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# Synergetic effect of aged garlic extract and methotrexate on rheumatoid arthritis induced by collagen in male albino rats

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Aged garlic extract (AGE) exhibit anti-inflammatory effect in many diseases, and methotrexate (MTX) as rheumatoid arthritis (RA) treatment drug shows adverse hepatotoxicity effect. Therefore, in this study, we evaluated the antioxidant and anti-inflammatory effects of AGE treatment alone or with MTX in collagen-induced arthritis (CIA) rats to diminish the hepatotoxicity. The study used eight groups of rats as one control non treated group and seven treated groups with CIA, AGE (200 mg/kg/PO), MTX (1.5 mg/kg/2 days/subcutaneous), CIA-AGE, CIA-MTX, AGE-MTX and CIA-MTX-AGE. All treatments started from day 21 after the symptoms of arthritis appeared to day 50. The CIA-AGE and CIA-MTX-AGE groups showed significantly decreased serum liver function markers ASAT, ALAT and ALP enzymes activities. In line with the significantly increased antioxidants, total glutathione and SOD and CAT enzymes activities and decreased MDA levels as compared to CIA and CIA-MTX treated groups' values. In addition, the CIA-AGE and CIA-MTX-AGE groups recorded significant decrease in the measured cytokines (CRP and TNF) and interleukins (IL-17, IL-6, and IL-1) values as compared to the corresponding values in CIA and CIA-MTX groups. Results suggested the safety of AGE for achieving a better control in treatment of RA with the conventional drug MTX to diminish its hepatotoxicity.

**Keywords:** *Allium sativum*, Antioxidants, Cytokines, Glutathione, Hepatotoxicity, Interleukins, Malondialdehyde

Rheumatoid arthritis (RA) is a multisystemic, chronic immuno-inflammatory disease characterized by chronic arthritis leading to progressive joint erosions. According to WHO, methotrexate (MTX), a folic acid antagonist, is the most potent anti-inflammatory anti-rheumatic drug for the treatment of RA[1]. Despite its proven effectiveness, MTX due to hepatotoxicity, results in poor compliance of therapy[2]. Moreover, long-term follow up of high-dose methotrexate may cause progressive fibrosis and liver injury that progressing to cirrhosis. It was previously suggested that the fibrosis is a consequence of elevated oxidant parameters and reduced antioxidants levels especially in the liver tissue[3,4]. Even at very low doses in long term therapy, MTX is reported to be toxic to liver, kidneys, respiratory and reproductive systems[5]. Methotrexate accumulation or its reactive metabolites may affect the gene expression and cellular homeostasis in liver function and structure[6]. However, studies suggest that supplementation of natural compounds having antioxidant properties may diminish the toxicity of MTX[7].

Garlic (*Allium sativum*) used for centuries as a prophylactic and therapeutic medicinal agent due to its organosulfur compounds. Aged garlic extracts had hepatoprotective, neuroprotective, genoprotective, immunoprotective and exert inhibitory effect on lipid peroxidation and oxidative factors. Moreover, it has a potent activity in scavenging the reactive oxygen species (ROS) and in enhancing the cellular antioxidant enzymes. Garlic extract also reported as a synergistic antitumor beside its pharmacological and biological effects on a variety of inflammatory diseases, including allergy, asthma, arthritis, etc.[8-11].

To establish an efficient and safe medication strategy for treatment of RA using natural medicine is a goal with conventional drug MTX to diminish its hepatotoxicity. Hence, in the present study, we tried to evaluate the antioxidant and antiinflammatory effects of AGE treatment alone on CIA rat model and its synergetic activity in the co-treatment with MTX to diminish the methotrexate hepatotoxicity.

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## Material and Methods

The experimental procedures were reviewed and approved by the research ethics committee at King Faisal University (Ref. No. KFU-REC/2019-05-05).

### Animals

Fifty six adult male albino rats (*Rattus norvegicus*), weighing (180-200 g) were used in the present study. Animals housed in environmentally controlled conditions (temperature of 22±2°C) with a 12 h light/dark cycle and had free access to commercial rodent pellets and water *ad libitum*.

### Experimental design

Rats were divided into eight groups (n= 7) and sacrificed after 50 days as follows: Group I, control, with healthy normal non-treated rats to the 50$^{th}$ day; Group II, collagen-induced arthritis rats (Arthritis was induced by approximately 0.2–0.4 mL of the collagen-CFA emulsion by intradermal injection at the base of the tail at day 0 and for 21 consecutive days as previously mentioned[12]. Then the group was managed without treatment to the 50$^{th}$ day); Group III, arthritis-induced rats as in Gr. II, but were treated subcutaneously with methotrexate (MTX) @1.5 mg/kg/2 days, from day 21 to the 50$^{th}$ day[13]; Group IV, arthritis-induced rats, which were treated orally with aged garlic extract (AGE) @200 mg/kg daily from day 21 to the 50$^{th}$ day, as previously mentioned[14]; Group V, arthritis-induced rats, which were treated with AGE + MTX from day 21 at the aforementioned doses, routs and period as in Gr. III & IV; Group VI, healthy rats treated with MTX from day 21 at the aforementioned dose and periodas in Gr. III; Group VII, healthy rats treated with AGE from day 21 at the aforementioned dose and period as in Gr. IV; and Group VIII, healthy rats, which were treated with MTX+AGE from day 21 as the aforementioned as in Gr. V.

### Chemicals

Aged garlic extract (AGE) as capsules (Kyolic® HI-POTM formula 100) from Wakunaga of America CO., LTD (Mission Viejo, CA, USA). Methotrexate (MTX), Complete Freund's adjuvant (CFA) and collagen type II purchased from Sigma-Aldrich (St. Louis, MO, USA). Aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) enzymes determined as described by the manufacturer's manual. Alkaline phosphatase activity (ALP) determined using calorimetric method[15]. Total glutathione (TGSH), superoxide dismutase (SOD) and catalase (CAT) were determined by colorimetric kits Cat. No: EIAGSHC, EIASOD and MBS006963, respectively, each as described by the manufacturer's manual. Malondialdehyde (MDA) level determined by the thiobarbituric acid test at 532 nm[16]. In addition to C-reactive protein (CRP), TNF-α, IL-17, IL-6 and IL-1 beta determined by rat ELISA kits Cat. No: 88-7501-28, BMS622, 88-7170-88, ER3IL65 and BMS630, respectively in accordance with the manufacturer's instructions.

### Induction of collagen-induced arthritis

The arthritis condition induced by injection with 0.2-0.4 mL of collagen-CFA emulsion intradermal into the base of tail of each rat; 5 mg collagen type II dissolved in 2.5 mL cold, 0.1 M acetic acid. This mixture emulsified with 2.5 mL CFA and the solution mixed using a glass homogenizer for ~15 min. The procedure for preparing this solution conducted on ice to ensure the proteins in the emulsion were not denatured. Prior to receiving the injection of the collagen-CFA mixture, the rats anesthetized with diethyl ether (Sigma-Aldrich, St Louis, MO, USA)[12].

### Serum collection

The animals were anesthetized by diethyl ether and sacrificed at the end of the experimental period (50 days) by cutting the neck at the jugulars by a sharp razor blade. Blood was collected into clean test tubes without anticoagulants. Then left to coagulate at room temperature (22± 2 °C) and centrifuged for 30 min at 3000 rpm for clot separation. Serum was separated immediately by long Pasteur pipette and stored at −70°C to for biochemical and ELISA assays.

### Statistical analysis

Reported values represent mean ± SE. Statistical analysis was evaluated by one-way ANOVA. Once a significant F test was obtained, LSD comparisons were performed to assess the significance of differences among all groups. Statistical Package for social sciences "SPSS" for Windows software, Release 21.0 (SPSS, Chicago, IL) was used.

## Results

Data illustrated in Fig. 1(A) showed significant increase in liver functions enzymes activities (ASAT, ALAT and ALP) in all treated groups except AGE group as compared to control values. The arthritis treated groups, CIA-AGE and CIA-MTX-AGE and the healthy treated ones (AGE, MTX and MTX-AGE) recorded significant decrease in the liver enzymes activities as compared to CIA and CIA-MTX treated groups values.

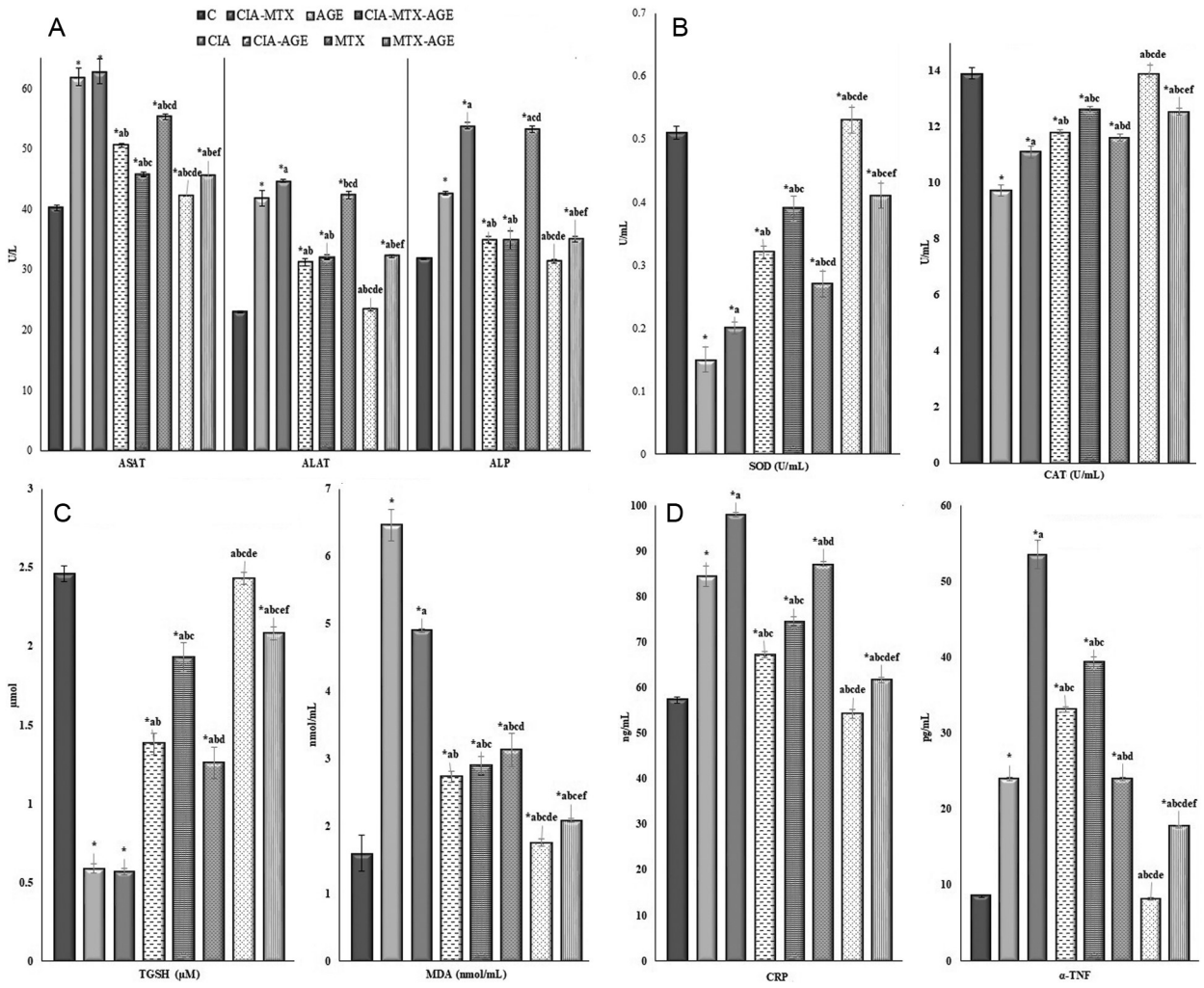


Fig. 1 — Effect of treatments on rat serum (A)ASAT, ALAT and ALP (U/L); (B) SOD (U/mL) and CAT (U/mL); (C) TGSH (µmol) and MDA (nmol/mL) levels and (D) CRP (ng/mL) and α-TNF (pg/mL) levels. [Data illustrated in mean ± SE, n =7. Significant at $P$ <0.05 indicated by asterisk (*) as compared to control, (a) as compared to CIA group, (b) as compared to CIA-MTX group, (c) as compared to CIA-AGE group, (d) as compared to CIA-MTX-AGE group, (e) as compared to MTX group, and (f) as compared to AGE group according to one-way ANOVA]

The results illustrated in Fig. 1 B & C showed significant decrease in serum TGSH contents as well as SOD and CAT enzymes activities, while revealed significant increase in MDA of arthritis group (CIA) as compared to control and all other treated groups. Meanwhile, AGE group recorded not statistically differences in the antioxidants parameters as compared to control values. Arthritis treated groups CIA-AGE and CIA-MTX-AGE as well as healthy treated ones (AGE and MTX-AGE) recorded significant increase in the antioxidants values and significant decrease in MDA levels as compared to CIA and CIA-MTX groups corresponding values.

Data in illustrated in Fig. 1 D and Fig. 2 presented significant increase in the proinflammatory cytokines (CRP and TNF) and interleukins (IL-17, IL-6, and IL-1) in CIA and all other treated groups as compared to control and AGE groups. Arthritis treated group with methotrexate (CIA-MTX) showed significant increase in the proinflammatory cytokines values as compared to all groups corresponding values. Results of the CIA-AGE and CIA-MTX-AGE groups recorded significant decrease in the measured cytokines and interleukins values as compared to the corresponding values in CIA and CIA-MTX groups.

## Discussions

Natural products have become more popular in the treatment of rheumatoid arthritis due to thier relief effect on symptoms and no side effects. Therefore,

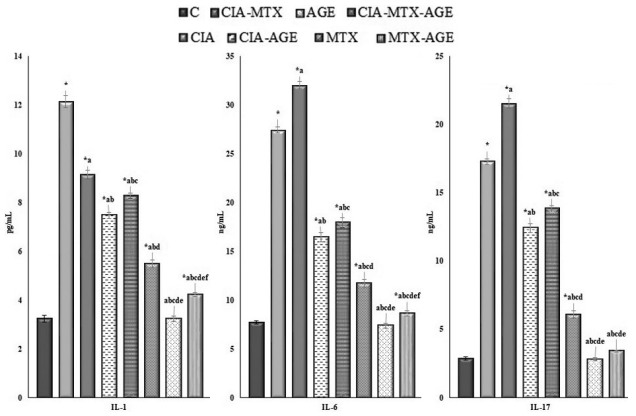


Fig. 2 — Effect of treatments on rat serum interleukins IL-1 (pg/mL), IL-6 (pg/mL) and IL-17 (pg/mL) levels. [Data illustrated in mean ± SE, n =7. Significant at $P$ <0.05 indicated by asterisk (*) as compared to control, (a) as compared to CIA group, (b) as compared to CIA-MTX group, (c) as compared to CIA-AGE group, (d) as compared to CIA-MTX-AGE group, (e) as compared to MTX group, and (f) as compared to AGE group according to one-way ANOVA]

combination of natural medicinal product and conventional medicine are approached to be useful in curing acute and chronic inflammatory arthritis[17-18]. The current work targeted the efficacy and safety of the combination of aged garlic extract and methotrexate in rat's animal model of RA.

The present study showed significant increase in ASAT, ALAT and ALP enzyme activities in CIA group confirming the impact of this model on liver function indicating hepatocellular injury which in line with previous studies[19,20]. For increased cytosolic enzyme activity, ALAT is the best indicator of liver damage and leakage in plasma membrane permeability associated with cell death and necrosis. Serum ASAT and ALAT have been reported to play vital roles in the formation of active chemical mediators such as bradykinins in inflammatory process. In addition, ALP is a marker liver enzyme of bone metabolism and its alteration can be indicative of increased bone metabolism and a consequence of ongoing destructive joint erosion[21,22]. The antioxidant defences diminished by arthritis as previously determined in arthritic animal model[23]. Over-production of oxidants leads to oxidative tissue damage at the molecular level. Many studies have implicated oxidative injury as the major pathogenic mechanism in RA. In overproduction of MDA, the lipid peroxidation marker plays a role in oxidative stress and the impaired SOD and CAT antioxidant enzymes, and the glutathione defence system as observed in the present study by TGSH, play a role of defence against oxygen-derived free radicals and support the hypothesis that defence mechanisms against reactive oxygen species impaired in RA[24-26]. The results of our study also showed the onset of the inflammatory response through the significant elevated levels of the proinflammatory cytokines (CRP and TNF) and interleukins (IL-17, IL-6, and IL-1) in CIA group, which are important in RA previously discussed in different studies[12,27,28].

Methotrexate monotherapy previously demonstrated hepatotoxicity through elevation of liver enzymes at low dose in experimental animals[6,29] and its potency for oxidative stress as a mechanism of methotrexate toxicity recorded in a rat model of RA[30]. In addition, high doses of methotrexate (MTX) may directly inhibit several folate dependent enzymes such as dihydrofolate reductase and thymidylate synthase that leading to disrupted purine and pyrimidine synthesis and extracellular release of adenosine which is a potent antiinflammatory agent[29,30-33]. The mechanisms underlying MTX hepatotoxicity may include intracellular accumulation of methotrexate polyglutamate and consequent folate depletion, generation of oxidative stress and activation of proinflammatory cytokines as well as genetic polymorphism[34,35].

As regard to MTX, suppression of inflammation potency recorded as dose response in a manner of decline in higher doses[36]. Hence, the role of MTX in the reduction of antioxidant defence mechanism as well as in the inflammatory cytokines as observed in our study, suggest its toxicity through the development of MTX-mediated tissue damage, and trigger to postulate the need for combination of treatment to advocate its toxicity and improve its therapeutic potency in RA. Several studies in this line were implemented combinations to ameliorate MTX toxicity using beta-glucan[32], Melatonin, ursodeoxycholic acid and balanites aegyptiacato extract[31,37] and to achieve better therapeutic benefit compared as to monotherapies using Leflunomide[29]. In addition to the posturated regulation of CoQ10 and its protective properties against liver fibrosis caused by methotrexate treatment[38]. The combination of AGE with MTX in this study showed ameliorating activity against the higher values of liver function enzymes activities, antioxidants and proinflammatory cytokines as compared to arthritic rats treated with either methotrexate (CIA-MTX) or MTX monotherapy. In line with our results, previous studies strongly suggested the immune modifier activity of AGE through the maintenance of homeostasis of immune

functions[39]. AGE oral treatment or pre-treatment against cisplatin hepatotoxicity proved antioxidant and protective effects in rats by its controlling on serum liver function enzymes and liver structure as well as antioxidants levels[40]. In addition to the predicted antioxidant and anti-inflammatory effects of AGE on rat gastric inflammation models[14], AGE modulated all the immunosuppressive adverse effects shown in rats treated with malathion and/or carbaryl[41]. Pathak *et al*.[42] validated the use of aged garlic extract in the treatment of diseases causing liver toxicity including hepatocarcinoma. The effect of AGE may be attributed to its active ingredients like water-soluble organosulfur compounds[43,44] which provide protection against oxidation, free radicals and demonstrating hepatoprotection by preventing the formation of thiobarbituric acid–reactive substances and alleviating oxidative liver damage[45].

## Conclusion

The results of the present study further substantiates the hepatoprotective efficacy of the aged garlic extract (AGE) and its immunomodulatory activity as observed by earlier researchers. It also suggests the safety of AGE for achieving a better control in the treatment of rheumatoid arthritis (RA) with the conventional drug methotrexate (MTX) to diminish its liver toxicity.

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## Conflict of interest

The authors declared no conflicts of interest.

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