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Functional role of myrtle on the regeneration of endothelial dysfunction and the activity of lipoxygenases in ovariectomized diabetic rats

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ABSTRACT

This study was performed to evaluate the beneficial role of myrtle plant as a natural product on the regeneration of endothelial cell dysfunction and the activity of lipoxegenases in diabetic- ovariectomized rats. Fifty female rats were divided into 5 groups, with 10 rats in the sham group, and 20 for each of ovariectomized (ovx) and ovariectomized diabetic groups (10 rats before and 10 rats after treatment with myrtle). Blood glucose, cholesterol, high density lipoprotein (HDL-cholesterol), low density lipoprotein (LDL-cholesterol), triglycerides, plasma insulin, estrogen, tumor necrosis factor alpha (TNF - α), interleukin-1beta (IL-1β), 5-lipoxygenase (5-LOX),15-lipoxygenase (15-LOX) and plasma level of lipoxin A4 (LXA-4) were estimated. Our results revealed that blood glucose level as well as triglycerides and cholesterol were significantly decreased in the treated groups with myrtle extract in the ovx and ovx diabetic groups. Also ovx and ovx diabetic groups showed progressive decrease in HDL level which improved after treatment with myrtle extract in ovx diabetic group. Significant increase in the activity of 5-LOX, tumor TNFα and the IL-1β in ovx and ovx diabetic groups, which decreased after treatment with myrtle. There were increase in the level of LA4 and 15 LOX after administration of myrtle in both ovx and ovx diabetic rats. We concluded that myrtle extract can alleviate the endothelial dysfunction and have the ability to reduce significantly the activity of 5-lipoxygenase during menopause or even in menopause associated with diabetes.

INTRODUCTION

Estrogens are essential regulators of different metabolic processes, including body weight, lipid and glucose metabolism, distribution of adipose tissue, caloric intake and energy expenditure **[1]**. Diabetes mellitus and estrogen deficiency as in menopause or ovariectomy are usually associated with atherosclerosis and endothelial dysfunction. Many postmenopausal women suffer from diabetes mellitus; however, few facts are known about how the changes that may occur during the period of menopause might uniquely directly or indirectly affect diabetes mellitus management **[2].** Postmenopausal diabetic women suffered from increased cardiovascular risk factors as a consequence of increase in their atherogenic lipid profile [3], as well as redox imbalance [4]**.**

Specific lipid mediators, such as lipoxins (LXs)], resolvins (Rvs), and protectins (lipoxygenase interaction products) have essential role in the resolution of inflammation [5]**.** Animal and cellular models proved that these oxidized lipids are induced under both diabetic and diabetic ovariactomy conditions, have strong proatherogenic effects on cell walls of blood vessels, and also act as mediators for the actions of cytokines and growth factors [6].

Several facts indicate that 2 lipoxygenases (12-LOX and 15-LOX), and their products, play essential roles in different organs and tissues, like kidney, the vasculature, brain, adipose tissue, and the pancreatic islet. One of the lipoxin family members, lipoxin A4 (LXA4) show antiestrogenic effect, significantly reducing the activity of the potent estrogen produced in the body-17-estradiol (E2) **[7].**

The incipience of menopause has been accompanied with spontaneous increase in the production of cytokines, mainly tumor necrosis factor- α (TNFα) and the interleukins, IL-1 and IL-6. Moreover, levels of cytokines are significantly lower in postmenopausal women treated with hormone replacement therapy as well as in estrogen-treated ovariectomized mice when compared with untreated controls [8]**.** In 2005 [9]Arenas et al., indicated a potent new role of TNFα in the vascular dysfunction development which may be considered another risk factor for CVD, in cases of estrogen deficiency.

One of the cytokines, interleukin-1β (IL-1β) is related to several inflammatory diseases including rheumatoid arthritis, gout, and diabetes mellitus, so targeting reduction of activity of IL-1β may be considered as a novel trend in the management of these diseases [10].

Nowadays a great concern is given to the efficacy and side effects of many synthetic drugs, that's why natural products are considered a good and safer alternative for the treatment of different health problems. Recently, *myrtle communis* has been shown to have antioxidant, antibacterial, analgesic and antifungal activities, anti-hemorrhagic, antimutagenic, and anti-hyperglycemic effects [11].

This study aims to elucidate the beneficial role of myrtle plant on the regeneration of endothelial cell dysfunction and the activity of lipoxegenases in diabetic- ovariectomized rats.

MATERIALS AND METHODS

Drugs and reagents

All drugs and chemicals for ex-vivo experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Biomedicals, LLC. (France).

Plant materials

Myrtus communis L. leaves were collected from farm of Faculty of Agricultural, Cairo University, and identified by Dr. Yasser Diab, Biochemistry Department, faculty of Agriculture, Fayoum University, Egypt. The collected samples were air dried, powdered and kept for chemical analysis.

Preparation of alcoholic extracts

A known weight of air dried powdered leaves was extracted at room temperature (28±2ºC) with successive chloroform and methanol. This extraction process was repeated at least five times until each organic solvent became colourless. The obtained extracts were filtered over Whatman No.1 filter paper and the combined extract (filtrate) was evaporated to dryness by vaccum rotary evaporator at 45ºC. The dried chloroform and methanol extracts (residues) were stored in a desiccator at 4ºC until use[12].

Animals & experimental design

Wistar strain albino rats, weighing 180 ± 20 g, were obtained from the Animal House, National Research Centre (NRC). The rats were individually housed in clean polypropylene cages and maintained in a controlled temperature room 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.

Fifty female rats were divided into 5 groups, with 10 rats in the sham group, and 20 for each of ovariectomized and ovariectomized diabetic groups (10 rats before and 10 rats after treatment with myrtle). They were classified as follows: group I (sham group), group II (ovariectomized group), group III (myrtle-treated ovariectomized group) ,group IV (ovariectomized diabetic group) and group V (myrtle-treated ovariectomized diabetic group) .Both groups (III & V) were received Myrtle extract orally daily in dose 100mg/kg body weight during the period of the experiment.

Induction of diabetes

Diabetes was induced by multiple intraperitoneal injections of freshly prepared streptozotocin (40 mg/kg of bodyweight) dissolved in 0.1 M chilled citrate buffer (pH 4.5) for 5 consecutive days according to the methods of **[13].**The sham group received the vehicle (water) alone. The animals allowed to drink 5% glucose solution over night to prevent initial streptozotocin-induced hypoglycemic mortality. Forty eight hours after last streptozotocin dose, fasting blood glucose levels will be monitored and animals with glucose levels > 200 mg/dl were considered diabetic and assigned for different treatment regimens.

Collection of plasma samples

After the experimental period(8 weeks), animals were kept fasting for 12 h. Blood samples were collected through the orbital sinus, under light ether anesthesia, centrifuged at 1000 × *g* for 15 min. using heparinized tubes. Plasma samples were separated and stored at − 20 °C .

Determination of blood glucose and lipids

Fasting blood glucose was done immediately by the glucose peroxidase method according to [14]Passing and Bablok (1983) (BioMerieux, Marcy l'Etoile, France). Cholesterol, high density lipoprotein (HDL-cholesterol) , low density lipoprotein(LDL-cholesterol) and triglycerides were measured by the colorimetric enzymatic assays according to [15]Allain et al. (1974), [16]Lopes-Virella et al. (1977), [17]Steinberg (1981) and [18]Glick et al. (1986) respectively. The kits were supplied from Biocon Diagnostic (Germany).

Determination of insulin

Plasma insulin levels were determined using ELISA according to the method of [19]Yallow and Bawman (1983) following the protocol given by the manufacturer (Crystal Chem Inc.).

Determination of estrogen

The concentration of plasma estrogen was measured using a commercial immunosorbent assay kit (Estradiol sensitive ELISA, DRG Instruments, Marburg, Germany) according to the method of [20]Taddei et al. (1996).

Determination of tumor necrosis factor alpha (TNF - α)

Tumor necrosis factor alpha (TNF - α) was determined by an enzyme amplified sensitivity immunoassay (EASIA) according to . [21]Aukrust et al.(1994), the kit was purchased from Biosource, Belgium.

Determination of plasma level of interleukin-1beta (IL-1 β)

Serum level of interleukin-1beta (IL-1 β) was determined using Enzyme-linked immunosrbent Assay (ELISA) for rat according to the manufacturer protocol (R&D systems) **[22].**

Determination of plasma level of 5-lipoxygenase (5- LOX) and 15-lipoygenase (15-LOX) activities

The levels of 5-lipoxygenase (5-LOX) and 15-lipoxygenase (15-LOX) were determined according to [13]Hardya et al .(2005) using Enzyme-linked immunosrbent Assay (ELISA) for rat according to the manufacturers protocols (R&D systems).

Determination of plasma level of lipoxin A4 (LXA-4)

Lipoxin A4 (LXA4) in the sample was determined according to the method of [23] David et al .(2011**)** using Enzyme-linked immunosrbent Assay (ELISA) for rat according to the manufacturers protocols (R&D systems).

Statistical Analysis

All data were expressed as mean ± SE. Distribution of the data were verified to be normal using Tests of Normality (SPSS package) (vesrion8). Statistical significance will be tested by one way analysis of variance (ANOVA) followed by Bonferroni *post hoc* analysis. $P< 0.05$ was considered to be statistically significant.

Table (1) Changes in plasma levels of glucose, insulin and estrogen in sham (SH), ovariectomized (OVX) and OVX diabetic (OVX-D) rats before and after treatment with *myrtle communis*

	SH (n=10)	OVX (n=20)		OVX-D (n= 20)	
		Before treatment	After treatment	Before treatment	After treatment
Glucose (mg/dl)	95.4± 2.8 [a]	114 ± 3.8 [b]	73 ± 1.58 [c]	220 ± 4.8 [d]	90.6 ± 6.7 [a]
Insulin (µIU/ml)	13.0 ± 2.6 [a]	14.0 ± 3. 2 [a]	12.6±3.13 [b]	9.0± 1.1 [c]	10.8 ± 0.56 [c]
Estrogen (Pg/ml)	43.3 ±1.87 [a]	12.5 ± 2.3 [b]	18.5± 3.5 [c]	10.9± 3.5 [d]	9.5 ± 3.2 [d]

Values sharing the same superscript means not significant. P> 0.05. Values are means ± standard error of mean (SEM). Different superscript letters within the row indicate significant differences by Duncan s multiple range test p< 0.05.

Table (2) Changes in plasma lipid profile in sham (SH) , ovariectomized (OVX) and OVX diabetic (OVX-D) rats before and after treatment with *myrtle communis*

	SH (n=10)	OVX (n=20)		OVX-D (n= 20)	
		Before treatment	After treatmen	Before treatment	After treatment
Cholesterol (mg/dl)	118 ± 3.1 [a]	220 ± 4.6 [b]	55 ± 3.0 [c]	165 ±5.5 [d]	125 ± 5.0 [e]
Triglyceride (mg/dl)	97.7 ± 1.4 [a]	126.2±10.6 [b]	67.7± 4.5 [c]	132 ± 6.7 [b]	77.5 ± 5.2 [d]
HDL-Cholesterol (mg/dl)	73.8 ± 9.5 [a]	34.6 ± 6.7 [b]	31 ± 2.7 [b]	28.7± 5.4 [c]	33± 3.2 [b]
LDL-Cholesterol (mg/dl)	26.8 ± 2.5 [a]	67.6 ± 6.7 [b]	41 ± 2.7 [c]	90.7± 5.4 [d]	65.0± 5.2 [b]

Values sharing the same superscript means not significant. P> 0.05. Values are means ± standard error of mean (SEM). Different superscript letters within the row indicate significant differences by Duncan s multiple range test p< 0.05.

Table (3) Changes in plasma levels of TNF-α , IL-Iß, 5-LOX, 15-LOX and LA-4 in sham (SH), ovariectomized (OVX) and OVX diabetic (OVX-D) rats before and after treatment with *myrtle communis*

	SH (n=10)	OVX (n=20)		OVX-D (n=20)	
		Before treatment	After treatment	Before treatment	After treatment
TNF-α (ng/L)	45.2 ± 2.5 [a]	170.0 ±11.0 [b]	162±12.3 [c]	255.0± 39.0 [d]	189.0± 12.1 [e]
IL1B (Pg/ml)	28.89 ± 1.20 [a]	45.41±1.71 [b]	36.83±1.9 [c]	48.16 ±1.17 [b]	39.30±1.05 [c]
5-LOX (U/L)	6.5 ± 1.36 [a]	14.3 ±1.15 [b]	8.4 ± 1.3 [c]	20.0 ± 1.3 [d]	10.9 ± 1.5 [e]
15-LOX (U/L)	11.4 ± 0.86 [a]	9.82±1.48 [b]	10.6 ± 1.0 [b]	11.1 ± 0.59 [a]	14.6 ± 2.5 [d]
LA-4 (Pg/L)	78.6 ± 12.5 [a]	94.0 ± 7.4 [b]	132 ±18.2 [c]	153.0 ± 13.1 [d]	181.0 ± 9.3 [c]

Values sharing the same superscript means not significant. P> 0.05. Values are means ± standard error of mean (SEM). Different superscript letters within the row indicate significant differences by Duncan s multiple range test p< 0.05 .

RESULTS AND DISCUSSION

The most inner surface of all blood vessels is lined with endothelial cells (EC) which are the cells that are in a direct contact with the blood stream. Endothelial dysfunction as well as atherosclerosis are almost among the common problems associated with diabetes and menopause [20]. A non-heme iron-containing dioxygenases called Lipoxygenases (LOXs) catalyze the process of dioxygenation of polyunsaturated fatty acids, which contain at least two isolated cis-double bonds. The LOX pathway's primary products are converted subsequently into a large number of bioactive lipid mediators including pro-inflammatory mediators one of them is the leukotrienes (LTs) which is synthesized by arachidonic liopxygenase-5 (ALOX5) [24] and also anti-inflammatory mediators such as lipoxins (LA4, LB4) [25]. Inflammation in diabetes is considered as a major contributor to β-cell, endothelial cell dysfunction as well as apoptosis specially in type 2 diabetes mellitus (T2DM) [26] IL-1β caused induction of β -cell apoptosis in the pancreas and death in type 1 and type 2 diabetes mellitus [27][28][29[(Schumann et al., 2007, Sumpter et al., 2011and Liu et al., 2012).

During menopause, inflammation may occur and this can be one of the causes of developing insulin resistance and type 2 diabetes [30]Treatment targeting replacement of estrogen can improve glucose intolerance and insulin resistance in post-menopausal women and therefore the whole body metabolism will be improved [31], and this proves that estrogen plays a very important role in glucose homeostasis.

The hormone replacement therapy is also very effective in improving the complications associated with menopause. However, increased risk for endometrial cancer and breast cancer can be a problem in case of prolonged use [32] Administration of non-steroidal anti-inflammatory drugs is associated with gastrointestinal side effect as well as severe cardiovascular adverse reactions. Therefore, development of new drugs of natural origin and of less side effects are urgently needed to be as an effective alternative for the drugs used nowadays. In this regard, extract from myrtle (*Myrtuscommunis*, myrtle, MC) has been reported to suppress the biosynthesis of eicosanoids by inhibition of 5-lipoxygenase and cyclooxygenase-1 in vitro.

In the current study blood glucose level was significantly decreased in the treated groups with myrtle extract in OVX and OVX diabetic groups (Table 1). Myrtle leaves are rich in turpenoids, alkaloids and flavonoids, which are biologically active compounds of known antidiabetic activity. The mechanism of action of these compounds can be either glycogenesis inhibition or glycolysis stimulation and also can act by stimulating the release of insulin and inhibiting the intestinal absorption of glucose. Another possible mode of action of these compounds is that they cause obstruction of ATP-dependent potassium channels in pancreatic beta cells and increase intracellular calcium by cell membrane voltage reduction and this in turn triggers the release of insulin and decrease glucose concentration. Significant reduction in glucose concentration was observed in treated groups [33]**.** Also [34]Sepici et al., (2004) showed that inhibition of small intestinal α-glucosidases was caused by myrtle extract leading to reduction of glucose intestinal absorption and delaying its release from complex carbohydrates.

Estrogen deficiency just like diabetes adversely affects the endothelial functions **[35].** This hormone is reduced normally in case of aging (menopause) or in women whose ovaries have been removed. Lately great concern has been given to management of menopausal patients in terms of epidemiology as well as public health and for medical research reasons. And this greatly improves life expectancy which allows the prediction that women may live a third of their lifetimes after menopause [36].

A decrease in both triglycerides and cholesterol levels was seen in the treated groups with myrtle extract in OVX and OVX diabetic groups and this decrease was statistically significant (Table 2). [37] Raffeq et al., (2014) proved that the aqueous extract of myrtle communis L-fruit has hypo-lipidemic activity when given to rabbits fed with cholesterol. The same study found that the dose of 50 mg/Kg of Myrtle extract is effective as hypolipidemic and also has an effect on parameters of blood coagulation. Another study done by [38] Ahmet, (2004) showed that myrtle communis L-berries contains compounds that have insulin like action and may be of similar structure to insulin and also they have a significantly decreasing effect on total lipids, cholesterol and glycogen content of liver tissues. And this can be explained by the fact that they cause inhibition of the regulatory enzyme β-hydroxy β-methyl glutaryl CoA reductase the one responsible for biosynthesis of cholesterol.

OVX and OVX diabetic groups showed progressive decrease in HDL level (Table 2) indicating abnormal fat metabolism which significantly improved after treatment with myrtle extract in OVX diabetic group.

The present study revealed a significant increase in the activity of 5-LOX enzyme in the OVX and OVX diabetic rats as compared to the sham group (Table 3).Our results are in agreement with[39][40].

The elevation of 5-LOX in all groups is significantly reversed after treatment with myrtle extract. *Myrtus communis*(MC) was found to cause suppression of the eicosanoids biosynthesis by directly inhibiting cyclooxygenase-1 (COX-1) and 5-lipoxygenase (5-LOX) both in vitro and in vivo. This ability to cause suppression of typical pro-inflammatory cellular responses made this plant of great importance and of useful therapeutic effect in treatment of any disease related to allergy and inflammation[41].

Indeed, like fish oil, the present study also showed that myrtle extract can down-regulate the activity of 5LOX in all rats either OVX or OVX-diabetic as compared to its activity before treatment (Table 3).Our results are in agreement with [41][42]Feisst et al., (2005) and Rossi et al., (2009). The suggested mechanism of action of MC in decreasing inflammation is the reduction of polymorphonuclear leukocyte (PMN) infiltration by down-regulating the adhesion molecules [42]. Other authors also suggested that MC may act by inhibiting generation of Leukotriene B4 (LTB4) in mice with tissue inflammation. In this regard[41],Feisst et al. (2005), showed that MC is a potential inhibitor of LT formation by interference with 5LOX metabolism. These findings proved that MC is considered as a potent anti-inflammatory agent due to its inhibitory effect on 5-LOX and other proinflammatory leukocyte functions.

Chronic inflammatory diseases such as diabetes, inflammatory bowel syndrome and rheumatoid arthritis are associated by different pathological responses which are found to be related to the high levels of TNF-α and IL-1β [43]**[44].** In agreement with Ana and Kathryn (2005) who found that circulating levels of the inflammatory cytokine TNF-α were 7- fold greater in ovariectomized rats, and Kiree et al., who suggested that pro-inflammatory cytokines TNF-α, 1L-β and IL-6 detected in liver homogenates were increased significantly in ovariectomized rats, our results (Table3) showed that, OVX and OVX-diabetic rats having a significant increment in the plasma level of IL-1β and TNF-α as compared to the sham group. Also administration of myrtle plant caused a significant reduction in the plasma level of TNF-α and IL-1β.

As anti-inflammatory, the extract of myrtus caused a reduction in plasma IL-1β, which may be due to reduction of leuckocyte migration to the damaged tissue caused by the anti-inflammatory activity of the essential oil of *Myrtus communis*.

Studies showed that there is a relation between endothelial dysfunction and reduced endogenous production of estrogens in cases where there is natural or surgical menopause and in women with premature ovarian failure (POF) [20][45].

In the present study, there is significant increase in the levels of LA4, 15-LOX after myrtle extract administration in each of OVX and OVX-diabetic rats (Table 3). Our results are in agreement with [35]**.**

Nowadays great concern is being given to drugs from plant origin which are a safer alternative to chemical drugs, and extracts from medicinal plants such as *myrtus communis L*. (common myrtle) have shown to be very useful **[11]** it contains anti-inflammatory and anti-hyperglycemic phenolic compounds and this can decrease the side effects caused by synthetic drugs usually used for treatment of undesirable effects of diabetes or menopause. Therefore, the present study aims to evaluate the beneficial effects of these natural products on the OVX and OVX-diabetic rats.
From the previous results we can conclude that myrtle extract can alleviate the endothelial dysfunction and have the ability to reduce significantly 5- lipoxygenase activity during menopause or even in menopause associated with diabetes.

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