



## STUDY OF THE EFFECTS OF EUGENIC ACID IN ARTHRITIS AND DIABETES MELLITUS IN EXPERIMENTAL RATS

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### Abstract

**Background:** Systemic inflammatory burden in rheumatoid arthritis (RA) has been shown to predispose to developing both insulin resistance and type 2 diabetes mellitus (DM). Polypharmacy with cumulative side effects are common problems met with in these medical situations of combined diseases.

**Aim of the work:** the present study evaluates the potential role of Eugenic Acid as a natural product with anti-inflammatory and hypoglycemic activities in combating both DM and RA in experimental animals.

**Materials and methods:** Twenty four male albino rats were used. Eight male rats were used as a normal control group received saline (group1). Arthritis was induced in 16 male albino rats by intradermal injection of 0.1 ml of Freund's Complete Adjuvant (FCA) in the right hind paw then diabetes induced by alloxan (arthritic and diabetic animals). Animals were divided into 2 groups. Arthritic and diabetic control received sesame oil oral (group 2). Arthritic and diabetic animals treated with Eugenic Acid 100 mg/kg/day orally (group 3). Treatment was started with Eugenic Acid after diabetes induction for two weeks.

**Results:** arthritic and diabetic control animals showed significant elevation in serum level of C-reactive protein (CRP), nitric oxide (NO), malondialdehyde (MDA) tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), hyperglycemia and dyslipidemia compared to normal control group. Treatment with Eugenic Acid exhibited significant reduction in serum level of CRP, NO, MDA, TNF- $\alpha$ , blood glucose level, triglycerides, total cholesterol and LDL compared with arthritic diabetic control group. There was insignificant change in HDL serum level.

**Conclusion:** These results suggested that dietary supplementation with Eugenic Acid could

beneficially treat hyperglycemia and dyslipidemia. Also, Eugenice Acid ameliorates RA and could be useful as a beneficial supplement in treatment of RA and DM.

**Keywords:** Rheumatoid arthritis, diabetes mellitus, Eugenice Acid, inflammatory mediators.

## INTRODUCTION

Rheumatoid arthritis is a chronic inflammatory disease associated with increased disability, morbidity and mortality (Giles et al., 2008). Both oxidative stress and inflammation are considered major role players in the pathogenesis of chronic degenerative diseases including cardiovascular diseases (Lüscher, 2015), DM (Odegaard et al., 2016) and RA (McInnes and Schett, 2011). Rheumatoid arthritis as an inflammatory autoimmune disorder has been found associated with development of insulin resistance and both IL-6 and TNF $\alpha$  contribute in developing insulin resistance (Chung et al., 2008). Currently, although several synthetic regimens are used to attenuate oxidative stress and inflammation-mediated degenerative diseases, none are free from side effects when utilized in the treatment of cardiovascular diseases (Alagona and Ahmad, 2015), DM (Kokil et al., 2015) or RA (Albrecht and Müller, 2010). Over the last two decades, tremendous experimental advancements have been made in the use of natural products against different types of degenerative diseases targeting oxidative stress and inflammation (Fischer and Maier, 2015). Many studies have also demonstrated that phytochemicals are important therapeutic agents targeting oxidative stress and inflammation, which are the major culprits in the pathogenesis of chronic degenerative diseases (Uttara et al., 2009, Aggarwal et al., 2011). Eugenice Acid (4-allyl-2-methoxyphenol) is the active principles of clove (*Zyzygium aromaticum*) also be found in basil and cinnamon. Eugenice Acid is known to possess antioxidant, analgesic and neuroprotective properties among others (Yogalakshmi et al., 2010; Park et al., 2011 and Prasad et al., 2016). In addition, it exhibit anti- inflammatory activities (Murakami et al., 2005) and antiulcer activity (Santin et al., 2011).

Chronic diseases such as cardiovascular disorders, DM, hyperlipidemia and RA are common situations met and represent serious causes of polypharmacy, morbidity and reduced longevity. They also pose tremendous economic burdens on individuals, families and societies.

The anti-inflammatory and hypoglycemic activities of Eugenice Acid in addition to the little side effects and low cost expected with its use therapeutically have gained interests for developing new therapy for arthritis and diabetes. Previous studies have been done on DM (Azza et al., 2011) and RA (Safwat et al., 2015) separately to show the effect of Eugenice Acid in each disease. So, the current study was designed to evaluate the possible therapeutic effect of Eugenice Acid on both FCA-induced arthritis and alloxan induced diabetes in rats.

## MATERIALS AND METHODS

### Animals

Male adult albino rats weighing 150-200 grams at the age of 3.0-4.0 months 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 $\pm$ 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) suspension of heat-killed *Mycobacterium tuberculosis* according to the method (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.

### Induction of diabetes

Diabetes was induced after induction of arthritis in overnight fasted rats by single intra-peritoneal injection of alloxan (60 mg/kg body weight) (Glauce et al., 2004) which dissolved in 0.5ml of physiological saline. Control rats received the same amount of saline. Development of hyperglycemia in rats was confirmed by fasting blood glucose measurement (blood samples from tail vein, 0.5 ml/each rat), 72 hours after alloxan administration with portable glucometer (Accu-Check, Roche, Germany). Animals having blood glucose level 200 mg/dl and above were considered diabetic and included in the study (Ojezele and Abatan, 2011).

### Treatment

Rats were divided into 3 groups of eight rats each. The treatment of animals began on the 3<sup>rd</sup> day after alloxan injection and this was considered as 1<sup>st</sup> day of treatment. The animals were treated for 2 weeks as follows:

**Group I:** saline treated normal control.

**Group II:** FCA arthritic and diabetic control received sesame oil 1.0 ml/rat oral.

**Group III:** FCA arthritic and diabetic animals treated with Eugenice Acid 100 mg/kg oral (Abraham, 2001).

At the end of treatment period, blood was collected from the heart and serum was separated by centrifugation and stored at – 80°C until analysis.

### Chemicals and solutions preparation

**Eugenice Acid:** (Sigma Aldrich Company, England). Eugenice Acid was pure oily solution, bottle contain 100 ml and freshly diluted with sesame oil (Abraham, 2001).

**Alloxan monohydrate:** (Pharco Co. for pharmaceuticals, Cairo, Egypt). It was available in powder, 25 gm. in bottle and has been dissolved in normal sterile saline (0.9%) before administration as intraperitoneal injection (Ojezele and Abatan, 2011).

**Sesame Oil:** (Nile Co. for pharmaceuticals, Cairo, Egypt). It has been used for dilution of Eugenice Acid, 1ml/rat/day (Pourgholami et al. 1999).

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

### Biochemical assessment

Serum samples collected were used to evaluate serum level of glucose as described by Caraway and Watts (1987); total cholesterol by Richmond (1973), triglyceride as described by Stein (1987), HDL cholesterol and LDL cholesterol as described by Friedewald et al. (1972).

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 dihydrochloride 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- $\alpha$  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.

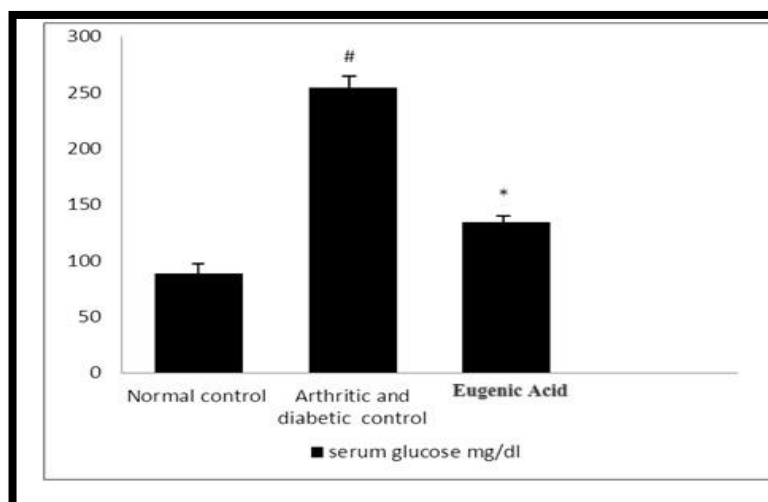
### Statistical analysis

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  $\pm$  standard error of the mean (SEM) and the level of significance between groups were considered significant (\*) at  $p < 0.05$ .

## RESULTS

### Effect of Eugenice Acid on serum glucose level

Serum glucose level of arthritic and diabetic control rats was significantly higher than corresponding normal control rats. There was significant reduction of serum glucose level in rats treated with Eugenice Acid as shown in figure (1).



**Figure (1):** Effect of Eugenice Acid (100 mg/kg) on serum glucose level in induced arthritis and diabetes in rats

Data represent mean  $\pm$  SE of 8 observations. <sup>#</sup> Significant result at  $p < 0.05$  from normal control.

\* Significant result at  $p < 0.05$  from arthritic and diabetic control group.

### Effect of Eugenice Acid on lipid profile

Lipid profile of arthritic and diabetic control rats was significantly higher than corresponding control normal rats. Results showed significant reduction in total cholesterol, triglyceride and LDL cholesterol after Eugenice Acid administration. There was no significant change in HDL level after Eugenice Acid treatment (table 1).

**Table (1):** Effect of Eugenice Acid (100 mg/kg) on lipid profile in induced arthritis and diabetes in rats

| Treatment                      | Triglyceride<br>mg/dl         | Total cholesterol<br>mg/dl     | HDL-cholesterol<br>mg/dl      | LDL-cholesterol<br>mg/dl      |
|--------------------------------|-------------------------------|--------------------------------|-------------------------------|-------------------------------|
| Normal control                 | 66.57 $\pm$ 2.59              | 87.49 $\pm$ 3.18               | 39.82 $\pm$ 2.01              | 27.88 $\pm$ 2.01              |
| Arthritic and diabetic control | 99.87 $\pm$ 4.52 <sup>#</sup> | 128.42 $\pm$ 6.87 <sup>#</sup> | 32.45 $\pm$ 2.99 <sup>#</sup> | 66.34 $\pm$ 4.32 <sup>#</sup> |
| Eugenice acid                  | 70.45 $\pm$ 2.17*             | 96.29 $\pm$ 6.16*              | 33.58 $\pm$ 2.68              | 45.87 $\pm$ 3.01*             |

Data represent mean  $\pm$  SE of 8 observations. <sup>#</sup> Significant result at  $p < 0.05$  from normal control.

\* Significant result at  $p < 0.05$  from arthritic and diabetic control group.

**Effect of Eugenice Acid on C-reactive protein**

The level of CRP was significantly elevated in the serum of arthritic and diabetic control group as compared to the normal control group. Treatment with Eugenice Acid significantly reduced CRP as compared to the arthritic and diabetic control group. Results showed that Eugenice Acid is effective in decreasing CRP (Table 2).

**Table (2):** Effect of Eugenice Acid (100 mg/kg) on C-reactive protein in induced arthritis and diabetes in rats

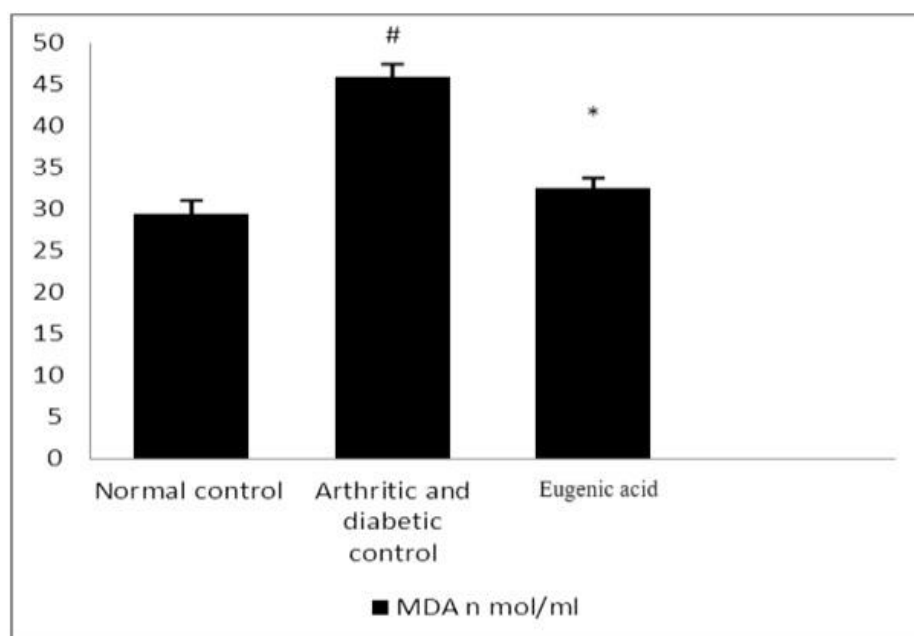
| Groups                         | CRP mg/L               |
|--------------------------------|------------------------|
| Normal control                 | 0.311±0.017            |
| Arthritis and diabetic control | 2.59±0.21 <sup>#</sup> |
| Eugenice acid                  | 1.72±0.14 <sup>*</sup> |

Data represent mean ± SE of 8 observations. <sup>#</sup> Significant result at p<0.05 from normal control.

<sup>\*</sup>Significant result at p<0.05 from arthritic and diabetic control group.

**Effect of Eugenice Acid on lipid peroxidation in induced arthritis and diabetes**

After induction of arthritis and diabetes, level of MDA in serum was significantly increased in arthritic and diabetic rats (group II) than that of control group (group I). After treatment with Eugenice Acid, MDA level was significantly decreased. Eugenice Acid is effective in reducing MDA level as shown in figure (2).



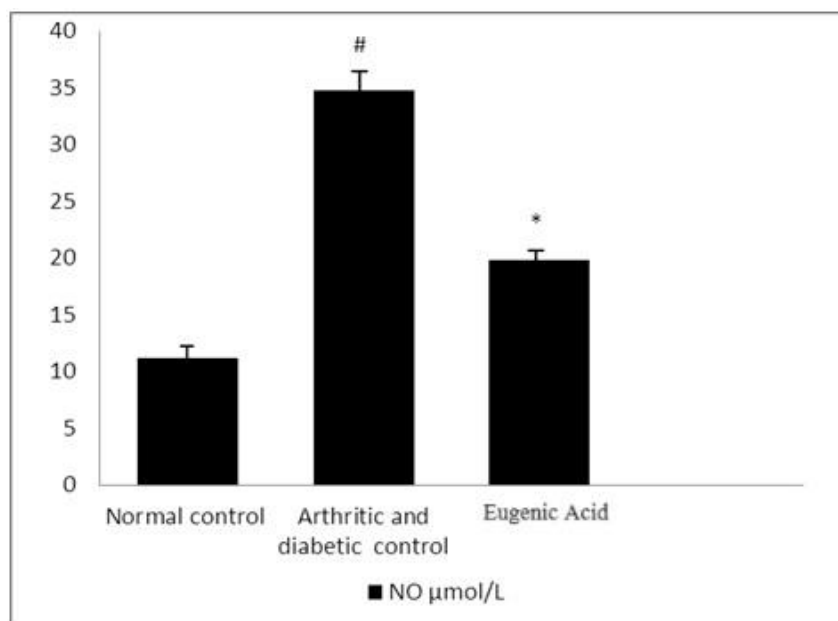
**Figure (2):** Effect of Eugenice Acid (100 mg/kg) on MDA in induced arthritis and diabetes in rats

Data represent mean ± SE of 8 observations. <sup>#</sup> Significant result at p<0.05 from normal control.

<sup>\*</sup>Significant result at p<0.05 from arthritic and diabetic control group

**Effect of Eugenice Acid on nitric oxide in induced arthritis and diabetes**

Serum level of NO was significantly elevated in arthritic and diabetic group compared to normal control group. Administration of Eugenice Acid significantly decreased serum NO level (Figure 3).



**Figure (3):** Effect of Eugenice Acid (100 mg/kg) on NO in induced arthritis and diabetes in rats. Data represent mean  $\pm$  SE of 8 observations. <sup>#</sup> Significant result at  $p < 0.05$  from normal control. <sup>\*</sup> Significant result at  $p < 0.05$  from arthritic and diabetic control group.

**Eugenice Acid effect on tumor necrosis factor alpha** Arthritic and diabetic control group revealed significant increase in serum level of TNF- $\alpha$  when compared with normal control group. Eugenice Acid administration modified the elevated serum level of TNF- $\alpha$  and produced significant decrease in its level. Results showed that Eugenice Acid is effective in decreasing TNF- $\alpha$  (Table 3).

**Table (3):** Effect of Eugenice Acid (100 mg/kg) on TNF- $\alpha$  in induced arthritis and diabetes in rats

| Groups                         | TNF- $\alpha$ pg/ml           |
|--------------------------------|-------------------------------|
| Normal control                 | 14.97 $\pm$ 1.38              |
| Arthritis and diabetic control | 49.31 $\pm$ 4.13 <sup>#</sup> |
| Eugenol                        | 25.78 $\pm$ 1.76 <sup>*</sup> |

Data represent mean  $\pm$  SE of 8 observations. <sup>#</sup> Significant result at  $p < 0.05$  from normal control. <sup>\*</sup> Significant result at  $p < 0.05$  from arthritic and diabetic control group.

## DISCUSSION

Diabetes is usually associated with inflammation and the latter contributes to the development of diabetes (Pradhan et al., 2001). Besides, evidence have shown that insulin resistance as a pro-inflammatory status may have existed for years before the occurrence of type 2 diabetes (Festa et al., 2000). Moreover, increased CRP and TNF- $\alpha$  are associated with nephropathy, retinopathy and cardiovascular disease in both types of diabetes (Goldberg, 2009).

Patients inflicted with various clustering chronic diseases (e.g. RA and DM) require treatment with multiple drugs with possible cumulative side effects of multiple drugs as well as drug-drug interactions. Therefore, the present study provides naturally-occurring compound for reducing or eliminating polypharmacy. Among the several constituents of plant essential oils, studies have shown that Eugenice Acid has antioxidant, anti-inflammatory, DNA-protective, analgesic and antimicrobial properties (Yogalakshmi et al., 2010 and Park et al., 2011) and neurorestorative effects (Prasad et al., 2016). Previous findings indicate that *Syzygium aromaticum*, whose major compound is Eugenice Acid, has an immune-modulatory effect (Carrasco et al., 2009).

Administration of Eugenice Acid produced significant decline in blood glucose level which is comparable to the arthritic and diabetic control rats. This is in harmony with previous reports about

hypoglycemic effect of Eugenice Acid treatment in streptozotocin (STZ)-induced diabetic rats (Tanaka et al. 2006; Atef and Talal, 2007 and Radhiah et al., 2010). Eugenice Acid has dual mode of action in combating diabetes; it lowers blood glucose by inhibiting  $\alpha$ -glucosidase and prevents advanced glycation end products formation by binding to  $\epsilon$ -amine group on lysine, protecting it from glycation, offering potential use in diabetic management (Singh et al., 2016). Prasad and co-workers (2016) hypothesize that Eugenice Acid may be employed as an adjuvant therapeutic molecule to alleviate complications under diabetic conditions. His *in vitro* studies under experimentally induced hyperglycemic conditions showed that exposure of cells to Eugenice Acid (5–10  $\mu$ M) improved their viability, reduced the glutathione levels and significantly decreased the glucose-associated oxidative stress (by diminishing reactive oxygen species (ROS) and peroxide levels).

The common pattern of dyslipidemia in diabetic patients shows elevated triglyceride, total cholesterol, LDL and decreased HDL cholesterol levels. (Pushparaj et al., 2000).

Administration of Eugenice Acid to arthritic and diabetic rats for two weeks produced significant reduction in serum level of triglyceride, total cholesterol, LDL cholesterol and insignificant change in HDL cholesterol level compared to control values. These results are in agreement with those obtained by Rajasekaran et al. (2006). Hypolipidemic effect of Eugenice Acid was reported by Chhanda et al. (2006), Atef and Talal. (2007) in STZ- induced diabetic rats, after two weeks treatment and (Karupphasamy et al., 2014). The previous studies showed that Eugenice Acid had hypolipidemic effect since it is probably mediated through inhibition of hepatic cholesterol biosynthesis, reduction of lipid absorption, enhanced catabolism of LDL-cholesterol and catabolism of TG (Mnafigui et al., 2013 and Venkadeswaran et al., 2014).

In recent decades, more studies have shown that inflammatory reactions and oxidative stress play critical roles in the pathogenesis of DM (Meng et al., 2013). Clove bud powder may represent potential functional food for the prevention and management of type 2 diabetes (Stephen et al., 2014). 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). It has been also implicated that increased levels of C-reactive protein could directly participate in amplifying the immune response leading to increased tissue damage (Yeh 2003). C-reactive protein can bind with various Fc receptors by forming complement activating complexes which generate antibody towards Fc fragment and causes cartilage degradation in RA (Jones et al., 2012). The present study demonstrated that Eugenice Acid treatment resulted in lowering of serum level of CRP in arthritic and diabetic rats compared to the control which confirms that Eugenice Acid suppress generation of autoantibody towards Fc fragments and protecting cartilage degradation.

Lipid peroxidation is well known as an important parameter for assessing oxidative stress. It leads to permeability and fluidity of the membrane lipid bilayer and can dramatically alter cell integrity (Dix and Aikens, 1993). As observed in figure 2, elevated serum MDA levels in arthritic and diabetic control rats suggest enhanced lipid peroxidation leading to tissue damage and inability of antioxidant defense mechanisms to prevent free radical attack. This peroxidative damage to membranes may lead to the leakage of enzymes and metabolites into the blood circulation (Stephen et al., 2014). The cloves treatment significantly reduced lipid peroxidation in STZ-induced diabetic rats by restoring the antioxidant enzyme levels (Radhiah et al., 2010) which is in accordance with the present results. 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 after Eugenice Acid administration and this is in harmony with Nagababu and his colleagues (2010) which showed that Eugenice Acid inhibits iron and OH radical initiated lipid peroxidation. Other studies evaluated the effect of Eugenice Acid on MDA and are in accordance with the present results (Fouad and Yacoubi,

2011; Gülçin, 2011). Gülçin found that Eugenice Acid inhibited 96.7% lipid peroxidation of linoleic acid emulsion at a 15 µg/ml concentration. According to the results of his study, he concluded that Eugenice Acid had the most powerful antioxidant activity and radical scavenging activity (Gülçin, 2011).

Oxidative stress inflicts damage to joints because of excessive generation of ROS and reactive nitrogenspecies in rheumatoid arthritis (Phillips et al., 2010). Oxidative stress leads to impaired β-cell function and reduced β-cell mass. Thus, there is a vicious cycle, in which hyperglycemia and increased free fatty acids induce oxidative stress, which disturbs β-cell function, and accelerates the hyperglycemia. In addition, oxidative stress is suggested to be one of the major causes of aberrant insulin signaling in target tissues, by activating Jun N-terminal kinase and Nuclear-factor-kappa-B pathways as well as by other mechanisms (Chang and Chuang, 2010).

Previous reports have shown that macrophages secrete inducible nitric oxide synthase (iNOS) involved in the production of large amount of NO (Ignarro, 2002). Hyperglycemia induces O<sub>2</sub>•<sup>-</sup> and ONOO<sup>-</sup> overproduction (Du et al., 2000). In the present study, NO serum level significantly increased in untreated arthritic and diabetic rats. It raised from the possibility that excessive NO production by iNOS induced by TNF-α and IL-1 and resulted in the formation of excessive amounts of superoxide (O<sub>2</sub>•<sup>-</sup>) (Hitchon and El- Gabalawy, 2004), which reacted with NO to generate peroxynitrite (ONOO<sup>-</sup>). It had been reported that peroxynitrite acting with tyrosine residues of protein to produce nitrotyrosine contributed to rheumatoid arthritis pathogenesis (Swindle and Metcalfe, 2007). Diabetes mellitus, via hyperglycemia-driven ONOO<sup>-</sup>, resulted in accelerated apoptosis (Ping et al., 2007).

The decrease in serum NO level by Eugenice Acid might be attributed to its inhibition of the lipopolysaccharides (LPS) -mediated production of NO by inhibiting the expression of iNOS protein without any toxic effects on cell viability, suggesting that Eugenice Acid can act as anti-inflammatory agents. Inhibiting action of Eugenice Acid on iNOS induction is independent of phosphorylation of IκB and further decrease the expression of COX-2 protein, implying that Eugenice Acid can act as principal anti- inflammatory mediators (Li et al., 2006).

Insulin resistance and type 2 DM are closely associated with chronic inflammation, characterized by abnormal cytokine production (mainly TNF-α) and the activation of a cascade of inflammatory signaling pathways (Wellen and Hotamisligil, 2005). TNF-α has been shown to enhance adipocyte lipolysis, which further increases free fatty acids and also elicits its own direct negative effects on the insulin signaling pathway by increasing serine/threonine phosphorylation of insulin receptor substrate 1 (Hotamisligil, 2000). The pro-inflammatory cytokines, TNF-α and IL-1 could promote the release of prostaglandins (e.g. PGE<sub>2</sub> 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). Results of the present study demonstrated that Eugenice Acid treatment in arthritic and diabetic rats caused a significant decrease in serum TNF-α. Eugenice Acid was shown to block the release of interleukin-1β, TNF-α and prostaglandin E<sub>2</sub> from LPS-stimulated macrophages. Eugenice Acid 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 Eugenice Acid (Lee et al., 2007).

## CONCLUSION

These results suggested that dietary supplementation with Eugenice Acid could beneficially treat hyperglycemia and dyslipidemia. In addition, these results insight into the previously described anti-inflammatory and hypoglycemic beneficial effects of Eugenice Acid. Eugenice Acid may be employed as an adjuvant therapeutic molecule to eliminate or reduce polypharmacy with cumulative side effects and possible drug-drug interactions. Also, Eugenice Acid may alleviate complications

under rheumatoid arthritis and diabetic conditions. Further study will be done clinically in patients have RA and DM.

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