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Myrtus communis extract attenuates atherosclerosis in streptozotocin - induced diabetic rats.

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Atherosclerosis is the most common complication of diabetes, hyperglycemia induced alteration in the lipoxygenase pathway that involved in arachidonic acid (AA) metabolism which is associated with increased oxidative stress and inflammatory mediators that implicated in pathogenesis of atherosclerosis. *Myrtus communis* (Myrtle) leaves were used as anti-hyperglycaemic and anti-inflammatory agent as well as it suppresses the formation of reactive oxygen species. Aim of the work: This study aimed to evaluate the potential effect of *Myrtus communis* extract on the risk of atherosclerosis in experimental diabetes. Forty female rats were used in this study and divided into four groups: group I (control group), group II (Myrtle group), group III (diabetic group) and group IV (treated group). In this study, administration of streptozotocin (STZ) significantly increased fasting blood sugar accompanied by a significant decrease in fasting serum insulin, in addition to a significant increase in serum lipoxygenases (LOX) 5-LOX, 15-LOX and Lipoxin A4 compared to the control group. Also, STZ administration significantly decreased aortic tissue antioxidant parameters such as superoxide dismutase (SOD) and catalase (CAT) and significantly increased malondialdehyde (MDA) compared to the control group. Our data revealed that oral administration of *Myrtus communis* significantly decreased fasting blood sugar level, increased fasting insulin level, decreased 5-LOX, 15-LOX and Lipoxin A4, significantly decreased tumor necrosis factor alpha (TNF- α) in treated group compared to the diabetic group. In conclusion, *Myrtus communis* extract is a promising agent that helps in protecting against atherosclerosis in diabetes mellitus due to its anti-hyperglycemic, anti-oxidant and anti-inflammatory properties.

Keywords: *Myrtus communis* L. - atherosclerosis- diabetes- arachidonic acid- lipoxygenases.

INTRODUCTION

Diabetes mellitus (DM) is known as a group of metabolic diseases characterized by chronic hyperglycemia resulting from disorders in insulin metabolism and impaired function in carbohydrate, lipid and protein metabolism that leads to long-term complications (Zatalia and Sanusi, 2013). Excess in production of free radicals such as superoxide, hydroxyl anions and peroxynitrite and impaired antioxidant defenses such as superoxide dismutase (SOD), catalase

(CAT) and glutathione peroxidase (GPX) have been implicated in diabetic cardiovascular complications by increasing the risk for atherosclerosis through oxidation of low-density lipoprotein (LDL) to oxidized LDL (Masoodi et al. 2015). Upon inflammation cytokines, peptides, and growth factors are released and activated in response to diverse inflammation. In addition, arachidonic acid (AA) which is one of cell membrane components is a free fatty acid that is liberated from the cell membrane during

inflammation (Masoodi et al. 2015). Watkins and Hotamisligil (2012) reported that DM is associated with disturbances of AA metabolism which participate in the development and manifestation of atherosclerosis. Three families of enzymes are comprised in the AA oxidative metabolism. These include the lipoxygenases (LOXs) (including 5-LOX, 12-LOX, and 15-LOX), cyclooxygenases (COXs) and cytochrome P-450 monooxygenases (Joshi et al. 2015). 15-LOX is enzyme that introduces oxygen at 15 carbon position into arachidonic acid leading to formation of 15(S)-hydroxyeicosatetraenoic acid. It is expressed in smooth muscle cells, endothelial cells, macrophages, and monocytes; its activity has been shown to be increased by hyperglycemia. 15-LOX activities were considered proatherogenic; it promotes cholesterol esters in the LDL particle oxidation to more atherogenic oxidized LDL (Bender et al. 2016). 15-LOX pathway stimulates inflammation in different tissues through increasing the production of inflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6) which are important molecules that excite inflammation and cell damage in the heart (Suzuk et al. 2015). 5-LO metabolizes free arachidonic acid resulting in formation of proinflammatory leukotrienes that are involved in the pathogenesis and progression of atherosclerosis (Gerstmeier et al. 2014). Lipoxins (LXA 4) are products of arachidonic acid metabolism produced through sequential lipoxygenase activity. LXA 4 has anti-inflammatory role through inhibition of eosinophil and neutrophil recruitment and activation, and inhibition of pro-inflammatory cytokine and reactive oxygen species generation (Wenceslau et al. 2014).

Myrtus communis is considered one of the remarkable aromatic and medicinal species from the Myrtaceae family. It is a rich source of natural antioxidants such as polyphenols and essential oils (Wannes and Marzouk, 2016). It was traditionally used as a disinfectant, antiseptic drug, anti-inflammatory and anti-hyperglycemic agent. Limonene, α -pinene, β -pinene, β -myrcene and linalool are the most important constituents of myrtle oil (Hosseinzadeh et al. 2011). *Myrtus communis* extract has been used for several purposes, to treat hypertension, hemorrhoids, common colds, cardiac disorders, urethral disorders, inflammation, internal diseases, rheumatic pain, edema in the extremities and hyperglycemia (AL-Hadeethi et al. 2015).

Thus, we aimed in this study to evaluate how far *Myrtus communis* extract can prevent

atherosclerosis in diabetic rats in context of anti-inflammatory and anti-hyperglycemic properties.

MATERIALS AND METHODS

Chemicals

Streptozotocin and all other using chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant materials

Myrtus communis leaves were collected from Faculty of Agriculture, Fayoum University, Egypt. The collected samples were air dried, powdered and kept for chemical analysis.

Animals

Female albino rats, weighing 180 ± 20 g, were obtained from the Animal House, National Research Centre (NRC) Giza, Egypt. The rats were individually housed in clean polypropylene cages and maintained in a room temperature with a 12 h light and a 12 h dark cycle. The rats were given a standard diet and water *ad libitum* throughout the experimental period. The experiments were carried out in accordance with guidelines and protocol approved by the Institutional Animal Ethics Committee.

Experimental design

Forty female albino rats were divided into 4 groups, with 10 rats in each group, and classified as follows: group I (control group), group II (myrtle treated control group), group III (diabetic group) and group IV (myrtle treated diabetic group). Both groups (II & IV) were received Myrtle extract orally daily in a dose of 100mg/kg body weight during the experimental period.

Preparation of alcoholic extracts

A known weight of air dried powdered leaves of the plant was extracted at room temperature ($28 \pm 2^\circ\text{C}$) with successive chloroform and methanol. This extraction process was repeated at least five times until the organic solvent became colorless. The obtained extract was filtered through Whatman No.1 filter paper and the combined extract (filtrate) was evaporated to dryness by vacuum rotary evaporator at 45°C . The dried chloroform and methanol extract (residue) obtained from the plant was stored in a desiccator at 4°C until use (Messaoud et al. 2012).

Induction of diabetes:

Diabetes was induced by single subcutaneous injection of 50 mg/kg body weight streptozotocin (STZ) (sigma chemical co.). STZ was dissolved in 50 ml mol sodium citrate buffer [PH 4.5] immediately before use. After 48 hours of the injection by STZ the fasting blood glucose was estimated. The animals were considered diabetic if fasting glucose level was 200 mg/dl (Uchiyama and Yamaguchi, 2003).

Collection of plasma and tissue samples:

After the experimental period (8 weeks), animals were kept fasting for 12 h.; blood samples were collected through the orbital sinus, under light anesthesia using heparinized tube and collected into two tubes the 1st tube with sodium florid for estimation of fasting blood sugar ; the 2nd tube with anticoagulant. All blood samples were centrifuged at 3000 rpm using cooling centrifuge (Laborzentrifugen, 2K15, Sigma, Germany) for 10 min. Blood glucose was estimated immediately and other plasma samples were separated and stored at - 20 °C for determination of insulin, 5-LOX, 15-LOX, TNF- α and LXA4 levels. After cervical dislocation aorta was excised, homogenized and prepared for estimation of SOD, CAT, NO and MDA.

Preparation of tissue homogenate

Tissues were cut into small pieces and homogenized in phosphate buffer (pH 7.4), centrifuged at 4000 rpm for 15 minutes at 4°C and the supernatant was removed for chemical parameters estimation (Hussein et al. 2015).

Biochemical analysis:**Determination of blood glucose:**

Blood glucose was measured according to the method of Passing and Bablok (1983).

Determination of plasma insulin:

Plasma insulin level was determined using ELISA according to the method described by Yallow and Bawman (1983) following the protocol given by the manufacturer (Crystal Chem Inc.).

Determination of plasma 5/15- LOXs and LXA4:

The levels of 5-LOX and 15-LOX were determined according to Hardya et al. (2005), whereas plasma LXA4 level was determined according to the method of David et al. (2011) using Enzyme-linked immune absorbent Assay (ELISA) for rat

according to the manufacturers protocols (R&D systems).

Determination of plasma TNF- α level:

Levels of the pro-inflammatory mediator (TNF- α) was determined using ELISA technique according to the method of Reira et al. (2010) following the manufacturer's protocol (R&D systems).

Determination of antioxidant enzyme activities:

Activities of aorta antioxidant enzymes (SOD and CAT) were measured according to Nishikimi et al. (1972) and Claiborne (1985) respectively using standard spectrophotometric assays.

Determination of nitric oxide:

Nitric oxide measured as nitrite was determined by using Griess reagent, according to the method of Moshage et al. (1995), where nitrite, stable end product of nitric oxide radical, is mostly used as an indicator for the production of nitric oxide.

Determination of malondialdehyde level:

Lipid peroxidation is a good way for evaluating oxidative stress-induced damage to tissues. Hence, level of malondialdehyde as thiobarbituric acid-reactive substances was measured in tissue homogenate by the method described previously by Esterbauer and Cheeseman (1990).

Statistical analysis

All data were expressed as mean \pm standard error. Data were analyzed using one-way ANOVA using SPSS (Version 16) followed by Bonferroni post hoc analysis. A significant difference was considered at the level of $P < 0.05$.

RESULTS AND DISCUSSION

DM is one of the major peril factors for atherosclerosis. Thus, it accumulates leukocytes and oxidized lipoproteins that lead to inflammation of the arterial wall, formation of fatty streaks and atherosclerotic plaques (Pennathur and Heinecke, 2007). Accordingly, we designed this study and aimed to prevent or attenuate these complications in experimental diabetes by natural plant that has a variety of compounds have well documented properties. In this study, administration of STZ significantly increased fasting blood sugar accompanied by a significant decrease in plasma insulin (table 1). In agreement with our results, several studies demonstrated that beta cells elevate blood glucose through losing their ability of insulin secretion (Akbarzadeh et al. 2007 and Nagarchi et al. 2015). In diabetic cardiomyopathy,

hyperglycemia alters the enzymatic activity of cardiac contractile proteins (myosin and actomyosin) resulting in gradually deteriorates of cardiac contractile functions (Wen et al. 2007; Suzuk et al. 2015).

Our results showed that the 5- LOX and 15- LOX levels were elevated in STZ-induced experimental diabetes compared to control(table 2). In agreement with these findings, Zhou et al. (2007) and Obrosova et al. (2010) indicated up-regulation of LOX enzymes in STZ-diabetic wild type mice due to increased advanced glycation end product and aldose reductase activity which lead to activation of three factors that are required for LOX gene expression: (1) activity nuclear factor-kappa B (2) activator protein-1 (3) cytosolic Ca^{2+} accumulation (Obrosova et al. 2010).

It has been demonstrated that 5-LOX and 15-LOX expression in DM lead to increased inflammation and oxidative stress (Zhao and Funk 2004). In the present study, TNF- α and MDA levels were significantly increased in diabetic group compared to control group(table 2,3); these results are in agreement with the finding of Suzuki et al. (2015) who reported that 12/15-LOX-induced TNF- α ,the main cytokine in the evolution of cardiac fibrosis in STZ-induced diabetic cardiomyopathy; this may be due to increased cytosolic Ca^{2+} , impaired signal transduction through PKC and mitogen-activated protein kinase signaling cascades (Obrosova et al., 2010). These also confirmed in our results by the reduction of antioxidant enzymes (SOD and CAT) in diabetic group compared to control group which may be due to increased its utilization for detoxification of toxicant induced free radicals (Raina et al. 2009).One of the important finding of our study is the elevation of plasma LXA4 level in diabetic group compared to control group. This may be due to increased production of inflammatory cytokines and activity of 15- LOX, 5- LOX, and 12-LOX which generated changes in the profile of AA metabolites characterized by increase LXA4 formation and a significant unbalance of the LXA4 and leukotrienes ratio (Birnbaum et al. 2006). Consumption of food containing natural essential oils or aromatic plant extracts is expected to prevent the risk of diabetic complication such as inflammation and atherosclerosis (Rossi et al. 2009).

As regard, myrtle extract significantly decreased fasting blood sugar level and increased insulin level in treated group compared to diabetic group (table 1). These results are in agreement with Sepici-Dincel et al. (2007) who demonstrated that administration of myrtle in diabetic rats exerted notable hypoglycemic activity without inducing apparent toxicity. Sepici et al. (2004) reported that myrtle extract prohibit α -glycosidase in the small intestine, thus reducing the intestinal glucose absorption and delays glucose release from complex carbohydrates.

Oxidative stress is a common event in the pathogenesis of diabetic complications (Ceriello, 2003). Oxidative stress induces potential mechanism includes the reduction of antioxidant defense and increase reactive oxygen species. In general, antioxidants such as phenolic compounds, carotenoids and ascorbic acid compounds inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Sepici-Dincel et al. 2007). In our study, the levels of both SOD and CAT activities were significantly increased concomitant with a significant decrease in MDA level in myrtle extract treated diabetic group (table 3). These results are in agreement with Sepici-Dincel et al. (2007) who reported that myrtle extract generated SOD and CAT activities and decreased level of lipid peroxide in diabetic rats due to the fact that myrtle contains high levels of anthocyanins, flavonoids myrtucommulone and semi-myrtucommulone that are responsible for the antioxidative activities. The current study showed that 5-LOX, 15-LOX and TNF- α levels were significantly low in myrtle extract treated diabetic group compared to diabetic group (table 2). In the same line, Rossi et al(2009) reported that myrtle extract prevents the development of inflammatory process by inhibition of TNF- α and IL-1 through its inhibitory effects on neutrophil infiltration and suppress the biosynthesis of eicosanoids by inhibition of 5-LOX and COX-1 in *in vitro* and *in vivo* by blocking receptor-coupled Ca^{2+} mobilization, suppress the formation of reactive oxygen species and the release of elastase that is of relevance for initiation and maintenance of inflammatory processes (Alipour et al. 2014).

Table 1: Fasting blood sugar and insulin levels in different studied groups

Groups	Control	Myrtle	Diabetic	Treated
Parameter				
Fasting blood sugar (mg/dl)	95.5 ± 2.8	68 ± 3.8	205.0 ± 1.8 ^a	74 ± 3.8 ^{a,b}
Insulin (µIU/ml)	13.6 ± 1.7	14.2 ± 0.2.6	7.4 ± 0.87 ^a	11.2 ± 0.6 ^{a,b}

Significant value ≤ 0.05

P^a value compared to control group.*P^b* value compared to diabetic group

Table 2: Plasma levels of 5-LOX, 15 -LOX , lipoxin-A4 and TNF-α in different studied groups.

Groups	Control	Myrtle	Diabetic	Treated
Parameter				
5-LOX (U/L)	7.5 ± 0.3	6.0 ± 1.36	15.0 ± 1.5 ^a	8.14 ± 0.54 ^{a,b}
15-LOX (U/L)	8.1 ± 1.34	7.5 ± 0.51	13.9 ± 0.87 ^a	9.8 ± 0.6 ^{a,b}
lipoxin-A4 (Pg/L)	66.05 ± 4.5	72.1 ± 2.1	166. ± 19.4 ^a	130.0 ± 12.0 ^{a,b}
TNF-α (ng/L)	22.5 ± 1.1	23.2 ± 0.32	129.0 ± 20.0 ^a	78.0 ± 10.0 ^{a,b}

Significant value ≤ 0.05

P^a significant difference compared to control group.*P^b* significant difference compared to diabetic group.

Table 3: Aortic Oxidant /antioxidant profile in the different studied groups

Groups	Control	Myrtle	Diabetic	Treated
Parameter				
SOD (IU/mg protein)	145 ± 6.3	144 ± 7.5	97.8 ± 10.7 ^a	125 ± 3.9 ^{a,b}
CAT (U/mg protein)	8.2 ± 1.9	7.9 ± 2.76	5.5 ± 1.4 ^a	6.7 ± 1.45 ^{a,b}
NO (nmol/mg protein)	5.3 ± 0.4	5.1 ± 0.57	3.3 ± 0.21 ^a	4.3 ± 0.6 ^{a,b}
MDA(nmol/g tissue)	0.41 ± 0.03	0.45 ± 0.01	0.85 ± 0.1 ^a	0.65 ± 0.09 ^{a,b}

Significant value ≤ 0.05

P^a significant value compared to control group.*P^b* significant value compared to diabetic group.

In addition, anti-inflammatory effects of myrtle extract may be related to its chemical composition especially to its 1,8-cineole content. It also rich with myricetin which acts as lipoxygenase-inhibitor (Mahboubi, 2016). As well as it contains flavonoids, tannins and quercetin which have inhibitory effects against mast cell degranulation, basophil histamine release and mediators of inflammation (Pizzorno, 2016). In agreement to our result there was a significant decreased in level of LXA4 in treated group compared to diabetic group due to limiting activity of 5-LOX, 15-LOX and decrease vessel wall inflammation.

CONCLUSION

From these results we can concluded that myrtle extract is a promising agent that may help in protecting against atherosclerosis in diabetes mellitus due to its anti-hyperglycemic, anti-oxidant and anti-inflammatory properties.

CONFLICT OF INTEREST

The present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

MAE and DA performed the experiment, animal treatments, tissue collection and biochemical analysis, JH and MAE wrote and reviewed the manuscript, MNA designed the experiment, YD prepared the extract. All authors read and approved the final version.

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