ABSTRACT

Chios Mastiha constitutes a resin secreted from *Pistacia lentiscus* L. *var. chia* (Chios Mastiha tree). *Pistacia lentiscus* L. *var. chia*, an endemic plant, is cultivated exclusively in the Northern part of Chios Greek island. Its cultivation last throughout the year with the Mastiha's secretion and collection taking place in the autumn. Chios Mastiha is being used as traditional medicinal product over 2500 years, even since ancient time. Nowadays, *Pistacia lentiscus* L. *var*. *chia* resin is widely used as food and beverages additive, with applications in cosmetology and dentistry as well. Interestingly, recent years many scientific studies have revealed Chios Mastiha biological properties, such as antimicrobial, anti-inflammatory, antioxidant, hypoglycemic, hypolipidemic, antiproliferative and anticancer properties. The above biological activities have been attributed to Chios Mastiha chemical composition, in which the major group are triterpenes (60-70%), while the rest 40-30% is occupied by the sticky polymer 1,4-poly-β-myrcene.

The steroid hormones glucocorticoids play fundamental role in basic functions of the human body, such as metabolism, development, growth and cognition. They exert their actions after binding to glucocorticoid receptor (GR). In the absence of hormone GR residues in the cytoplasm in its inactive state. After hormone binding, GR changes stereo-conformation and translocates to the nucleus. Within the nucleus, GR regulates gene expression after direct or indirect binding with DNA, inducing or repressing expression of its target gene. Synthetic glucocorticoids, such as dexamethasone, are the most common prescribed medication due to their immunosuppressive and anti-inflammatory effects. However, long-term or high doses of them cause severe side effects, such as hyperglycemia and diabetes, osteoporosis and muscle atrophy, glaucoma and hypertension. The above side effects are attributed to GR transcriptional activation, while beneficial anti-inflammatory actions caused by suppression of GR transcriptional activation.

Recently, triterpenes, which have been identified as the major chemical group in Chios Mastiha extract, have been studied as potential selective glucocorticoid receptor agonists, inducing its beneficial anti-inflammatory actions, while minimizing its harmful effects. In this context, the purpose of this research was to evaluate the biological actions and their biochemical mechanisms of actions, and specifically the anti-proliferative, the anti-inflammatory, anti-hyperglycemic actions of different polarity fractions from *Pistacia lentiscus* L. *var. chia* resin and leaves. Due to Chios Mastiha enrichment with triterpenes, emphasis was given on fractions interference with glucocorticoid receptor signaling. Specifically, three different polarity fractions of *Pistacia lentiscus* L. *var*. *chia* leaves cultivating in the South Chios and *Pistacia lentiscus* L. *var*. *chia* leaves growing in the Northern part but without secreting Mastiha, were examined. Moreover, three different polarity fractions of Chios Mastiha and eight subfractions (F1, SF2A, SF2B, SF3A, SF3B, SF3C, SF4A, SF5A) from neutral fraction of Chios Mastiha were also evaluated. Biological assays were performed in the human

embryonic kidney cell line HEK293, which overexpresses endogenously the glucocorticoid receptor.

Phytochemical analysis of *Pistacia lentiscus* L. *var. chia* leaves and resin were conducted by Prof. Giovanni Appendino and Associate Prof. Federica Pollastro (Department of Pharmaceutical Sciences, University of Piemonte Orientale, Novara, Italy). These results showed that apolar fraction from Southern tree consists of fatty acid triglycerides and traces of triterpenoids, while in the medium-polar fraction the triterpenes lupeol and α-amyrenone were identified. In polar fraction from Southern tree phenolic compounds and traces of triterpenoids were detected. Similarly, apolar fraction from Northern tree consists of fatty acid triglycerides and traces of triterpenoids, while in medium-polar fraction the triterpenes lupeol and β-sitosterol were found. In polar fraction from Northern tree phenolic compounds and traces of triterpenoids were detected, as well. Apolar fraction from *Pistacia lentiscus* L. *var. chia* resin was rich in the sticky polymer 1,4-poly-β-myrcene, and the triterpenes keto-oleanolic aldehyde και oleanolic aldehyde, too. In medium-polar fraction of Chios Mastiha the triterpenes lupeol, oleanolic aldehyde, 24Z-masticadienonic acid methyl ester and 24Z-isomasticadienonic acid methyl ester were identified. In polar fraction of Chios Mastiha phenolic compounds were found, without any triterpenoids. As far as neutral subfractions from *Pistacia lentiscus* L. *var. chia* resin, fraction 1 contains the sticky polymer 1,4-poly-β-myrcene and the triterpene keto-oleanolic aldehyde. Keto-oleanolic aldehyde was also found in subfraction 2A, while the triterpene β-amyrin was identified in subfraction 2B. The triterpene lupeol was found in subfractions 3A, 3B and 3C. In subfraction 3C keto-oleanolic aldehyde was the major compound. A mixture of triterpenes and other aliphatic compounds were found in subfraction 4A. Lupeol was also identified in subfraction 5A, while β-amyrin was the major compound.

In the present study, medium-polar and polar fractions from *Pistacia lentiscus* L. *var. chia* resin and leaves caused a dose-dependent suppression of the DEX-induced GR transcriptional activation. This suppression was followed up by reduction in GR protein levels, and its target phosphoenolpyruvate carboxykinase (PEPCK), glutamine synthetase (GS) and peroxisome proliferator-activated receptor alpha (PPARα) protein levels, revealing anti-hyperglycemic and regulatory actions on lipid metabolism. Medium-polar leaves fraction from the Southern tree was more active than the Northern one, as far as suppression of GR transcriptional activation and reduction of PEPCK protein levels. Reduction in GR protein levels by the medium-polar and polar fractions from *Pistacia lentiscus* L. *var. chia* leaves is mediated through proteasomal proteolytic pathway. The apolar fractions did not cause any remarkable change in GR transcriptional activation, possibly due to their low solubility in solvents compatible with HEK293 culture and the presence of the sticky polymer 1,4-poly-β-myrcene, which hinders the biological activity of the other compounds. Fraction 1, as well as subfractions 2B, 3B and 5A from Chios Mastiha neutral fraction caused suppression in the DEX-induced GR transcriptional activation, although to a lower extent compared to medium-polar and polar fractions. This suppression was not followed up by reduction in GR and PEPCK protein levels, indicating regulation of GR transcriptional activation

by the neutral subfractions, without affecting its protein levels. Interestingly, suppression of the DEX-induced GR transcriptional activation was followed up by reduction of PPARα protein levels, indicating the potential regulatory actions of neutral subfractions on lipid metabolism.

Moreover, fractions from *Pistacia lentiscus* L. *var*. *chia* leaves and resin were examined regarding their effect on energy metabolism regulation. AMPK constitutes a major energy balance regulator, sensing changes in ATP levels and restoring them. Medium-polar and polar fractions from *Pistacia lentiscus* L. *var*. *chia* leaves caused reduction in AMP-activated protein kinase (AMPK) protein levels and its phosphorylated state. Comparative studies showed that medium-polar fraction of Southern tree was more active than the Northern one. However, medium-polar and polar fractions *Pistacia lentiscus* L. *var*. *chia* resin increased phosphorylated AMPKα protein levels, without any impact on its non-phosphorylated state, indicating induction of catabolic versus anabolic pathways.

Furthermore, in this research anti-inflammatory actions of fractions from *Pistacia lentiscus* L. *var*. *chia* were investigated. Our results showed that medium-polar and polar fractions from *Pistacia lentiscus* L. *var*. *chia* leaves caused a dose-dependent suppression of the TNFα-induced NF-κB transcriptional activation. Leaves fractions from Southern tree were more active than the Northern ones. The absence of any remarkable changes in p65 subunit of NF-κB by leaves fractions indicates regulation only at transcriptional activation level. Apolar fractions from *Pistacia lentiscus* L. *var*. *chia* leaves suppressed the NF-κB transcriptional activation, but to a much lower extent than medium-polar and polar ones. Medium-polar and polar fractions from *Pistacia lentiscus* L. *var*. *chia* resin reduced both the TNFα-induced NF-κB transcriptional activation and the p65 protein levels, while apolar fraction did not cause any remarkable changes. Neutral subfractions from *Pistacia lentiscus* L. *var*. *chia* resin also suppressed the NF-κB transcriptional activation, in a dose-dependent manner and reduced the p65 subunit of NF-κB protein levels, with subfraction 5A being the most active one.

Finally, in this study the effect of fractions from *Pistacia lentiscus* L. *var*. *chia* on HEK293 cell proliferation and apoptotic mechanism were assessed. Specifically, fractions from *Pistacia lentiscus* L. *var*. *chia* reduced HEK293 cell viability in a time and dose-dependent manner. Leaves fractions from the Southern tree were more cytotoxic than the Northern ones. Medium-polar fraction from resin was more cytotoxic than the apolar and polar ones, indicating possible apoptotic effects of the triterpenes lupeol, methyl ester of 24Z-masticadienonic acid and methyl ester of 24Z-isomastichadienonic acid. Neutral subfractions 2A, 2B, 3A, 3B and 5A caused reduction in HEK293 cell viability, with subfraction 5A being the most active one. Moreover, medium-polar and polar fractions from resin and leaves induced apoptosis in HEK293 cells, as indicated by reduction in procaspase-3, procaspase-9 and bcl-2 protein levels, in a dose-dependent manner. Reduction in procaspse-9 protein levels indicates that apoptosis is mediated by the mitochondrial pathway. Similarly, neutral subfractions from resin reduced procaspase-3 and bcl-2 protein levels, but without

affecting procaspase-9 protein levels, indicating that apoptosis is not mediated by mitochondria. Subfraction 5A was the most apoptotic and the only one which reduced procaspase-9 protein levels.