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Immunomodulatory Effects of Quercetin in Patient with Active Rheumatoid Arthritis

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ABSTRACT

Rheumatoid arthritis is an autoimmune systemic inflammatory disease primarily affecting the joints as well as other extra-articular tissues. This study was designed to evaluate the immunomodulatory effect of different doses of quercetin (500mg, 1000mg, 1500mg/day) for rheumatoid arthritis in patients treated with the conventional disease modifying antirheumatic drugs (DMARDs). This project was performed on 160 rheumatoid arthritis patients who fulfilled the American College of Rheumatology (ACR) criteria for the diagnosis of rheumatoid arthritis. In addition to 30 healthy volunteers were invited to participate in the study and served as a control group. The selected patients were allocated randomly into four groups, group (A) treated with azathioprine (100 mg/day) plus a placebo (starch containing capsules), group (B) treated with azathioprine (100mg/day) plus quercetin (500mg/day), group (C) treated with azathioprine (100mg/day) plus quercetin (1000mg/day) and group (D) patients treated with azathioprine (100mg/day) plus quercetin (1500mg/day). Serum level of interleukin-6, interleukin-10, intercellular adhesion molecule I (ICAM-1), complements proteins pretreatment and after eight weeks of treatment. It was found that quercetin at doses of 1500mg/day, when added to 100mg azathioprine, significantly ($p < 0.05$) reduced interleukin-6, complement protein 3 (C3) & complement protein 4 (C4) levels and elevated interleukin-10 level more than when azathioprine had combined with placebo or with lower doses of quercetin (500, 1000mg). However, all of tested doses of quercetin in this study were able to significantly ($p < 0.05$) reduce the level of intercellular adhesion molecule-1 as compared to the quercetin-free treated group. Oral administration of different daily doses of quercetin (500, 1000, 1500mg) in combination with azathioprine (100 mg) produced an immunomodulatory action through the reduction of interleukin-6, intercellular adhesion molecule-1 and complement proteins while elevating the serum level of interleukin-10.

Keywords: Immunomodulatory, Quercetin, Rheumatoid Arthritis, Interleukins, Complements protein.

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INTRODUCTION

The focusing on plant research has been increased all over the world in last decade and a large amount of evidences has been collected to show the potential of medicinal plant in various traditional systems¹. Most of the immunomodulatory agents are of plant origins which are appealed to induce paraimmunity, the nonspecific immunomodulation of essentially granulocyte, macrophage, and natural killer cells and complement function². It is now being documented that immunomodulatory therapy could provide an alternative to conventional chemotherapy to a variety of disease conditions³. Quercetin is a member of the class of flavonoids called flavanols and forms the backbone for many other flavonoids including the citrus flavonoids like rutin, hesperidins, naringenin and tangeritin⁴. The best described property of quercetin is its ability to act as antioxidant⁵. Quercetin-induced suppression of TNF- α can result in the stimulation of anti-inflammatory cytokines via inhibiting the activation of NF- κ B, and therefore, one can anticipate that quercetin could be widely used as an anti-TNF- α therapy⁶. It has also found that quercetin reduced visceral adipose tissue TNF- α and nitric oxide production and downregulated nitric oxide synthase expression in obese zucker rats⁷. Quercetin exerts immune and inflammation modulating effect in several murine models of autoimmunity. In experimental allergic encephalomyelitis(EAE) a T-helper 1 (Th1) cell-mediated inflammatory demyelinating autoimmune disease model of multiple sclerosis-quercetin ameliorated EAE by blocking interleukin-12(IL-12) signaling and Th1 differentiation⁸ Kaneuchi et al showed that quercetin has anti-proliferative activity and the mechanisms of quercetin action may be through modulation of cell cycle and cell growth regulatory genes⁹. Quercetin has also been shown to limit the function of adhesion molecules on endothelial cells¹⁰. When activated inappropriately, the complement system may evoke pathologic reactions in a variety of inflammatory and degenerative diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), as well as acute respiratory distress syndrome (ARDS)¹¹ Therefore, inhibition of individual complement is a promising approach for the prevention and treatment of these diseases and numerous natural products have been reported to possess anti-complementary effect¹². In this study , we investigated the effect of different doses of quercetin on interleukin-6 (IL-6), interleukin-10 (IL-10), intracellular adhesion molecule-1(ICAM-1) and complement proteins, C3 & C4 in patient with rheumatoid arthritis treated with conventional DMARD, azathioprine .

MATERIALS AND METHOD

This study was performed on (190) subjects. 30 (10 male and 20 female) apparently healthy control and 160 randomly selected RA patients (55 males and 105 females) with active

rheumatoid arthritis, at the out patient clinics in Al-Hakeem and Al-Sader hospitals in Najaf-Iraq during the period December-2008 - October-2009, with age range (32 -71) years, mean age \pm SEM; (52.9 \pm 2.2), mean disease duration is 12 years (range 7-20 years). (111) patient only completed the study. All patients have active rheumatoid arthritis and fulfill the 1987 revised criteria for the diagnosis of rheumatoid arthritis, set by the American College of Rheumatology (ACR) (13). All selected patient are informed about the nature and aim of the study. None of the patients had received any other specific anti-rheumatoid therapy during the three months prior to the present study. Some of them were on intermittent use of one or more NSAIDs, and those were informed to leave one week after the last dose of those medications to ensure complete clearance. This research was approved by postgraduate research committee at college of pharmacy Baghdad University.

Study design

The selected RA patient were allocated into 4 groups ,each of 40 patients , groups A, B, C and D that received azathioprine 50mg(Glaxo Wellcome Inc.) plus placebo(starch containing capsules), quercetin 250mg (Jarrow formulas, USA) plus azathioprine 50mg , quercetin 500mg plus azathioprine 50 mg and quercetin 750mg plus azathioprine 50 mg, respectively. All treatments are given twice daily for eight weeks. In addition to 30 age- and sex- matched healthy subjects that did not received any medications including those used in the study. This group served as a control. Fasting blood samples were taken from patients and control. Serum was obtained and analyzed for measurements of sIL-6, sIL-10, sICAM, C3 and C4, Patients samples were analyzed at zero time (pre-treatment) and after 8 weeks (the end of the study).

Statistical analysis

SPSS (version 20.0 IBM, USA) software for windows was used to analyze the results of this study. All results are expressed as Mean \pm SEM. Student t-test and ANOVA was used to examine the difference in the mean of parameters tested between studied groups. P value <0.05 was considered significant.

RESULTS AND DISCUSSION

Effects of 8 Weeks Treatment of RA Patients with Azathioprine alone and its Combination with Different Doses of Quercetin on Serum levels of IL-6, IL-10 and ICAM-1:

The data presented in table (1) clearly showed that both IL-6 and ICAM-1 levels in all RA patients groups enrolled in this study were significantly ($P<0.05$) elevated as compared to healthy untreated volunteers. However, IL-10 level seems to be not altered significantly ($P<0.05$) in patients baseline samples after 8 weeks. Table (1) also demonstrated a significant ($P<0.05$) reduction in the serum level of IL-6 in all patients groups after treatment with

respect to the pre-treatment values of each group. It was also found that only patients treated with 1500 mg quercetin plus azathioprine showed a significant ($P < 0.05$) reduction in serum level of IL-6 with respect to both azathioprine only treated group or quercetin 500mg/azathioprine 100mg treated patients. Regarding the level of IL-10, it was indicated that there was a significant ($P < 0.05$) increase in this cytokine in patients receiving either azathioprine alone or combined with quercetin and, similar to IL-6, except for patients receiving the highest quercetin dose showed a significant difference with respect to both azathioprine only treated patients or those treated with quercetin 500mg/azathioprine 100 mg regimen. Treatment with azathioprine alone did not significantly affect the level of ICAM-1 with respect to the pre-treatments values. Whereas, other groups that were treated with different doses of quercetin showed significant reduction ($P < 0.05$) in this adhesion molecule compared with azathioprine only treated group, with the highest dose among them was significantly different from other doses. (Figure 1)

Effects of 8 Weeks Treatment of RA Patients with Azathioprine alone and its Combination with Different Doses of Quercetin on Serum Levels of Complement Proteins, C3 and C4.

Table 1: Effects of treatment of RA patients with azathioprine alone and its combination with different doses of quercetin on serum level of IL-6, IL-10 and sICAM.

Group		IL-6(pg/ml)	IL-10(pg/ml)	sICAM(pg/ml)
Healthy control	Untreated	146.9 ± 8.3	140.1 ± 6.44	106.4 ± 4.45
Azathioprine (100mg/day)	Pre-treatment	354.1 ± 14.4*	159.1 ± 8.74	446.3 ± 21.5*
	Post-treatment	238.7 ± 14.0*†	175.7 ± 16.4*†	431.7 ± 20.7*
Azathioprine + Quercetin (100/500mg/day)	Pre-treatment	332.1 ± 15.2*	156.9 ± 8.27	485.7 ± 21.0*
	Post-treatment	218.8 ± 14.5*†	242.6 ± 14.7*†	388.0 ± 15.9*†a
Azathioprine+ Quercetin (100/1000mg/day)	Pre-treatment	352.0 ± 13.3*	160.4 ± 7.74	435.5 ± 19.7*
	Post-treatment	208.0 ± 14.7*†	244.6 ± 14.0*†	310.6 ± 15.9*†a
Azathioprine+ Quercetin (100/1500mg/day)	Pre-treatment	341.6 ± 11.81*	150.5 ± 5.77	448.6 ± 18.89*
	Post-treatment	171.6 ± 11.52†a,b	237.0 ± 13.6*†a,b	279.6 ± 15.5*†a,b

-Data are expressed as mean ± SEM

-* $P < 0.05$ with respect to healthy control group.

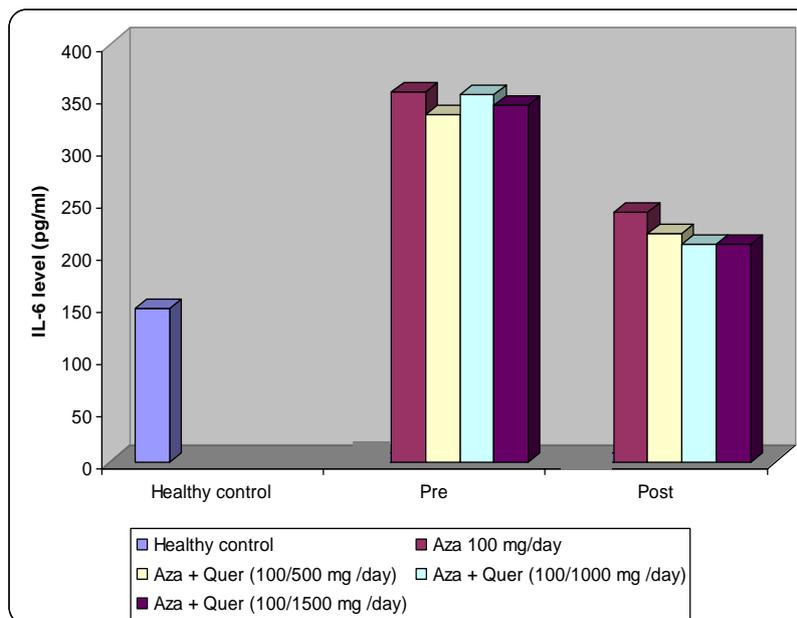
-† $P < 0.05$ with respect to pretreatment value.

-a $P < 0.05$ with respect to azathioprine treated group.

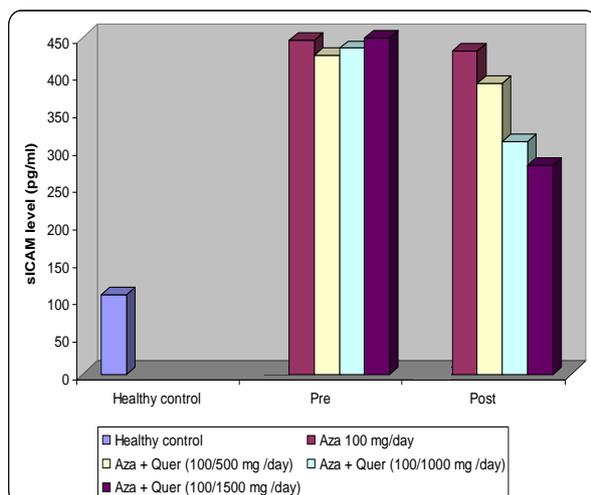
-b $P < 0.05$ with respect to azathioprine + Quercetin (100/500mg/day) treated group.

Data presented in table (2) showed no significant differences in the levels of complement proteins, C3 and C4, in RA patients compared to the healthy untreated control group. Table (2) also indicated that treating RA patients with azathioprine alone or combined with 500 mg /day quercetin for eight weeks resulted in slight, non-significant reduction in the serum level of both complement proteins, C3 and C4. Data also demonstrated a significant reduction in serum level of both C3 and C4 in patient treated with 1000 or 1500 mg/day of quercetin plus

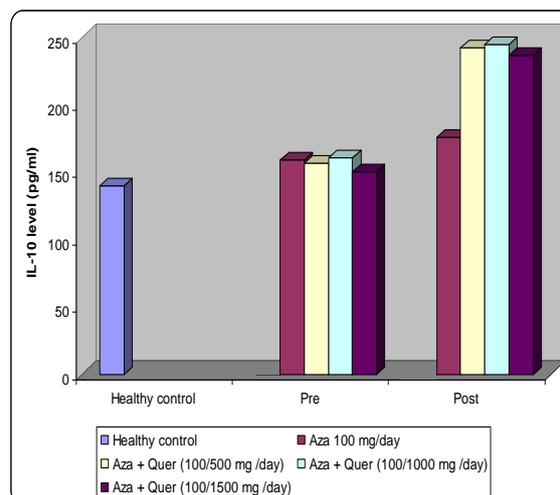
azathioprine with respect to both other groups treated with azathioprine only or azathioprine plus 500 mg/day.(Figure 2)



(A)



(B)



(C)

Figure (1): Serum level of IL-6 (A), ICAM-1 (B) and IL-10(C) in RA patients after 8 weeks treatment with azathioprine and its combination with different doses of quercetin.

Both IL-6 and ICAM-1 levels in all RA patients groups enrolled in this study were significantly ($P < 0.05$) elevated as compared to healthy untreated volunteers. However, IL-10 level seems to be not altered significantly ($P < 0.05$) in patients baseline samples after 8 weeks. Significant ($P < 0.05$) reduction in the serum level of IL-6 in all patients groups after treatment with respect to the pre-treatment values of each group. It was also found that only patients treated with 1500 mg quercetin plus azathioprine showed a significant ($P < 0.05$) reduction in serum level of IL-6 with respect to both azathioprine only treated group or quercetin 500mg/azathioprine 100mg treated patients. Regarding the level of IL-10, it was indicated

that there was a significant ($P<0.05$) increase in this cytokine in patients receiving either azathioprine alone or combined with quercetin and, similar to IL-6, except for patients receiving the highest quercetin dose showed a significant difference with respect to both azathioprine only treated patients or those treated with quercetin 500mg/azathioprine 100 mg regimen. Treatment with azathioprine alone did not significantly affect the level of ICAM-1 with respect to the pre-treatments values. Whereas, other groups that were treated with different doses of quercetin showed significant reduction ($P<0.05$) in this adhesion molecule compared with azathioprine only treated group, with the highest dose among them was significantly different from other doses.

Table 2: Effects of treatment of RA patients with azathioprine alone and its combination with different doses of quercetin on serum level of complement proteins (C3 & C4)

Group		C3 (mg/dl)	C4 (mg/dl)
Healthy control	Untreated	136.3 ± 7.71	77.4 ± 7.08
Azathioprine (100mg/day)	Pre-treatment	140.9 ± 8.44	93.6 ± 4.62
	Post-treatment	115.5 ± 6.77	88.0 ± 4.21
Azathioprine+ Quercetin (100/500mg/day)	Pre-treatment	133.4 ± 7.68	89.3 ± 4.39
	Post-treatment	111.3 ± 5.28*	86.9 ± 3.28
Azathioprine+ Quercetin (100/1000mg/day)	Pre-treatment	120.5 ± 4.38	91.8 ± 3.55
	Post-treatment	93.2 ± 3.66*†b	67.8 ± 3.12†ab
Azathioprine+ Quercetin (100/1500mg/day)	Pre-treatment	121.6 ± 4.45	91.7 ± 3.50
	Post-treatment	88.5 ± 3.66*†ab	62.3 ± 3.95†ab

Data are expressed as mean ±SEM

* $P<0.05$ with respect to healthy control group.

† $P<0.05$ with respect to pretreatment value.

a $P<0.05$ with respect to azathioprine treated group.

b $P<0.05$ with respect to azathioprine +Quercetin (100/500mg/day) treated group.

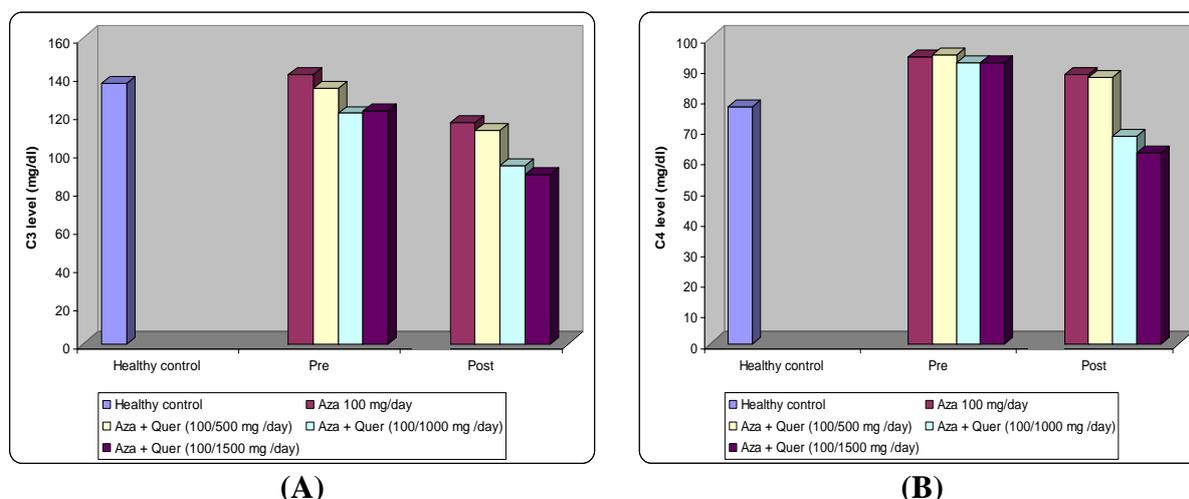


Figure (2): Serum level of C3 (A) and C4 (B) in RA patients after 8 weeks treatment with azathioprine and its combination with different doses of quercetin.

No significant differences in the levels of C3 and C4, in RA patients compared to the healthy untreated control group. A significant reduction was found in serum level of C3 and C4 in patient treated with 1000 or 1500 mg/day of quercetin plus azathioprine with respect to both other groups treated with azathioprine only or azathioprine plus 500 mg/day. It has been well documented that synovial tissues are able to synthesize the mediators of inflammatory cascade in the joint, which may play an important role in the pathophysiology of RA. While no known biological marker to be definitive for the diagnosis or prognosis of RA, the levels of biomarkers such as cytokines, chemokines, and metalloproteinases can potentially reflect the inflammatory state in joints¹⁴. Several studies have addressed the feasibility of biomarker analysis in serum and synovial fluids in arthritis¹⁵. IL-6 is derived from not only immunocompetent cells but also from other cells, such as neoplastic cells, endothelial cells, and muscle fibers¹⁶. It is well known that IL-6 plays an important role in acute-phase inflammatory reaction through accelerating release of PMN from the bone marrow, enhancing PMN adhesiveness and recruitment, activating PMN, and enhancing release of cytotoxic contents¹⁷. Neutrophils are terminally differentiated cells and most important inflammatory cells. On one hand, activated neutrophils defense against pathogenic micro organisms; on the other hand, activated neutrophils may aggravate constitutive damage of auto-tissues through producing various inflammatory mediators¹⁸. Therefore, the level of IL-6 in body or in local inflammatory sites may affect the solution of inflammation. In normal situation of body, bone marrow, plasma and peripheral blood serum have low levels of IL-6 and this is clear from the analysis of the serum of the normal healthy volunteers invited for this study that showed to be significantly lower than the level seen in RA patients. However, in infectious situation of pathogenic bacteria, IL-6 producing cells synthesize and release IL-6 in response to the stimuli. LPS, a potential pro-inflammatory factor derived from Gram negative bacteria, induces IL-6 production by neutrophils and expedites neutrophil recruitment by activating Toll-like receptor 4 (TLR4)¹⁹. In the present study we found that treating RA patient with 1500mg/day of quercetin combined with 100mg azathioprine resulted in a significant reduction in the level of the pro-inflammatory cytokines, IL-6, and such reduction was significantly lower than that produced by azathioprine alone or when combined with lower doses of quercetin(Table 1). It was also found that quercetin to exhibit inhibitory effect on IL-6 production by LPS-stimulated neutrophils. They indicate that quercetin might decrease the susceptibility of neutrophils to pro-inflammatory factors (e.g., LPS), or interfere with the response of neutrophils to pro-inflammatory factors. The inhibitory effect of quercetin on LPS-induced IL-6 production by neutrophils might partially eliminate the accumulation of neutrophils into the inflamed sites through inhibiting

neutrophil activation and neutrophil adhesion to endothelial cells. Thus, this process is much beneficial to the resolution of inflammation²⁰. Conducting such findings with the pro-inflammatory activity of IL-6 level may explain the results of our study which involved a significant dose dependent reduction in IL-6 in the serum of RA patient treated with quercetin combined with azathioprine. On the other hand, IL-10 has been showed to inhibit the production of pro-inflammatory cytokines by monocytes, as well as, inhibiting its own production. While IL-10 is undetectable in the synovial fluid, it is clearly present in RA synovial membrane and is detectable in macrophage and T-cells isolated from RA synovial tissue²¹. Therefore, modulation of IL-10 production in RA synovial tissue could have a significant effect on the chronic inflammatory process and may represent a potential therapeutic target both for the existing and new treatments for RA²². Kojo *et al* (2005) had found that IL-10 produced by iNKT cells induces regulatory dendritic cells, which in turn generates CD4+ regulatory T cells known to suppress immune responses²³. It is clear from the results of this study that IL-10 is significantly increased in response to quercetin administration in a dose independent manner (Table 1). Furthermore, Sakaguchi (2000) demonstrated that such increment may be related to the immunoregulatory T cells which are characterized by their capability to synthesize and secrete latent TGF-1 and IL-10(24). Additionally, Siegfried Ansorge *et al* (2003) had found that propolis and some of its constituents like quercetin down-regulate DNA synthesis and decrease inflammatory cytokine production but induce TGF-1 and IL-10 production of human immune cells Thus, the bee product propolis can be considered as a powerful natural anti-inflammatory medicine influencing different types of immune-responses probably *via* immune regulatory T cells²⁵. Azathioprine was found to had no significant effect on the level of IL-10(Figure.1 C). This finding is in agreement with the result of Aurore *et al* (2005)²⁶ and Chiba *et al* (2004)²⁷. The adhesion of leukocytes to the vascular endothelial cells is a critical step in the inflammatory response and involves recruitment and infiltration of leukocytes to the site of tissue injury, infection, or lesion formation. These processes are mediated by a wide variety of adhesion molecules. Intercellular adhesion molecule-1 (ICAM-1, CD54) expressed on endothelial cells is one of the major cell surface glycoproteins that contribute to the cell adhesion processes²⁸. Although ICAM-1 is constitutively expressed on endothelial cells, it can be significantly induced in response to proinflammatory mediators such as tumor necrosis factor- α (TNF- α) and interleukin-1²⁹, as well as phorbol 12-myristate 13-acetate (PMA)³⁰, oxidants³¹, and human immunodeficiency virus-1 tat proteins³². Elevated levels of ICAM-1 expression have been shown to be critically involved in the development of a variety of autoimmune diseases and pathologic inflammatory disorders, e.g., rheumatoid arthritis, psoriasis, and

atherosclerosis³³. This fact also documented in RA patient participated in this study where they showed a significantly increased level of this adhesion molecule compared with the healthy control group (1). This table also indicated that combination of quercetin with azathioprine results in a dose dependent reduction in ICAM-1 serum level with respect to both, their pre-treatment values and azathioprine-only treated group. As shown in Figure (1 B), the highest quercetin dose in this study (1500mg/day) resulted in a significant reduction in ICAM with respect to the lowest dose (500mg/day). Recently, ICAM-1 expression by tumor cells has been reported to be a major contributing factor that facilitates metastatic progression³⁴. The modulation of ICAM-1 expression is therefore an important therapeutic target, as shown by the beneficial effects of anti-ICAM-1 antibodies and other pharmacological agents on the progression of inflammatory responses in several *in vivo* studies³⁵. The inhibitory effect of flavonoids on the expression of adhesion molecules has been suggested to be mediated by down regulation of the induced NF-B activation³⁶. Other studies that support our findings were those indicated by Binwu Ying (2009) who reported that quercetin attenuated IL-1 beta-induced expression of ICAM-1 mRNA and protein in a dose-dependent manner. His experiment suggested that quercetin actively inhibited the inhibitory protein of nuclear factor-kappa B (I kappa B) degradation, and nuclear factor-kappa B (NF-kappa B) activity³⁷. This inhibitory effect of quercetin on inducible ICAM-1 expression may represent a mechanism that contributes to the anti-inflammatory property of this dietary flavonol. The elevated expression of sICAM-1 could also play a role in increasing the risk of atherogenesis and cardiovascular diseases in patient with RA³⁸. Table (2) had showed that although azathioprine alone or its combination with low dose (250mg/day) quercetin had lowered the level of both complement proteins (C3&C4), such reduction did not reach the significant level. However, quercetin in higher doses (1000 or 1500mg/day), in combination with azathioprine, resulted in significant reduction in both C3 and C4 with respect to azathioprine only treated group or to a group receiving lower dose of quercetin(Figure 2). These findings may be explained by the role of complement system in an inflammatory process and immune response.

CONCLUSION

It is concluded that the use of quercetin in patient with rheumatoid arthritis that treated with azathioprine resulted in dose dependent immunomodulatory actions regarding its effect on cytokines, sICAM and complement proteins.

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