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EUGENOL AMELIORATES FREUND'S ADJUVANT INDUCED ARTHRITIS IN MALE RATS

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ABSTRACT

Background: Rheumatoid arthritis (RA) is a chronic relapsing autoimmune disorder. Eugenol, a main component of clove oil, possesses potent anti-inflammatory and antioxidant activity. Present investigation was designed to evaluate anti-arthritic potential of eugenol in Freund's Complete Adjuvant (FCA) induced arthritis in male albino rats. **Materials and Methods:** Thirty two male albino rats were used. Eight male rats were used as a normal control group received saline (group 1). Arthritis was induced in the 24 male albino rats by intradermal injection of 0.1 ml of FCA in the right hind paw (arthritic animals). Animals were divided into 3 groups. Arthritic control received sesame oil oral (group 2). Arthritic animals treated with indomethacin, it was used as a standard, 2 mg/kg/day orally (group 3).. Arthritic animals treated with eugenol 250 mg/kg/day orally (group 4). Fourteen days after immunization, treatment was started with eugenol till 28 days. **Results:** arthritic control animals showed significant elevation in serum level of C-reactive protein (CRP), nitric oxide (NO), malondialdehyde (MDA) and tumor necrosis factor-α (TNF-α) compared to normal control group. Treatment with eugenol exhibited significant reduction in serum level of CRP, NO, MDA and TNF-α compared with arthritic control group. **Conclusion:** Therefore, eugenol ameliorates experimental arthritis and could be useful as a beneficial supplement in treating human arthritis.

KEYWORDS: Rheumatoid arthritis, eugenol, adjuvant arthritis, inflammatory mediators.

INTRODUCTION

Rheumatoid arthritis is a chronic inflammatory disorder characterized by pain, synovial membrane inflammation and restricted joint movement due to tissue damage. In RA inflammatory process is targeted towards the synovium which causes destruction of the articular cartilage, peri-articular bone erosion and eventual alteration of joint integrity and function (Yuan et al., 2012). Recent study has demonstrated that non-enzymatic factors like reactive oxygen species (ROS), reactive nitrogen species (RNS) and other inflammatory mediators largely contribute in the degeneration of the cartilage and bone (Hemshekhar et al., 2012). Therapeutic management of RA is based on two principal approaches, symptomatic and disease modifying anti-rheumatic treatment with non-steroidal anti-inflammatory drugs (NSAIDs) and disease modifying anti-rheumatic drugs (DMARDs) respectively (Wang et al., 2011). However, sever adverse effects, potential toxicity and high cost of NSAIDs and DMARDs limit their effectiveness (Shen et al., 2011). Accordingly, it is important to search for an alternative therapy which is safe and effective for RA treatment.

Eugenol (4-allyl-2-methoxyphenol) is the major component in the essential oil of many aromatic plants, including clove (*Zyzygium aromaticum*) and it is known to possess antioxidant, analgesic and neuroprotective properties among others (Yogalakshmi et al., 2010 and Park et al., 2011.) In addition, eugenol and related compounds exhibit anti-inflammatory activities, *e.g.* inhibition of lipopolysaccharide-stimulated nuclear factor-kappa B activation, cytokine release and cyclooxygenase-2 expression by macrophages *in vitro* (Murakami et al., 2005) and inhibition of 5-lipoxygenase activity in polymorphonuclear cells (Raghavenra et al., 2006). Eugenol possesses antiulcer activity based on its ability to stimulate the synthesis of mucus, an important gastroprotective factor (Santin et al., 2011). Therefore, screening of new therapeutic agents from natural products which usually have little side effects and low cost have gained interests for developing new therapy for arthritis. So, the current study was designed to evaluate therapeutic effect of eugenol on FCA-induced arthritic rats.

MATERIAL AND METHODS

Animals: Male adult albino rats weighing 150-200 grams at the age of 3.0-4.0 months were obtained from the animal house, Faculty of Medicine, Assiut University and were housed in animal house 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 food and water *ad libitum.*

Induction of arthritis and treatment: 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) suspension of heat-killed Mycobacterium tuberculosis according to the method by literatures (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 then rats were divided into 3 groups of eight rats each. Treatment with eugenol 250 mg/kg/day and indomethacin 2 mg/kg/day was started 14th day after arthritis induction and continued till 28 days. Experimental animals were divided into 4 groups, each of 8 animals

Group I: saline treated normal control oral.
Group II: FCA arthritic control received sesam oil 1.0 ml/rat oral.
Group III: FCA arthritic animals treated with indomethacin oral.
Group IV: FCA arthritic animals treated with eugenol oral.
On the 29th day, blood was collected from the heart and serum was separated by centrifugation and stored at –80˚C until analysis.

Drugs and chemicals: Eugenol**:** (Sigma Aldrich Company, England). Eugenol was pure oily solution, bottle contain 100 ml and freshly diluted with sesame oil Indomethacin: (Nile Co. for pharmaceuticals, Cairo, Egypt).
Sesame Oil: (Nile Co. for pharmaceuticals, Cairo, Egypt). It has been used for dilution of eugenol. Freund's complete adjuvant (FCA) was purchased from Sigma-Aldrich

Biochemical assessment: The level of C-reactive protein 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.

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 posthoc Newman-keuls test. Data were expressed as means ± standard error of the mean (SEM) and the level of significance groups were considered significant (*) at $p<0.05$.

RESULTS

Effect of eugenol on C-reactive protein

The level of CRP was significantly elevated in the serum of arthritic control group as compared to the normal control group. Treatment with eugenol significantly reduced CRP as compared to the arthritic control group. Results showed that eugenol is effective in decreasing CRP as compared with indomethacin (Table 1).

Table (1): Effect of eugenol (250 mg/kg) on C-reactive protein in FCA-induced arthritis in rats

Groups	CRP mg/L
Normal control	0.321±0.018
Arthritis control	2.39±0.22#
Indomethacin 2mg/kg	1.63±0.13*
Eugenol 250 mg/kg	1.74±0.15*

Data represent mean ± SE of 8 observations. # Significant result at $p<0.05$ from normal control * Significant result at $p<0.05$ from arthritic control group.

Effect of eugenol on lipid peroxidation in induced arthritis: On day 29 after induction of arthritis, level of MDA in serum was significantly increased in arthritic After treatment with eugenol, MDA level was significantly decreased.In comparison with indomethacin, eugenol is effective in reducing MDA level as shown in figure (1).

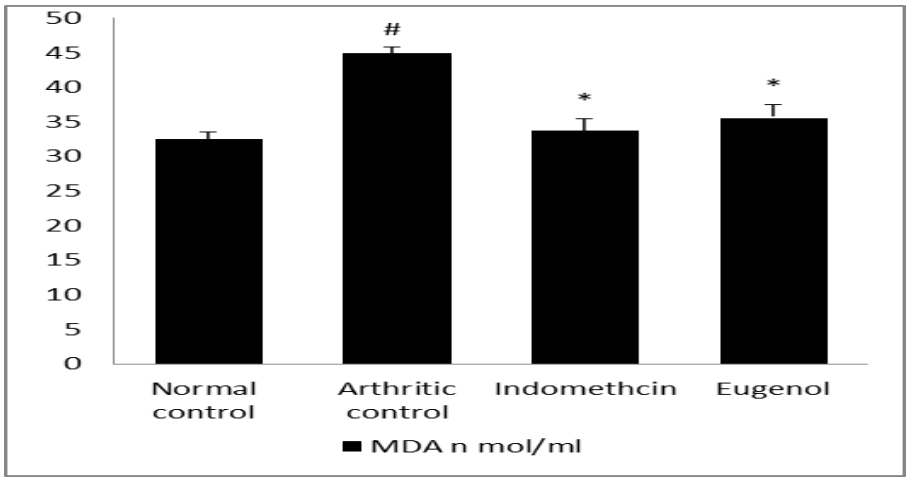

Figure (1): Effect of eugenol (250 mg/kg) on MDA in FCA-induced arthritis in rats

Data represent mean ± SE of 8 observations. # Significant result at p<0.05 from normal control * Significant result at p<0.05 from arthritic control group.

Effect of eugenol on nitric oxide in FCA-induced arthritis: Serum level of NO was significantly elevated in arthritic group compared to normal control group. Administration of eugenol significantly decreased serum NO level compared to the arthritic group and is effective in comparison with the standard drug indomethacin (Figure 2).

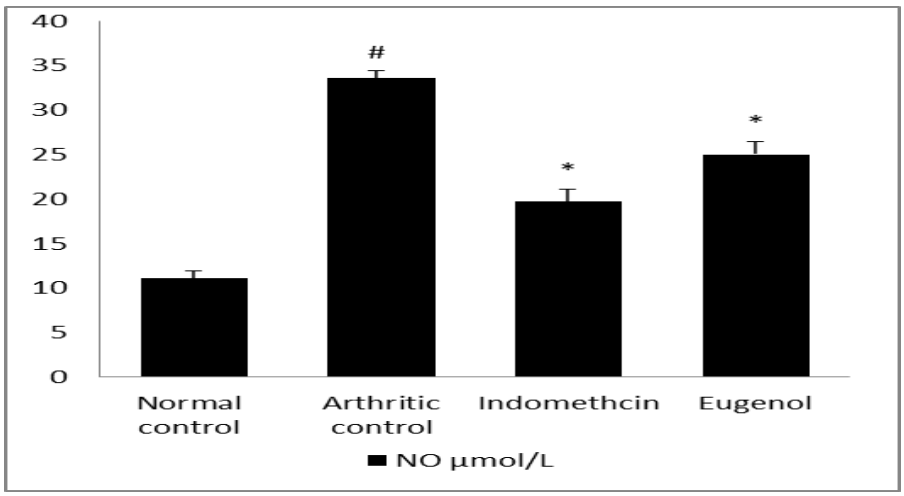

Figure (2): Effect of eugenol (250 mg/kg) on NO in FCA-induced arthritis in rats

Data represent mean ± SE of 8 observations. # Significant result at p<0.05 from normal control * Significant result at p<0.05 from arthritic control group.

Eugenol effect on tumor necrosis factor alpha: Arthritic control group revealed significant increase in serum level of TNF-α when compared with normal control group. Eugenol administration modified the elevated serum level of TNF-α and produced significant decrease in its level. Results showed that eugenol is effective in decreasing TNF-α as compared with indomethacin (Table 2).

Table (2): Effect of eugenol (250 mg/kg) on TNF-α in FCA-induced arthritis in rats

Groups	TNF-α pg/ml
Normal control	14.67±1.37
Arthritis control	48.32±4.03#
Indomethacin 2mg/kg	19.51±1.57*
Eugenol 250 mg/kg	24.68±1.87*

Data represent mean ± SE of 8 observations. # Significant result at p<0.05 from normal control * Significant result at p<0.05 from arthritic control group.

DISCUSSION

Freund's complete adjuvant-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).

Among the several constituents of plant essential oils, studies have shown that eugenol has antioxidant, anti-inflammatory, DNA-protective, analgesic and antimicrobial properties (Yogalakshmi et al., 2010 and Park et al., 2011). Previous findings indicate that Syzygium aromaticum, whose major compound is eugenol, has an immunomodulatory effect (Carrasco et al., 2009). Eugenol exhibited potent inhibitory activity against HCl/ethanol-induced gastric lesion and the reduction of gastric secretion. Therefore, eugenol is expected to have potential protective effect against gastritis (Jung et al., 2011).

C-reactive protein is an inflammatory marker, which is a member of the group of acute phase proteins and the level of CRP increases in response to inflammation (Kamezaki et al., 2008, Rhodes et al., 2011). The CRP assay is used as an optimal laboratory test for the observation of inflammation resulting from RA and other inflammatory diseases. It is an effective indicator of tissue damage and the concentration of CRP in serum is associated with disease activity (Poole et al., 2008, Rhodes et al., 2011). In the present study, an increased CRP level was observed in rats with arthritis and treatment with eugenol significantly inhibited the arthritis-induced CRP changes observed. The higher level of CRP observed in the arthritis group confirmed the pathology of the joint and the CRP production may have increased as a result of the activated macrophages and fibroblasts within the synovium of the inflamed joints. The production of CRP is also controlled by inflammatory mediators within the joints including IL-1 and IL-6, thus the reversal of CRP level following treatment indicates a significant decrease in the activation of synovial macrophages and fibroblasts (Jones et al., 2011). It is previously reported that concentration of C-reactive protein in the blood positively correlates with disease severity and progression of rheumatoid arthritis similar to rheumatoid factor (Jung et al., 2005). In rheumatoid arthritis, CRP can bind with various Fc receptors by forming complement activating complexes which generate antibody towards Fc fragment and causes cartilage degradation (Jones et al., 2012). In the present study, eugenol treatment in arthritic rats significantly reduced serum level of CRP as compared with arthritic control rats which confirms that eugenol showed anti-arthritic activity by suppressing generation of autoantibody towards Fc fragments and protecting cartilage degradation.

Ogata and his colleagues (2000) studied the antioxidant activity of eugenol and its related compounds, and found that the mechanism inhibiting lipid peroxidation has two steps:

1) It interferes with the chain reactions by trapping the active oxygen, such as superoxide anion and hydroxyl radicals.

2) It is metabolized to a dimer, and the dimeric compound (dieugenol) inhibits lipid peroxidation at the level of propagation of free radical chain reaction like α –tocopherol (Ogata et al., 2000). However, the peroxidation was induced by Fe^{+2}-ascorbic acid system, and the Fenton reaction was used in the generation of the hydroxyl radical. Eugenol trapped hydroxyl radicals directly, because it had no iron chelating action (Ogata et al., 2005). Malondialdehyde was a peroxidation product produced because of lipid attacked by free radicals and the level of MDA represented the intensity of body injury (Su et al., 2015). Results of present study showed significant reduction in MDA serum level and this is in harmony with Nagababu and his colleagues (2010) which showed that eugenol inhibits iron and OH radical initiated lipid perioxidation. Other studies evaluated the effect of eugenol on MDA and are in accordance with the present results (Fouad and Yacoubi, 2011; Gülçin, 2011). Gülçin found that eugenol inhibited 96.7% lipid peroxidation of linoleic acid emulsion at a 15 µg/ml concentration. According to the results of his study, eugenol had the most powerful antioxidant activity and radical scavenging activity (Gülçin, 2011).

Oxidative stress inflicts damage to joints because of excessive generation of reactive oxygen species and reactive nitrogen species in rheumatoid arthritis (Phillips et al., 2010). Previous reports have shown that macrophages secrete inducible nitric oxide synthase (iNOS) involved in the production of large amount of NO (Ignarro, 2002). Since the adhesion of neutrophils to the endothelial cells is mediated by NO and acts as a pro-inflammatory mediator in arthritic inflammation (Kubes et al., 1991). Nitric oxide is a critical biochemical mediator of inflammation and involved in autoimmune mediated tissue damage and inflammation (Bogdan, 2011). In the present study, nitric oxide serum level significantly increased in untreated adjuvant arthritis rats. It raised from the possibility that excessive nitric oxide production by iNOS induced by TNF-α and IL-1 and resulted in the formation of excessive amounts of superoxide (O^{2-}) (Hitchon and El-Gabalawy, 2004), which reacted with NO to generate peroxynitrite ($ONOO^{-}$). It had been reported that peroxynitritere acting with tyrosine residues of protein to produce nitrotyrosine contributed to rheumatoid arthritis pathogenesis (Swindle and Metcalfe, 2007). However,

determination of serum nitrotyrosine might provide an evidence for this proposed action mechanism. The decrease in serum nitric oxide level by eugenol might be attributed to its inhibition of the LPS-mediated production of NO by inhibiting the expression of iNOS protein without any toxic effects on cell viability, suggesting that eugenol can act as anti-inflammatory agents. Inhibiting action of eugenol on iNOS induction is independent of phosphorylation of IκB and further decrease the expression of COX-2 protein, implying that eugenol can act as principal anti-inflammatory mediators (Li et al., 2006). Other studies are in accordance with the results of the present study (Kar Mahapatra et al., 2011; Fouad and Yacoubi, 2011; Jung et al., 2011; Kaur and Sultana, 2012).

Taking into account that cytokines involved in direct cell-to-cell communication and in the tissue damage observed in rheumatoid arthritis (Dong et al., 2010). The pro-inflammatory cytokines, TNF-α and IL-1 could promote the release of prostaglandins (e.g. PGE2 causes synovial inflammation), leukotriene, and oxygen free radical and generate collagenases and neutral protease, which induced the cartilage matrix breakdown, cartilage resorption and bone destruction (Lee et al., 2009). Tumor necrosis factor -α appears to act principally on induction of osteoclast differentiation (Kobayashi et al., 2000). Tumor necrosis factor -α and IL-1 have been shown to play a pivotal role in the pathogenesis of the synovitis in RA and, as above, are regulators of osteoclastic resorption. TNF-α is an important cytokine in the initiation and perpetuation of inflammatory arthritis (Keffer et al., 1991). In the present study, serum level of TNF-α was significantly elevated in FCA-induced arthritis control. On the other hand, rats treated with eugenol showed a significant reduction in serum TNF-α level. Other studies are in accordance with the present results (Kar Mahapatra et al., 2011; Kaur and Sultana, 2012).

Eugenol was shown to block the release of the bone resorbing mediators, including interleukin-1β, TNF-α, and prostaglandin E2 from LPS-stimulated macrophages. Consistent with down regulation of bone-resorbing mediators, eugenol suppressed the messenger RNA expression of LPS-induced IL-1β, TNF-α, and COX-2 in macrophages. The results suggest a potential anti-inflammatory effect of eugenol (Lee et al., 2007).

In conclusion, the results presented herein give additional insight into the previously described anti-inflammatory beneficial effects of eugenol, suggesting that this compound may be an alternative and/or supplemental treatment to chronic inflammatory disease such as rheumatoid arthritis.

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