New approaches in protecting against atherosclerosis in experimental model of postmenopause

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ABSTRACT

This work is aimed to evaluate the ability of Myrtus communis leaves extract in attenuating endothelial dysfunction as well as risk of atherosclerosis in ovariectomized rats as a common model of postmenopause. Total 60 female albino rats, weighing 180 g were used and assigned to four groups (sham, sham and Myrtus communis leaves extract, ovariectomized rats (OVX) and ovariectomized treated rats with Myrtus communis leaves extract). Plasma estrogen, lipid profile, asymmetric dimethylarginine (ADMA), von Willebrand factor (vWF), interleukin 1beta (IL-1β), Lipoxin A4 (LXA4), aortic oxidant and antioxidant in addition to erythrocyte membrane fatty acids were determined. OVX rats showed a significant increase in inflammatory and oxidant parameters while, Myrtus communis extract administration (100 mg/kg body weight) for two months attenuates these values in treated group. Myrtus communis leaves extract confirmed our idea in protecting from atherosclerosis and endothelial dysfunction in ovariectomized rats due to its high content of anti-oxidant and anti-inflammatory compounds in addition to its high content of ω-3 fatty acids.

INTRODUCTION

Atherosclerosis and its complications are a great global challenge. Coronary artery diseases are the main cause of mortality worldwide. Myocardial infarction or stroke and atherosclerosis are lethal consequences that cause morbidity which related to heart failure resulting from ischemic heart disease or neurological impairment due to stroke (Go et al., 2014). Postmenopausal women are more susceptible to cardiovascular disease than in age-matched premenopausal women. Estrogen vasoprotective actions include activation of nitric oxide production and decreased in vascular smooth muscle cell proliferation while menopause are associated with atherogenic risk factors such as hyperlipidemia, hypertension, obesity, and insulin resistance (Akishita, 2004).

Inflammation is the main key player in atherosclerosis from the formation of lesions to the incidence of a coronary take place. Chronic vascular inflammatory mechanism is related to the endothelial ability to release pro-inflammatory cytokines, such as Interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) that produced by the endothelium, stimulating adhesion molecules and increasing vascular risk (Teixeira et al., 2014).

Nitric oxide (NO) controls the proliferation of vascular smooth muscle cell and reduced the expression of endothelial leukocyte adhesion molecules and the deposition of lipids. Endothelial dysfunction appears to inhibit the biological activity of NO leading to increases in the expression of pro-thrombotic factors, pro-inflammatory adhesion molecules, chemotactic factors, cytokines that stimulate the expression of monocyte chemotactic protein-1 (MCP-1), which recruits monocytes and other chronic inflammatory cells. Monocytes and macrophages were important factors in the development of atherosclerotic lesions (Demir et al., 2012).
Several studies used food derived anti-oxidant and anti-inflammatory properties as chemopreventive agents in many diseases including diabetes (Hussein et al., 2012), osteoporosis (El-Khayat et al., 2010) and liver injury (Hussein et al., 2016).

Myrtus communis is a recognized medicinal plant and traditionally applied for treatment of urethritis, diarrhea, peptic ulcers, hemorrhoids, inflammation, bleeding, headache, palpitation, leucorrhoea, epistaxis, conjunctivitis, excessive perspiration, pulmonary and skin diseases (Jabri et al., 2016). Myrtus communis essential oil and all extracts from its leaves, branches, fruits and flowers are rich in terpenoid compounds including 1,8- cineole, α-pinene, myrtenyl acetate, limonene, linalool and α-terpinolene (Wannes et al., 2010; Berka-Zougali et al., 2012). The leaves also rich in tannins and flavonoids (Akin et al., 2010).

Also Wannes and Marzouk (2016), reported that the fatty acid content of Myrtus communis oil offers the lipids as a valid source of polyunsaturated fatty acids (PUFA) and clarified the usefulness of PUFA in ameliorating heart diseases, inflammation, autoimmune disorders, atherosclerosis, diabetes, and other diseases.

AIM OF THE WORK

This study aimed to evaluate the ability of the natural supplement of plant origin (Myrtus communis leaves extract) to attenuate endothelial dysfunction as well as atherosclerosis complications in ovariectomized rats as a suitable model for postmenopausal women.

MATERIALS AND METHODS

Materials

Chemicals

Fatty acids standards (HPLC grade), Sigma-Aldrich (St. Louis, MO, USA). Chloroform, methanol and ethyl ether all are HPLC grade and were purchased from (ALDRICH, Germany).

Experimental animals

Sixty female albino rats, weighing 180 ± 20 g from Animal House, National Research Centre (NRC), Giza, Egypt. Rats were individually kept in clean cages of polypropylene and maintained in controlled room temperature with light and dark cycle, given a standard diet and water ad libitum along the experimental period. The experiment was carried out in accordance with guidelines and protocol approved by the Institutional Animal Ethics Committee of National Research Centre (NRC), Giza, Egypt.

Methods

Myrtus communis alcoholic extract

Myrtus communis leaves were collected, identified, air dried, powdered and extracted at 28±2°C by the method described by Messaoud et al. (2012).

Ovariectomy Surgical Procedure

The ovariectomy and sham operations were done according to the method described by Goseki et al. (1995).

Experimental design

Rats were assigned to four groups (15 rats each) as follow:

Group (I): Sham group: Healthy rats were sham-operated and received a vehicle.

Group (II): Sham + Myrtus communis group: sham rats receive alcoholic extract of Myrtus communis in a dose of 100 mg/kg, b.w. daily for two months by stomach tube.

Group (III): OVX group: Rats were surgically ovariectomized and receive a vehicle.

Group (IV): Treated group: OVX rats received alcoholic extract of Myrtus communis in a dose of 100 mg/kg daily for two months by stomach tube.

After the end of experimental period (two months), animals were kept fasting overnight (12 hour). Under anesthetic blood withdrawn in heparinized tubes; centrifuged at 3000 rpm using cooling centrifuge (Laborzentrifugen, 2K15, and Sigma, Germany). Plasma was separated and stored at -20 °C for biochemical analysis. Packed RBCs were used for determination of cell membrane parameters. Aorta from each rat was removed quickly; washed with ice-cold saline and used for determination of oxidant and antioxidant parameters.

Determination of lipid profile

Cholesterol and high density lipoprotein (HDL) were measured according to Allain et al. (1974). Triglycerides was measured according to Glick et al. (1986). Low density lipoprotein (LDL) was calculated from the equation mentioned by Ahmadi et al. (2008).

Determination of aortic nitric oxide (NO)

Aortic NO level was estimated according to Mosnaj et al. (1995).

Determination of aortic superoxide dismutase (SOD); catalase (CAT) and malondialdehyde (MDA)

Aortic SOD and CAT activities were determined according to the method described previously (Nishikimi et al., 1972 & Johansson and Bory, 1988) respectively and MDA was determined colorimetrically as described before (Uchiyamara et al., 1978).

Determination of total protein

Total protein in tissue was estimated using BICON Kit diagnostic, Germany according to Passing and Balok (1993).
**Plasma estrogen, asymmetric dimethylarginine (ADMA), interleukin 1 beta (IL-1β), Lipoxin A4 and von Willebrand factor (vWF)**

We determined vWF concentrations. The concentrations in samples were obtained from samples, and their peak areas were estimated by HPLC. Different concentrations of each standard were estimated by HPLC chromatography (HPLC) according to El-Khayat et al. (2013), Briefly, cell membrane was homogenized in 2% acetic acid / ethyl ether mixture (2:1 volume ratio). The solution mixed well using vortex and centrifuged at 3000 rpm, the organic phase was evaporated to dryness; the residue was dissolved in 500 μl acetonitrile and filtered by PVDF 0.45 μm filter before injection onto HPLC. Erythrocyte membrane fatty acids (FA) were fractionated and analyzed by high performance liquid chromatography (HPLC) according to El-Khayat et al. (2013), Deanfield et al. (2007), David et al. (2007), Deanfield et al. (2000) respectively.

**Erythrocyte membrane fatty acids**

Erythrocyte membrane fatty acids (FA) were fractionated and analyzed by high performance liquid chromatography (HPLC) according to El-Khayat et al. (2013), Briefly, cell membrane was homogenized in 2% acetic acid / ethyl ether mixture (2:1 volume ratio). The solution mixed well using vortex and centrifuged at 3000 rpm, the organic phase was evaporated to dryness; the residue was dissolved in 500 μl acetonitrile and filtered by PVDF 0.45 μm filter before injection onto HPLC.

**HPLC Condition**

Fatty acids were determined using reversed phase HPLC, column ODS (250 X 4.6 X particle size 5 μl ) and mobile phase acetonitrile / water mixture (70/30) v/v by isocratic elution with flow rate 1 ml / min. UV detector adjusted at 200 nm wave length. Different concentrations of each standard were estimated by HPLC and their peak areas were determined. Standard curve was constructed by plotting peak areas versus the corresponding concentrations. The concentrations in samples were obtained from the standard curve.

### Statistical Analysis

All data were expressed as mean ± SE. Statistical significance was tested by one way analysis of variance (ANOVA).

### RESULTS

Results of this study revealed that cholesterol and triglycerides levels were significantly increased in OVX group compared to SH group; whereas the treatment by *Myrtus communis* significantly decreased these parameters in treated group compared to the OVX group. On the other hand, plasma HDL-cholesterol was significantly decreased in OVX group compared to SH while this value was significantly increased in treated group compared to OVX group (table :1).

Ovariectomized rats showed a significant decrease in aortic NO, SOD and CAT and a significant increase in MDA level compared to SH group while in treated group there was a significant increase in aortic SOD and CAT accompanied by a significant decrease in MDA level compared to OVX group (table : 2).

Our results indicated that in OVX group there was a significant decrease in plasma estrogen and LA-4 levels comonitmit with a significant increase in plasma ADMA, vWF and IL-1β levels. However *Myrtus communis* supplementation significantly increased plasma estrogen and LA-4 and decreased levels of ADMA, vWF and IL-1β in treated group compared to OVX group (table:3).

### Table 1: Plasma lipid profile in different studied groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SH</th>
<th>SH + <em>Myrtus communis</em></th>
<th>OVX</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mg/dl) Mean±SE</td>
<td>110 ± 0.4</td>
<td>93.2 ± 0.6</td>
<td>125.8 ± 4.0</td>
<td>50.6± 0.3</td>
</tr>
<tr>
<td>Triglyceride (mg/dl) Mean±SE</td>
<td>95.1 ± 1.4</td>
<td>71.7* ± 0.60</td>
<td>89.2* ± 1.0</td>
<td>60.1± 0.5</td>
</tr>
<tr>
<td>HDL-Cholesterol (mg/dl) Mean±SE</td>
<td>78.8 ± 9.5</td>
<td>74.6 ± 7.2</td>
<td>21.6* ± 6.7</td>
<td>34.6± 2.7</td>
</tr>
<tr>
<td>LDL-Cholesterol (mg/dl) Mean±SE</td>
<td>24.8±1.3</td>
<td>20.1±0.45</td>
<td>86.4*±1.0</td>
<td>36.4±0.98</td>
</tr>
</tbody>
</table>

Significant value ≤0.05
P< value compared to SH group, P> value compared to ovariectomized group.

### Table 2: Aortic Nitric oxide (NO), Superoxide dismutase (SOD), Catalase (CAT) and Malondialdehyde (MDA) in different studied groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SH</th>
<th>SH + <em>Myrtus communis</em></th>
<th>OVX</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO level (µmol/gm tissue) Mean±SE</td>
<td>40.6 ± 5.9</td>
<td>41.1 ± 2.16</td>
<td>22.3* ± 2.1</td>
<td>30.5± 1.87</td>
</tr>
<tr>
<td>SOD activity (U/mg protein) Mean±SE</td>
<td>11.1 ± 0.6</td>
<td>12.1 ± 0.8</td>
<td>7.6* ± 2.1</td>
<td>16.7± 0.2</td>
</tr>
<tr>
<td>CAT activity (U/mg protein) Mean±SE</td>
<td>6.8 ± 0.67</td>
<td>6.2 ± 0.1</td>
<td>4.1*±0.6</td>
<td>11.1±0.22</td>
</tr>
<tr>
<td>MDA level (µmol/mg protein) Mean±SE</td>
<td>0.31±0.03</td>
<td>0.29 ± 0.01</td>
<td>0.63*±0.05</td>
<td>0.5±0.06</td>
</tr>
</tbody>
</table>

Significant value ≤0.05
P< value compared to SH group, P> value compared to Ovariectomized group.

### Table 3: Plasma estrogen, ADMA, vWF, IL-1β and lipoxin-A4 levels in different studied groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SH</th>
<th>SH + <em>Myrtus communis</em></th>
<th>OVX</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen (Pg/ml) Mean±SE</td>
<td>40 ± 1.87</td>
<td>42.4 ± 2.6</td>
<td>10.5±2.3</td>
<td>19.2±3.5</td>
</tr>
<tr>
<td>ADMA (µmol/L) Mean±SE</td>
<td>3.36 ± 0.4</td>
<td>2.86 ± 0.7</td>
<td>4.2*±0.8</td>
<td>3.0±0.85</td>
</tr>
<tr>
<td>vWF (ng/L) Mean±SE</td>
<td>534.0 ± 62.0</td>
<td>510.0 ± 40.0</td>
<td>753*±30.3</td>
<td>610.0±40.0</td>
</tr>
<tr>
<td>IL-1β (Pg/ml) Mean±SE</td>
<td>25.7 ± 1.2</td>
<td>27.1±1.6</td>
<td>48.9*±1.7</td>
<td>39*±1.0</td>
</tr>
<tr>
<td>LA-4 (Pg/L) Mean±SE</td>
<td>88 ± 2.0</td>
<td>90 ± 2.3</td>
<td>64*±2.1</td>
<td>129*±4.7</td>
</tr>
</tbody>
</table>

Significant value ≤0.05
P< value compared to SH group, P> value compared to ovariectomized group.
Results of this study indicated that there was a significant increase in erythrocyte membrane arachidonic acid (AA) and linoleic acid (LA) along with a significant decrease in α-linolenic acid (ALA) in OVX group compared to SH group while administration of myrtle significantly attenuated these levels in treated group compared to OVX group (table 4).

### DISCUSSION

Menopause is a significant risk factor for cardiovascular diseases (CD) that were considered one of the main reasons of mortality and morbidity in women (Demir et al., 2012). Estrogen plays an important role in heart protective mechanism, thus it reduces LDL level in addition to its antioxidant properties in lipids oxidative degradation (Subbiah, 2002). Oxidation of LDL in the vessel wall appears to be the first step in lipid deposition on the arterial wall (Østerud and Bjørklid, 2003). Oxidized LDL (oLDL) is a chemotactic agent that increases the flow of monocytes and macrophages into the endothelium which rapidly evolve into lipid laden foam cells of an atheromatous plaque (Singh et al., 2002).

In this study, it was observed that OVX rats showed a significant decrease in plasma estrogen level accompanied by significant increase in plasma cholesterol, triglycerides and LDL levels, in addition to a significant decrease in HDL-cholesterol compared to sham group. Also Yung et al. (2011) demonstrated that increase cardiovascular hazard is associated with estrogen insufficiency that characterized by atherogenic modulation of plasma lipid profile, stimulation of the renin-angiotensin system and excess production of reactive oxygen species (ROS) which affects nitric oxide (NO) as was found in the current study. Also, Rossouw et al. (2002) suggested that increased levels of total cholesterol and LDL were associated with atherosclerosis lesions in human and animals. Estrogen affects lipoprotein and triglyceride profiles in such a way that indirectly influences atherosclerosis as well as more direct affects the vascular endothelium through estrogen receptors. The most important effect of estrogen was through estrogen receptor alpha (ER-α) which stimulates endothelial nitric oxide synthase and promote endothelium-dependent relaxation (Mitchell et al., 2005).

Our results indicated that there was a significant increase in ADMA level accompanied by a significant decrease in aortic nitric oxide in OVX group compared to the sham group indicates endothelial dysfunction. Several studies suggested a positive correlation between ADMA and cholesterol levels. ADMA exhibited a positive association with LDL cholesterol; LDL oxidation in the vascular wall may be responsible for the effect of this lipoprotein on ADMA elevation, through inhibition of dimethylarginine dimethylaminohydrolases (DDAH) activity (Landim et al., 2009). Kitova et al. (2007) demonstrated that endogenous nitric oxide synthetase (eNOS) activation decreased in hypercholesterolemic patients, resulting in alteration of endothelium-dependent vasodilation and decreased adhesion of platelets and monocytes, these events were related to the elevation of ADMA levels, which was a competitive inhibitor of eNOS, thus LDL cholesterol increases the expression of ADMA precursor protein and also inhibits the activity of DDAH enzyme, which degrades ADMA. In addition, activation of eNOS is controlled by estrogen via modulation of the eNOS/caveolin-1 (Cav-1) complex formation while in menopause, which was accompanied by loss of estrogen and inactivation of estrogen receptors, it correlates with a reduction in eNOS expression (Loyer et al., 2007).

Elevation of vWF level which secreted from endothelial cells and released into the circulation plays functional roles in homeostasis through mediating platelet adhesion to the vascular wall and appeared to be strongly associated with major cardiac events (Kucharska-Newton et al., 2009). It was associated with the increase in LDL and atherogenesis (van Galen et al., 2012). Our results confirmed these suggestions, we found elevation in LDL and vWF levels in OVX group. Our data revealed that in ovariectomized rats there was a significant decrease in erythrocyte membrane ALA along with increase in AA and LA compared to sham group. Alterations in the composition of erythrocyte membrane fatty acid, specifically ω-3, were a significant indication of increased cardiovascular risk. In agreement, Harris et al. (2007) indicated that, low levels of plasma n-3 PUFA is strongly correlated to vascular inflammation, endothelial activation and increase of coronary heart disease in postmenopausal women. Recently, Hussein et al. (2016b) suggested that increasing n-3 fatty acids in ovariectomized rats decrease both production and releasing of pro-inflammatory cytokines such as IL-1β as was found in our study. Contrarily, n-6 fatty acids have the opposite effect.

In the current study, a significant increase in IL-1β along with a significant decrease was observed in LXA4 in OVX group compared to the sham group. Concomitant with our results Hussein et al. (2016b) indicated that experimental OVX was followed by osteoporotic features participating in activation of osteoblastic causing release of pro-inflammatory cytokines like IL-6, IL-1 and TNF-α. The correlation between estrogen and inflammatory cytokines was demonstrated by Compston (2001). Estrogen decreases bone resorption directly via inhibiting

### Table 4: Erythrocyte membrane fatty acids in different studied groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>SH</th>
<th>SH + Myrtus communis</th>
<th>OVX</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA (mg/ml RBCs) Mean±SE</td>
<td>0.05±0.004</td>
<td>0.05±0.008</td>
<td>0.19±0.001</td>
<td>0.06±0.001</td>
<td></td>
</tr>
<tr>
<td>LA (mg/ml RBCs) Mean±SE</td>
<td>0.73±0.03</td>
<td>0.68±0.03</td>
<td>0.85±0.04</td>
<td>0.73±0.02</td>
<td></td>
</tr>
<tr>
<td>ALA (mg/ml RBCs) Mean±SE</td>
<td>0.48±0.008</td>
<td>0.68±0.015</td>
<td>0.49±0.03</td>
<td>0.69±0.02</td>
<td></td>
</tr>
</tbody>
</table>

Significant value ≤ 0.05.

P<sup>a</sup> value compared to SH group, P<sup>b</sup> value compared to ovariectomized group.
osteoclasts and indirectly via suppressing osteoplastic production of various proresorptive paracrine factors such as IL-1β, IL-6, and TNF-α. In addition estrogen reduces the degree of inflammation and tissue damage by decreasing the expression of the inflammatory markers and inhibiting their reaction (Faloni et al., 2007). Lipoxins (LX), the products of arachidonic acid metabolism, were produced through sequential lipoygenase activity. LX4 affects endothelial cells by the stimulation of cytoprotective pathways (Fiorucci et al., 2003). It upregulates the expression of heme-oxygenase 1 (HO-1), which is responsible for the inhibition of cellular activation, including decreased expression of adhesion molecules (Nascimento-Silva et al., 2005). Also LXA4 inhibits pro-inflammatory cytokine and reactive oxygen species (ROS) generation in addition to suppression of NAD (P) H oxidase-mediated ROS generation in endothelial cells (Gozzelino et al., 2010). It was reported that, inflammatory diseases were characterized by neutrophil infiltration, release of proinflammatory cytokines such as TNF-α and IL-1β and bioactive mediators e.g., leukotrienes (LTs) and prostaglandins, and expression of adhesion molecules [intracellular adhesion molecule (ICAM)-1 and P-selectin]; these mediators are involved in tissue damage (Fiátkow et al., 2007). In addition, the increase in concentration of intracellular Ca²⁺ leads to a production of proteases (e.g., leukocyte elastase or cathepsin G) and formation of ROS (Parekh and Penner, 1997), which destroy invading particles and damage cells and tissues of the host. Moreover, ROS generation induced by intracellular Ca²⁺ elevation caused lipid peroxidation and DNA single-strand damage (Pérez-De La Cruz et al., 2008). Concomitant with our results, Muthusami et al. (2005) indicated the increase of LPO and H₂O₂ levels and decrease in antioxidants enzymes such as SOD, GPx and GST in OVX rats compared to sham-operated control group. Endothelial dysfunction results in increasing the pro-inflammatory and prothrombotic phenotype of the endothelium and thus induce attachment and subsequent migration of leukocytes, events that were associated with the commencement of the formation of atherosclerotic lesion (Landmesser et al., 2004). Taken together, these findings suggest that ovariectomy caused systemic inflammation and increasing in free radicals that is related to the progression of atherosclerotic lesion.

In this study, administration of Myrtus communis extract significantly attenuated inflammation and oxidative stress. Myrtus communis is a vital aromatic species with medicinal benefits from the Myrtaceae family and was traditionally used in several diseases due to its antioxidant and anti-inflammatory properties (Aleksic et al., 2013). The antioxidant function of Myrtus communis was due the active compounds in its essential oil and phenolic fraction that contribute to the overall antioxidant and anti-mutagenic potential. The ROS scavenger activity and inhibition of the expression adhesion molecules expression of Myrtus communis was mainly related to the interaction with intracellular Ca signaling. In addition, it had a direct effect on oxidative enzymes that induced the antioxidant enzyme activities, reduced lipid peroxidation and scavenger free radicals (Rosa et al., 2008 & El-Bana et al., 2017). Myrtus communis inhibits the synthesis of eicosanoids through suppression of 5-lipoxygenase, cyclooxygenase-1and inhibition of elastase and ROS production via inactivation of receptor-coupled camobilization (Feisst et al., 2005). Sepici et al. (2004) reported that plant extract rich in polyunsaturated fatty acids (PUFA) such as myrtle had an important role in relieving cardiovascular diseases, inflammation, atherosclerosis and other diseases. It was postulated that n-3 PUFAs hypotriglyceremic properties including, decrease synthesis of TG and saturated fatty acids through the reduction of hepatic sterol regulatory element-binding protein 1, acetyl-CoA carboxylase 1, fatty acid synthase, and diacylglycerol acyltransferase 2. In agreement, our results indicated the attenuation of fatty acids disturbance in ovariectomized rats by supplementation of Myrtus communis extract. Increasing danger of cardiac events was negatively correlated with blood and tissue content of ω-3 fatty acids (Harris et al., 2007). Beneficial roles of PUFA include enhanced vasodilatation through keeping the balance between vasoconstrictor and vasodilator endothelium-derived factors, activation and of cyclooxygenase-2 expression, inhibition of nuclear factor kappa B, reduction of expression of endothelial adhesion molecules, suppression of leukocyte adhesion to endothelial cells (Kris-Etherton et al., 2003) and modulation of oxidative stress (Harris et al., 2007).

CONCLUSION

We concluded that Myrtus communis extract could be a promising source of natural anti-oxidants, anti-inflammatory and omega-3 fatty acids that attenuates the risk of endothelial dysfunction and atherosclerosis in an experimental model of postmenopausal women.

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