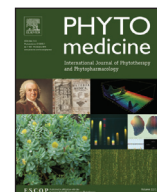




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“*Pistacia lentiscus* L.” reduces the infarct size in normal fed anesthetized rabbits and possess antiatheromatic and hypolipidemic activity in cholesterol fed rabbits

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ARTICLE INFO

Article history:

Received 11 December 2015

Revised 23 May 2016

Accepted 3 June 2016

Available online xxx

Keywords:

Pistacia lentiscus

Mastic gum

Myocardial infarction

Atheromatosis

Lipid peroxidation

ABSTRACT

Hypothesis/Purpose: The aim of the present study was to evaluate *in vivo* the potential anti-ischemic and antiatheromatic activity of Chios Mastic gum, the resin of the trunk and branches of “*Pistacia lentiscus* var. *chia*”, used since antiquity in traditional Greek medicine. The main compounds of mastic are triterpenes, possessing phytosterol-like structures. This led to the hypothesis that mastic and particularly its neutral fraction, enriched in phytosterol-like compounds, possess antiatheromatic activities.

Methods: Total Mastic Extract without Polymer (TMEWP) and the neutral mastic fraction (NMF) were administered orally for 6 weeks to normal fed and to cholesterol fed rabbits in the form of sunflower oil solution. All the animals were randomly divided into 6 groups, anesthetized and subjected to 30 min ischemia of the heart, followed by 3 h reperfusion: At the end of the experiment the area at risk and the infarct zone were determined with the aid of fluorescent particles and triphenyl tetrazolium chloride staining, and small segments of the ascending and descending aorta and the heart were taken for histologic examination. Blood samples were collected at different time points of ischemia and reperfusion, for malondialdehyde (MDA) evaluation as an index of lipid peroxidation, for total and LDL cholesterol determination and for evaluation of oxidized LDL.

Results: In the normal fed animals the NMF and the TMEWP reduced significantly the infarct size, while in the hypercholesterolemic rabbits both treatments were ineffective. Atherosclerosis was detected in all the animals fed cholesterol enriched diet in the form of subintimal accumulation of lipids and foamy macrophages. There was no detection of atherosclerosis in Groups treated with TMEWP and NMF, which both reduced the total cholesterol levels by 47 and 88% respectively, whilst had not effect on LDL oxidation. TMEWP and NMF reduced the MDA concentration in normal fed rabbits, but had no effect on MDA levels in cholesterol fed animals. TMEWP and NMPF reduce the infarct size in normal animals and possess significant antiatheromatic and hypolipidemic activities in rabbits fed cholesterol enriched diet.

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Introduction

Acute myocardial infarction (AMI) is one of the leading causes of human death worldwide (Li et al. 2012). The

sure liquid chromatography-atmospheric pressure chemical ionization-high resolution mass spectrometry; VCAM-1, Vascular cell adhesion molecule 1.

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Abbreviations: AMI, Acute myocardial infarction; CVD, Cardiovascular disease; CMGGA, Chios mastic gum growers' association; GC–MS, Gas chromatography–mass spectrometry; HMPC, Committee on herbal medicinal products; EMA, European medicines agency; HDL, High density lipoprotein; ICAM-1, Intercellular adhesion molecule 1; iPC, Ischemic preconditioning; LDL, Low density lipoprotein; MDA, Malondialdehyde; TMEWP, Total mastic extract without polymer; NMF, Neutral mastic fraction; oxLDL, Oxidised low density lipoprotein; PBMC, peripheral blood mononuclear cells; TNF- α , Tumor necrosis factor α ; UHPLC-APCI-HRMS, Ultra-high pres-

<http://dx.doi.org/10.1016/j.phymed.2016.06.002>

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Please cite this article as: I. Andreadou et al., “*Pistacia lentiscus* L.” reduces the infarct size in normal fed anesthetized rabbits and possess antiatheromatic and hypolipidemic activity in cholesterol fed rabbits, *Phytomedicine* (2016), <http://dx.doi.org/10.1016/j.phymed.2016.06.002>

continually increasing number of patients around the world suffering from cardiovascular disease (CVD) indicates the need of innovative strategies for more effective CVD prevention and treatment. Multiple clinical studies have focused on the impact of diet and functional foods specifically in CVD (Sofi et al. 2010). It is considered that the intake of certain functional food, rich in ω -3 polyunsaturated fatty acids, is capable of protecting the myocardial tissue against ischemia-reperfusion injury (Abdukeyum et al. 2008).

Chios Mastic gum is the resin of "*Pistacia lentiscus* var. *chia*", acquired through the incisions made on the trunk and branches of the shrub. Commonly known as mastic, it is uniquely produced in the southern part of the Greek Aegean island of Chios, probably because of the exceptional combination of climate and soil conditions found there. Mastic has been used since antiquity in traditional Greek medicine mainly to treat gastrointestinal disorders, but also for its anti-inflammatory, emollient and astringent activities. The healing properties of the gum are mentioned by many medical writers of the classic era (Galenus, Dioscorides). During the last decades, many researchers have studied the anti-ulcer activity of mastic (Al-Said et al. 1986; Heo et al. 2006), as well as its ability to kill *Helicobacter pylori* (Huwez et al. 1998; Marone et al. 2001) and to diminish its colonization grade in the stomach (Paraschos et al. 2007). Today, mastic is used in the production of natural chewing gum by the Chios Mastic Gum Growers' Association (CMGGA), and for dental and culinary purposes. In August 2015 the Committee on herbal medicinal products (HMPC) to the European Medicines Agency (EMA) recognized mastic of Chios as a medicinal product within the EU and EEA countries and classified it to the category of traditional herbal medicines in two therapeutic indications: dyspeptic problems, skin inflammation and wound healing (EMA/HMPC/46.756/2015 Herbal Medicinal Products Committee. Assessment report on "*Pistacia lentiscus* L.", resin) (mastic)].

Additionally to the above, it has been shown that mastic exhibited beneficial activity against low density lipoprotein (LDL) oxidation in peripheral blood mononuclear cells (Dedoussis et al. 2004). Furthermore, it showed hypolipidemic activity in human volunteers (Triantafyllou et al. 2007), by exhibiting a decrease in a series of serum cardiologic and hepatic biochemical indices, such as total cholesterol, LDL, total cholesterol/HDL ratio, lipoprotein (a), apolipoprotein A-1, apolipoprotein B etc.

The phytochemical analysis of the mastic gum has revealed that it contains essential oil, a high percentage (30%) of an insoluble polymer (poly- β -myrcene), an acid fraction containing mainly triterpenic acids and a neutral mastic fraction (NMF) (Paraschos et al. 2007). The latter, as previously reported (Paraschos et al. 2007) contains several neutral triterpenic compounds possessing phytosterol-like structure. Several phytosterols and plants extracts rich in phytosterols are widely used as additives in dietary products aiming to reduce total cholesterol levels. This discovery, in connection with the above mentioned antioxidant and hypolipidemic activities of Chios mastic gum led us to the hypothesis that mastic and particularly its neutral fraction (NMF) containing phytosterol-like compounds, could possess antiatheromatic activities, because of the well established effect of plant sterols on the atheromatic disease (Ling and Jones, 1995). Furthermore, although it is speculated that mastic gum may exert cardioprotective properties in humans (Triantafyllou et al. 2007), there are no bibliographic data concerning the effect of mastic gum on myocardial infarct size in vivo.

Due to the above considerations, in the present study we sought 1) to determine if the Total Mastic Extract without Polymer (TMEWP), acquired from the crude resin by decantation of the insoluble poly- β -myrcene (Van den Berg et al. 1998) and the neutral fraction (NMF) enriched in phytosterol-like compounds, reduce the infarct size in both normal and cholesterol fed animals in vivo, 2) to evaluate the potential antiatheromatic activity by histopatholog-

ical examination and 3) to evaluate the potential activity of both extracts against lipid peroxidation and LDL oxidation.

Materials and methods

For complete methods, see Supplementary material.

Dosage protocol

According to recently published results (Lemonakis et al. 2011) in order to evaluate the role of the natural polymer in the absorption process, the bioavailability of two major compounds of TMEWP was examined in comparison with these of the natural mastic gum extract. The bioavailability of the compounds in the TMEWP was higher, probably due to the fact that the sticky, insoluble polymer prolongs the absorption of the active compounds. In accordance to the above, we have concluded that the TMEWP would be the most appropriate mastic extract for per os administration in the in vivo rabbit model.

Since TMEWP represents roughly 70% of the crude mastic gum (Paraschos et al. 2007), the dose for TMEWP was calculated to be 46 mg/kg, according to the nutraceutical dose proposed in the mastic capsules' leaflet, being issued by the Chios Mastic Gum Growers Association and which are suitable for humans.

In more detail, the chosen dose is calculated to be equivalent to a dose of 1278 mg/person/day (Reagan-Shaw S. et al., 2008) which is in compliance with the range of the optimal dose per day for humans (http://www.gummastic.gr/library/Capsules_Description_ENG.pdf). In order to evaluate the effect of an enriched extract originating from the mastic gum, we have chosen to administer the same dose as previously, 46 mg/kg of the NMF. Considering that the NMF constitutes the 27% of the crude mastic gum, the equivalent dose of mastic gum for humans would be 3300 mg/person/day (Reagan-Shaw S. et al., 2008) which is in agreement with the therapeutic dose, ranging from 1 to 5 g/person/day (Triantafyllou A. et al., 2007). Moreover it's interesting to point out that the mastic gum is not a toxic compound, even in a dose of 28 g/person/day (Kang et al., 2007).

Experimental protocol

Mastic Total Extract without Polymer (TMEWP) and the Neutral Mastic Fraction (NMF), were administered to rabbits in the form of sunflower oil solution, orally via a small feeding tube. Characterization of both extracts using Ultra-High Pressure Liquid Chromatography-Atmospheric Pressure Chemical Ionization - High Resolution Mass Spectrometry (UHPLC-APCI (\pm)-HRMS) as well as Gas Chromatography-Mass Spectrometry (GC-MS) is given in the supplementary materials. Several marker compounds characterizing the analyzed extracts have been identified (Fig. 1). Forty three anesthetized rabbits were subjected to 30 min regional ischemia of the heart, followed by 3 h of reperfusion and randomized into 6 groups as follows:

Normal fed animals:

Control group (n = 8): sunflower oil solution was administered for 6 weeks.

Group TMEWPN (n = 7): animals treated with 46 mg/kg/day of TMEWP for 6 weeks.

Group NMFN (n = 7): animals treated with 46 mg/kg/day of NMF for 6 weeks.

Animals fed with cholesterol-enriched diet for 6 weeks:

CHOL group (n = 7): sunflower oil solution was administered for 6 weeks.

Group TMEWPH (n = 7): animals treated with 46 mg/kg/day of TMEWP for 6 weeks.

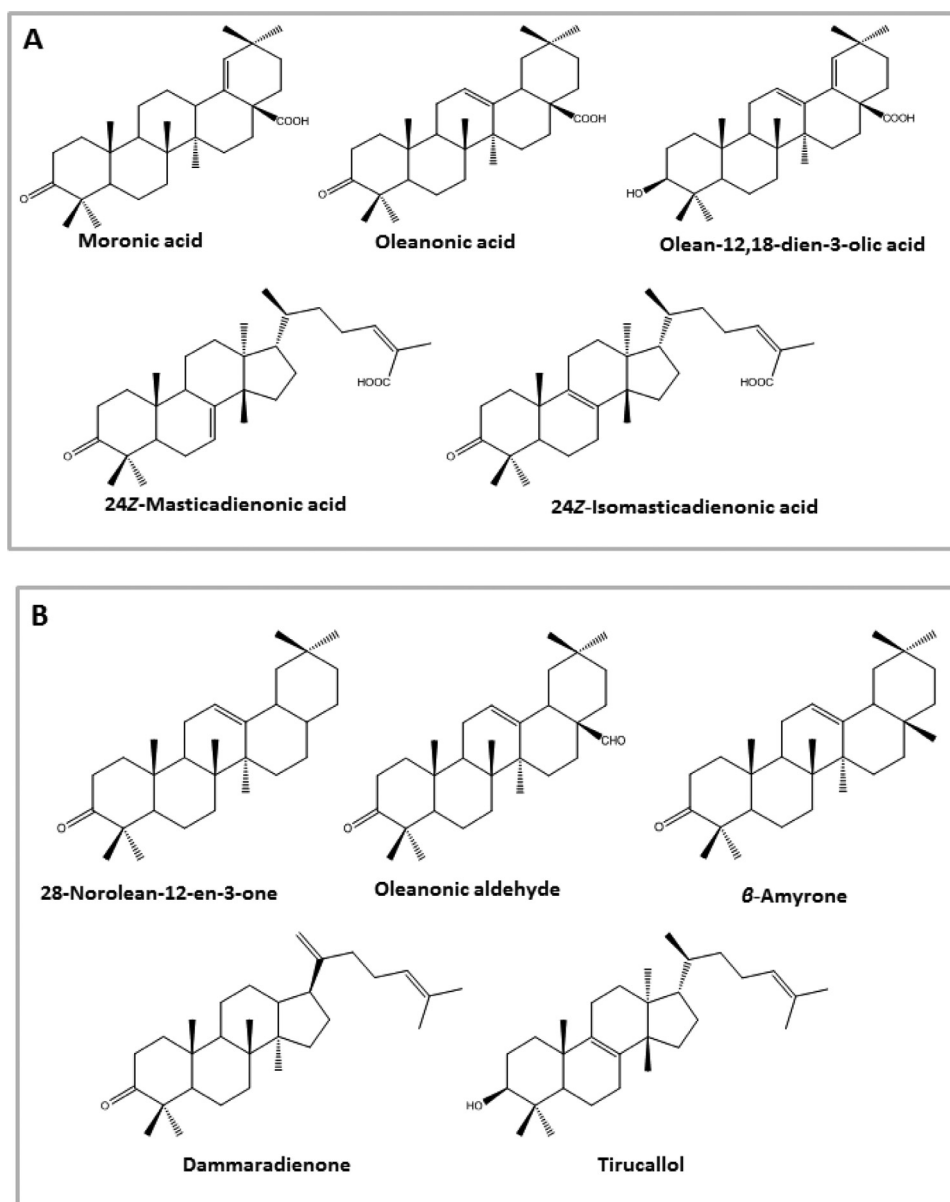


Fig. 1. Characteristic constituents of (A) Total Mastic Extract without Polymer (TMEWP) and (B) Neutral Mastic Fraction (NMF).

Group NMFH (n = 7): animals treated with 46 mg/kg of NMF for 6 weeks.

The experimental protocol is presented in Fig. 2. At the end of the experiment the area at risk and the infarct zone were determined. Blood samples were collected at several time points, for malondialdehyde (MDA) evaluation as an index of lipid peroxidation, for total cholesterol and LDL cholesterol determination and for the evaluation of LDL oxidation.

Results

Hemodynamic variables

The hemodynamic measurements were performed at baseline, at the 20th min of ischemia and at the end of reperfusion period. No significant differences in mean heart rate and mean blood pressure were observed between the groups (data not shown).

Infarct size

No significant differences were detected in risk areas between the studied groups (Fig. 3B). The I/R% was $46.7 \pm 2.4\%$ in the Control group and $50.5 \pm 2.35\%$ in the CHOL group as shown in Fig. 3A. The mastic total extract (TMEWP) and the phytosterolic fraction (NMF) reduced the infarct size ($18.3 \pm 3.4\%$, and $25.7 \pm 2.8\%$ vs. $46.7 \pm 2.4\%$ in the Control group, $P < 0.05$). In the hypercholesterolemic rabbits no reduction on infarct size was observed by both treatments ($43.1 \pm 3.3\%$ and $44.7 \pm 3.5\%$ in TMEWPH and NMFH groups respectively, $P = \text{NS}$), (Fig. 3A).

Measurement of circulating MDA

The effect of the interventions in each study group on MDA production at baseline, at the 20th min of ischemia, at the 1st and 20th min of reperfusion as an index of lipid peroxidation is shown in Fig. 4. The MDA concentration was significantly higher at the

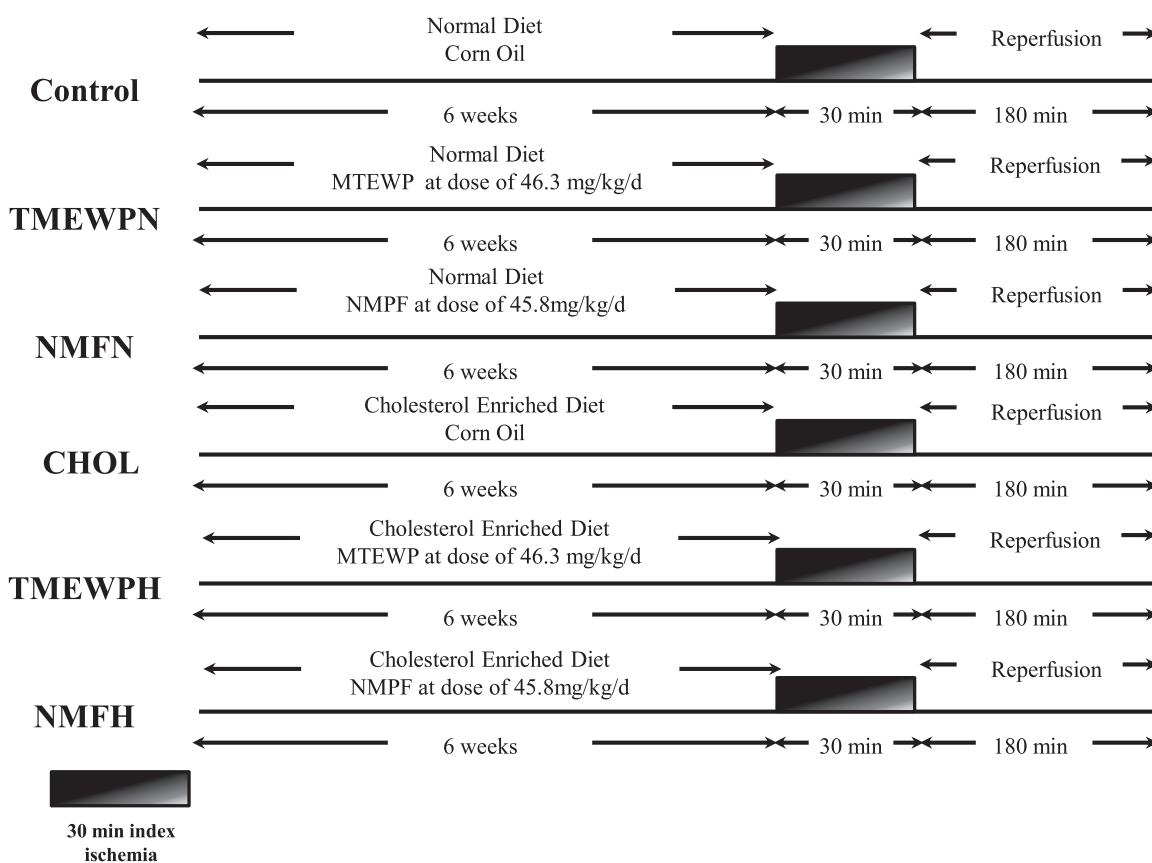


Fig. 2. Schematic presentation of the study protocol.

20th min of reperfusion in the Control, CHOL, TMEWPH and NMFH groups compared with baseline ($P < 0.05$). Treatment for 6 weeks with TMEWP and NMF reduced the MDA concentration in normal fed rabbits ($P < 0.05$ vs. Control), but had no effect on lipid peroxidation in cholesterol fed animals (Fig. 4).

Cholesterol and histology

After centrifugation of the blood, cholesterol levels were assessed. Mean cholesterol level was 998.3 ± 46 mg % in the CHOL group and 62.9 ± 2.6 mg% in the control group. Treatment for 6 weeks with TMEWP and NMF reduced the total cholesterol concentration by 47 and 88% respectively ($P < 0.01$), (Fig. 5A). The mean LDL levels are shown in Fig. 5B. TMEWP and NMF treatment for 6 weeks reduced the plasma LDL levels in the cholesterol fed animals. However both fractions had no effect on LDL oxidation as it is shown in Fig. 5C.

Atherogenesis was detected in the form of subintimal accumulation of lipids and foamy macrophages. In the CHOL group, both the ascending and the descending aorta showed atheromatous changes (Fig. 6A). In the aorta of rabbits fed for 6 weeks with TMEWP and NMF no atheromatosis was detected in any of the samples examined (Fig. 6B). The median perimeter ratio was 23% in CHOL group, and the lesions were patchy and interspersed by normal in intima (Fig. 6A). In the heart, in the specimens analyzed from intramural sections, subintimal deposition of lipids in coronary branches were identified in the Chol group. In the case of the rabbits treated with both extracts, there was no detection of atheromatous coronary vessels.

Discussion

The present study demonstrates that treatment for 6 weeks of Mastic Total Extract without Polymer (TMEWP) and the neutral

fraction (NMF) reduced significantly the infarct size in the normal fed rabbits. Both extracts showed significant antiatheromatic and hypolipidemic activities in the hypercholesterolemic rabbits in vivo without decreasing the oxidation of LDL and the lipid peroxidation during reperfusion.

The protective effect of mastic gum against in vitro LDL oxidation has been documented as it was shown that different fractions of mastic gum exhibited antioxidant/antiatherogenic effect on LDL (Andrikopoulos et al. 2003). Additionally, it has been documented that the polar extract from *P. lentiscus* resin increased the survival of oxLDL-treated peripheral blood mononuclear cells (PBMC). More specifically, cells exposed to oxLDL underwent apoptosis and necrosis, whereas when cells were cultured with oxLDL and the polar extract concurrently, inhibition of both necrosis and apoptosis was observed, suggesting that *P. lentiscus* triterpenes exert antioxidant/antiatherogenic effect in vitro (Dedoussis et al. 2004). In our case we did not observe inhibition of oxidation of LDL or inhibition of MDA formation in cholesterol fed rabbits after administration of TMEWP and NMF. According to our knowledge this is the first time that *P. lentiscus* extracts are evaluated in vivo on LDL oxidation.

In the present study we observed a significant reduction in total cholesterol and LDL circulatory levels in cholesterol fed rabbits after administration of both extracts. This is in accordance with previous findings that showed a beneficial action of mastic gum powder on serum total and LDL-cholesterol in human subjects (Triantafyllou et al. 2007). The difference of the reduction of total cholesterol levels by TMEWP and NMF (47 and 88% respectively), is in accordance with the fact that NMF is an enriched in phytosterol-like compounds extract. Moreover, herein we showed that both TMEWP and NMF not only reduced the total cholesterol and LDL levels in vivo but also reduced the subintimal accumulation of lipids and foamy

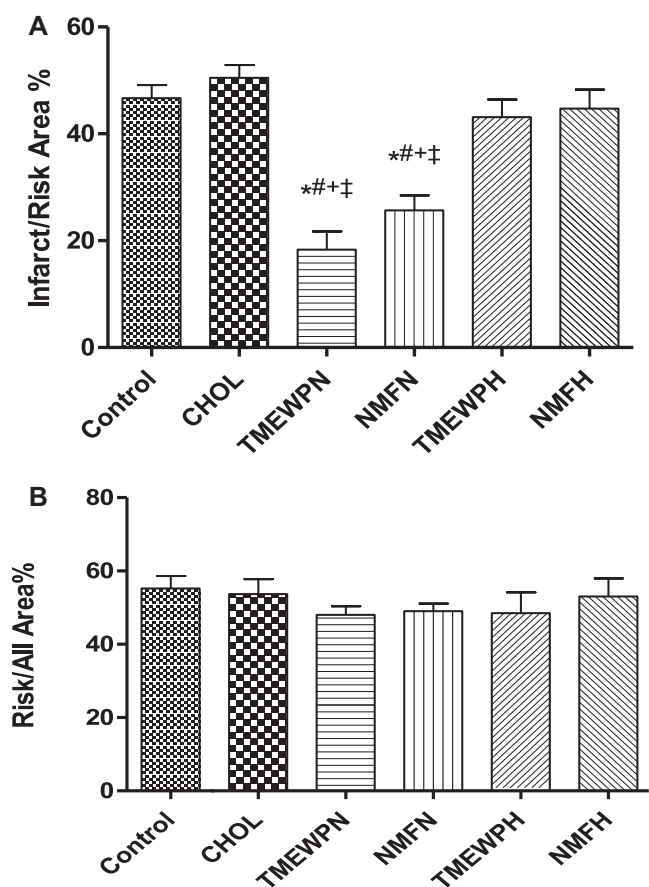


Fig. 3. A The effect of various interventions on infarct size (expressed as a percent of risk zone) in rabbit heart following 30 min of ischemia and 180 min of reperfusion. Control group: sunflower oil solution was administered for 6 weeks; CHOL group: cholesterol and sunflower oil solution was administered for 6 weeks; TMEWPN group: animals treated with 46 mg/kg⁻¹/day of TMEWP for 6 weeks; NMFN group: animals treated with 46 mg/kg⁻¹/day of NMF for 6 weeks; TMEWPH group: animals treated with 46 mg/kg⁻¹/day of TMEWP and cholesterol for 6 weeks; NMFH group: animals treated with 46 mg/kg⁻¹/day of NMF and cholesterol for 6 weeks. **P* < 0.05 vs. all the other study groups. B % Areas at risk/whole myocardial area in rabbits subjected to ischemia/reperfusion.

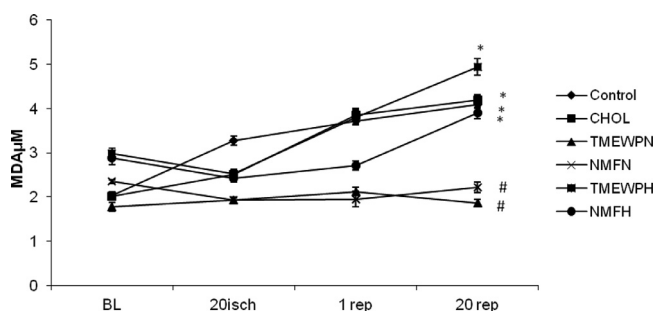


Fig. 4. MDA formation during ischemia/reperfusion at baseline, 20th min of ischemia, 1st and 20th min of reperfusion (**p* < 0.05 vs. baseline, #*p* < 0.05 vs. the control, CHOL and TMEWPH groups).

macrophages, indicating a strong antiatherogenic effect of both extracts.

MDA formed by the breakdown of lipid peroxides is considered as a biochemical marker for lipid peroxidation end product, and it also had been used to assess oxygen free radical mediated injury of myocardium (Shi et al. 2013). Therefore, we measured a time course of MDA production at baseline, the 20th min

of ischemia, the 1st and 20th min of reperfusion in order to access the effect of both extracts on lipid peroxidation in normal and cholesterol fed rabbits. MDA increased in both control groups early in reperfusion, confirming our previous reports concerning an increased formation of lipid peroxidation products in the circulation at the beginning of reperfusion (Andreadou et al. 2004, 2006). Both extracts reduced MDA levels at the 20th min of reperfusion compared to the control groups, whereas in cholesterol fed rabbits, such beneficial effect was not observed. Previous studies have demonstrated that different components of *P. lentiscus* inhibited lipid peroxidation in vitro. The digallic acid obtained from the fruit *P. lentiscus* inhibited the lipid peroxidation induced by H₂O₂ in the K562 cell line (Bhouri et al. 2010). Extracts prepared from *P. lentiscus* were also effective in suppressing Fe²⁺-induced lipid peroxidation (Ljubuncic et al. 2005). In agreement to the above in vitro findings we showed for the first time in an in vivo model of ischemia/reperfusion that extracts from *P. lentiscus* suppressed lipid peroxidation. However this was not observed in the cholesterol fed rabbits, although the administration of both extracts reduced significantly atheromatosis. It is well established that oxidative stress and inflammation play important roles in atheromatosis (Efentakis et al. 2015), and our group has recently shown that mastic gum neutral extract can directly inhibit inflammatory process in human aortic endothelial cells by inhibition of TNF- α -induced endothelial activation and expression of ICAM-1 and VCAM-1 adhesion molecules (Loizou et al. 2013). Thus, the inhibition of inflammatory mediators may be the mechanism by which mastic gum reduced atherogenesis in our in vivo model.

Acute coronary syndromes, including myocardial infarction, are the most serious and lethal manifestation of cardiovascular heart diseases. The major determinant of prognosis in acute myocardial infarction is infarct size. Although it has been proposed that Mastic Gum powder could have a cardioprotective role in humans (Triantafyllou et al. 2007) no study has been performed until now to investigate the effects of Mastic Gum on infarct size. We found that both extracts reduced significantly the infarct size in the normal fed anesthetized rabbits compared to the Control group. However, this beneficial effect on myocardial infarction was lost when atheromatosis is present, parallel to lipid peroxidation inhibition. The reduction in infarct size by both TMEWP and the NMF can be achieved by several mechanisms such as the elimination of reperfusion injury (because of lesser lipid peroxidation and oxidative stress), or by activation of intracellular mediators that are crucial to protect the heart against lethal reperfusion injury.

Protection of the myocardium can be achieved by mechanical vascular occlusions such as ischemic preconditioning (IPC), which leads to cardioprotection characterized by reduction of infarct size (Andreadou et al. 2015a). Although, the effectiveness of preconditioning in animals is beyond dispute, the demonstration in clinical practice has been surprisingly elusive (reviewed in Iliodromitis et al. 2015). Nutritional preconditioning is considered to be a form of pharmacological preconditioning, mediated by the intake of nutraceuticals through diet (Andreadou et al., 2015b). We have previously shown that a six weeks treatment with oleuropein, (a polyphenol from olive tree) reduced significantly the infarct size in normal fed rabbits, and only at higher doses in hypercholesterolemic rabbits, although it reduced significantly the total cholesterol levels (Andreadou et al. 2006). Later on, we showed that oleuropein reduced the infarct size by activating cellular signaling cascades similar to those of preconditioning. Although the effectiveness of preconditioning has been verified under normal conditions in every species studied, there are several concerns regarding its effectiveness under pathological circumstances. It has been shown that preconditioning limits the infarct size in hypercholesterolemic animals (Iliodromitis et al. 2006); however, other studies have documented that the cardioprotective effect of

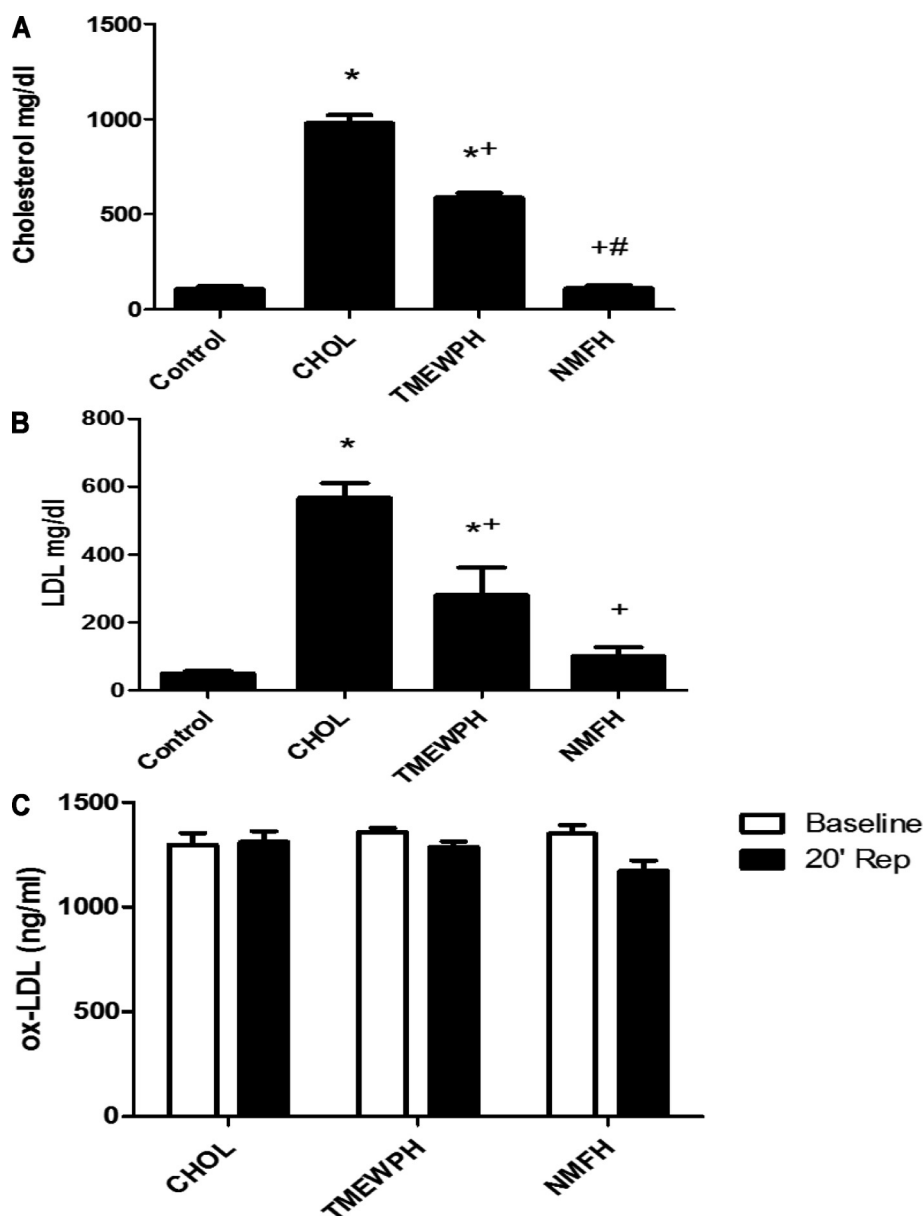


Fig. 5. A Plasma total cholesterol concentrations in rabbits fed a hypercholesterolemic diet and administered TMEWP and NMF fractions. * $P < 0.05$ vs. Control group, + $P < 0.05$ vs. CHOL group and # $P < 0.05$ vs. TMEWPH group. B Plasma LDL levels at the baseline concentrations in rabbits fed a hypercholesterolemic diet and administered TMEWP and NMF fractions. * $P < 0.05$ vs. Control group, + $p < 0.05$ vs. CHOL group. C. Plasma ox-LDL (ng/ml) levels at the baseline and 20th min of reperfusion in rabbits fed a hypercholesterolemic diet and administered TMEWP and NMF fractions. $P = NS$.

preconditioning is attenuated in various pathological conditions, such as hyperlipidemia, (Gircz et al. 2006). Alterations in the signaling mechanism in the cardioprotection afforded by preconditioning may be the possible mechanisms involved in the abrogated cardioprotective potential of preconditioning in pathological conditions (Ferdinandy et al. 2014). Hypercholesterolemia impairs endothelial function potentially by increasing the production of several oxidants such as peroxynitrite and lipid peroxidation compounds (Napoli et al. 2006). Our study showed that although both mastic gum extracts reduced infarct size and lipid peroxidation in normal fed rabbits they did not exert the same beneficial effect in hypercholesterolemic condition. It seems that both fractions might must be used in a higher concentration in order to reach in sufficient levels and reduce the oxidative stress and the myocardial infarct size in atheromatosis. The elucidation of the dosage which

is necessary to reach the proper pharmacokinetic properties in order to reduce the myocardial infarction in atheromatosis should be addressed by further studies.

In conclusion, Chios Mastic gum can be considered as a nutraceutical that induces nutritional preconditioning in healthy conditions. Furthermore, it reduces atheromatosis, and the circulatory levels of total and LDL cholesterol in hyperlipidemic conditions. Thus, it can be suggested that Chios Mastic Gum may be a possible application as natural medicine or food supplement with prospective cardioprotective effects.

Conflict of interest

The authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no

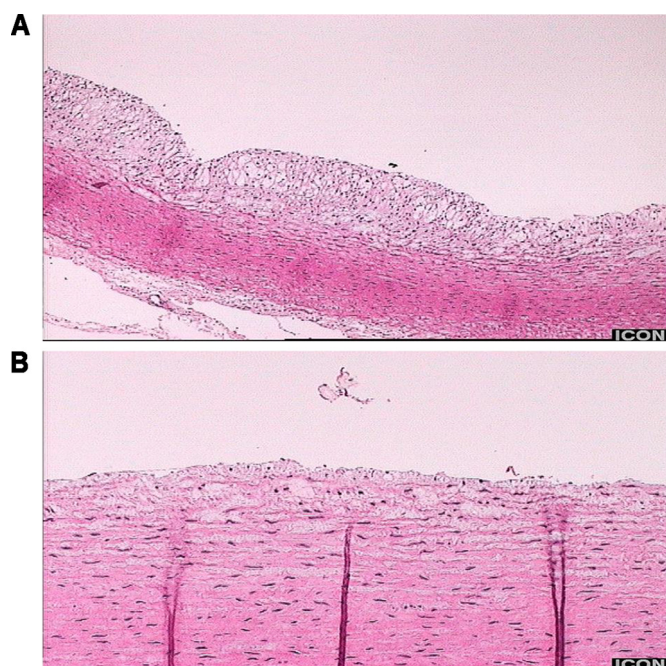


Fig. 6. A CHOL group: Cross section of the ascending aorta showing increased atherosclerosis. B TMEWPH group: Cross section of the ascending aorta showing no evidence of atherosclerosis.

significant financial support for this work that could have influenced its outcome.

Acknowledgment

We thank the mastic producers from Chios island, Greece for supplying the commercially available mastic gum.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.phymed.2016.06.002](https://doi.org/10.1016/j.phymed.2016.06.002).

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