress induced by FCA in rat model. Therefore, metformin may have a therapeutic potential for the treatment of RA.

Conflict: No conflict of interest.

REFERENCES

1. Abhimanu Pandey and Vijay L. Kumar (2016): Protective Effect of Metformin against Acute Inflammation and Oxidative Stress in Rat. Drug development research, 77: 278–284.
2. Ansari MM, Neha, Khan HA. (2015). Effect of cadmium chloride exposure on the induction of

collagen induced arthritis. Chem Biol Interact, 238*:* 55–65.
3. Bax M, van Heemst J, Huizinga TW and Toes RE (2011): Genetics of rheumatoid arthritis: what have we learned? Immunogenetics. 63: 459–466.
4. Biswas SK. (2016). Does the interdependence between oxidative stress and inflammation explain the antioxidant paradox? Oxid Med Cell Longev 2016: 5698931.
5. Bradford M Ms.(1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. **72**: 248-54,
6. Chaabo K., Kirkham B.(2015): Rheumatoid arthritis-anti-TNF. Int. Immunopharmacol.; 27: 180–184. doi: 10.1016/j.intimp.2015; 04: 051.
7. Cho JG1, Song JJ1, Choi J1, Im GJ1, Jung HH, Chae SW. (2016): The suppressive effects of metformin on inflammatory response of otitis media model in human middle ear epithelial cells. Int J Pediatr Otorhinolaryngol. Oct; 89:28-32. Epub 2016 Jul 25.
8. Choi J.K., Oh H.M., Park J.H., Choi J.H., Sa K.H., Kang Y.M., et al., (2015): Salvia plebeia extract inhibits the inflammatory response in human rheumatoid synovial fibroblasts and a murine model of arthritis. Phytomedicine.; 22: 415–422. doi: 10.1016/j.phymed..01.007.
9. Coelho, M. C. et al. (2004)Anti-arthritic effect and subacute toxicological evaluation of Baccharis genistelloides aqueous extract. Toxicol. Lett. 154: 69–80.
10. Doan T., Massarotti E. (2005): Rheumatoid arthritis: An overview of new and emerging therapies. J. Clin. Pharmacol. 2005; 45: 751–762.
11. Ferrannini E.(2014): The target of metformin in type 2 diabetes. N Engl J Med.; 371: 1547–8.
12. Ghadge A1, Harsulkar A1, Karandikar M2, Pandit V3, Kuvalekar A1.(2016) Comparative anti-inflammatory and lipid-normalizing effects of metformin and omega-3 fatty acids through modulation of transcription factors in diabetic rats. Genes Nutr. Mar 17; 11: 10. doi: 10.1186/s12263-016-0518-4.
13. Gheorghe KR, Thurlings RM, Westman M, Boumans MJ, Malmström V, Trollmo C, et al., (2011): Prostaglandin E2 synthesizing enzymes in rheumatoid arthritis B cells and the effects of B cell depleting therapy on enzyme expression. PLoS One. 6:e16378 2011.
14. Imboden J.B. (2009) The immunopathogenesis of rheumatoid arthritis. Annu. Rev. Pathol. Mech. Dis. 2009; 4: 417–434. doi: 10.1146/annurev.pathol.4.110807.092254.
15. Isoda K, Young JL, Zirlik A, MacFarlane LA, Tsuboi N, Gerdes N, et al., (2006). Metformin inhibits pro-inflammatory responses and nuclear factor-kappa B in human vascular wall cells. Arterioscler Thromb Vasc Biol 26: 611–617.
16. Jin J.H., J.S.Kim, S.S.Kang, K.H. Son, H.W. Chang and H.P.Kim (2010): Anti-inflammatory and anti-arthritic activity of total flavonoids of the roots of *Sophora flavescens.* J. Ethnopharmacol. 127: 589-595.
17. Kalariya NM, Shoeb M, Ansari NH, Srivastava SK, Ramana KV.(2012). Anti-diabetic drug metformin suppresses endotoxin induced uveitis in rats. Invest Ophthalmol Vis Sci 53: 3431– 3440.
18. Kaur G. and Sultana S. (2012): Evaluation of anti-arthritic activity of isoeugenol in adjuvant induced arthritis in murine model. Food Chem Toxicol. Aug; 50(8): 2689-95.
19. Khazaei M, Roshankhah S, Ghorbani R, Chobsaz F. (2011) Sildenafil effect on nitric oxide secretion by normal human endometrial epithelial cells cultured in vitro. Int J Fertil Steril. 5: 142–147.
20. Kim HG, Han EH, Jang WS, Choi JH, Khanal T, Park H, et al.,.(2012). Piperine inhibits PMA-induced cyclooxygenase-2 expression through down-regulating NF-kappa B, C/EBP and AP-1 signaling pathways in murine macrophages. Food Chem Toxicol 50*:* 2342–2348.
21. Lichtenberger LM, Barron M and Marathi U. (2009). Association of phosphatidylcholine and NSAIDs as a novel strategy to reduce gastrointestinal toxicity. Drugs Today (Barc). 45: 877–890.
22. Liu, Q.; Yuan, W.; Tong, D.; Liu, G.; Lan, W.; Zhang, et al., (2016)"Metformin represses bladder cancer progression by inhibiting stem cell repopulation via COX2/PGE2/STAT3 axis" Oncotarget, Vol. 7, No. 19. *PCOM Scholarly Papers.* Paper 1685.
23. Lu QY, Han QH, Li X, Li ZC, Pan YT, Liu L, et al., (2014): Analysis of differentially expressed genes between rheumatoid arthritis and osteoarthritis based on the gene co-expression network. Mol Med Rep.; 10: 119–124.
24. Majithia V., Geraci S.A.(2007): Rheumatoid arthritis: Diagnosis and management. Am. J. Med.; 120: 936–939. doi: 10.1016/j.amjmed.2007.04.005
25. Malínskà H, Oliyarnyk O, _Skop V, _Silhav_y J, Landa V, Z_ıdek V, et al. (2016). Effects of metformin on tissue oxidative and dicarbonyl stress in transgenic spontaneously hypertensive rats expressing human C-reactive protein. PLoS ONE 11: e0150924.
26. Mariaselvam C.M., Aoki M., Salah S., Boukouaci W., Moins-Teisserenc H., Charron D., et al., (2014): Cytokine expression and cytokine-based T cell profiling in South Indian rheumatoid arthritis. Immunobiology.; 219: 772–777. doi: 10.1016/j.imbio..06.004.
27. Martin-Montalvo A., Mercken E. M., Mitchell S. J., et al.(2013): Metformin improves health span and lifespan in mice. *Nature Communications.*;4, article 3192 doi: 10.1038/ncomms3192.

28. McInnes IB, Schett G.(2007): Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol. 2007; 7: 429–42.
29. Ohkawa H, Ohishi N, Yagi K. (1979) Assay of lipid peroxide in animal tissue by thiobarbituric acid reaction. Ann Biochem. 95: 351–358.
30. Phadke K., R.L.Fouts, J.E. Parrish and L.D. Butler (1985): evaluation of the effects of various anti-arthritic drugs on type II collagen-induced mouse arthritis model. Immunopharmacology, 10: 51-60.
31. Rajesh Rajaiah, David Y.-W. Lee, Zhongze Ma, Arthur Y. Fan, Lixing Lao, Harry HS Fong, et al., (2009): Huo-Luo-Xiao-Ling Dan modulates antigen-directed immune response in adjuvant-induced inflammation. J. Ethnopharmacol. May 4; 123(1): 40–44.
32. Shindler KS, Ventura E, Dutt M, Elliott P, Fitzgerald DC and Rostami A(2010): Oral resveratrol reduces neuronal damage in a model of multiple sclerosis. J Neuroophthalmol. 30: 328–339.
33. Tikoo K1, Sharma E2, Amara VR2, Pamulapati H2, Dhawale VS2. (2016): Metformin improves metabolic memory in high fat diet (HFD)-induced renal dysfunction. J Biol Chem. Aug 22. pii: jbc.C116.732990.
34. Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F.(2012): Cellular and molecular mechanisms of metformin: an overview. Clin Sci (Lond); 122: 253–70.
35. Yao Q. Pi Z, Liu S, Song F, Lin N, Liu Z. (2014): A metabonomic study of adjuvant-induced arthritis in rats using ultra-performance liquid chromatography coupled with quadruple time-of-flight mass spectrometry. Mol Biosyst. Oct; 10(10): 2617-25.
36. Zheng C.J., Zhao X.X., Ai H.W., Lin B., Han T., Jiang Y. Pet al., (2014): Therapeutic effects of standardized *Vitex negundo* seeds extract on complete Freund's adjuvant induced arthritis in rats. Phytomedicine.; 21: 838–846. doi: 10.1016/j.phymed. 2014; 02: 003.