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Chemical analysis and antioxidant activities of resin fractions from *Pistacia Lentiscus* L. *var. Chia* in neuroblastoma SH-SY5Y cells

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Abstract: Chios mastiha is the natural aromatic resin of Pistacia lentiscus L. var. Chia, which is exclusively cultivated in the southern part of the Greek island Chios. Chios mastiha is well-known for its distinctive taste and aroma and has been known since ancient times due to its healing properties in gastrointestinal and inflammatory disorders but also because of its anti-bacterial and anti-fungal activities. In this study the chemical composition, applying LC-QTOF-MS/MS analysis, and the antioxidant activity of three different polarity mastiha fractions, apolar, medium polar and polar, were characterized in human neuroblastoma SH-SY5Y cells. Chemical analysis of the fractions unveiled new components of mastiha resin composition, mainly fatty acids compounds, known for their antioxidant activity and regulatory effects on lipid metabolism. Applying MTT assay and confocal microscopy analysis we showed that mastiha fractions, especially the apolar and medium polar fractions, enriched in triterpenes and fatty acids, caused suppression of the H_2O_2-induced reduction in cell viability, ROS production and depolarization of the mitochondrial membrane potential, in SH-SY5Y cells. Moreover, Western blot analysis revealed that apolar fraction, enriched in fatty acids, induced expression of the PPARα, which is well-known for its antioxidant activities and its crucial role in lipid metabolism. Induction of PPARα, a GR target gene, was also accompanied by increase in GR protein level. Enhanced antioxidant activities of the apolar fraction may be correlated with its chemical composition, enriched in fatty acids and triterpenoids. Thus, our results indicate neuroprotective actions of mastiha fractions, highlighting their potential application as neuroprotective agents in neurodegenerative diseases.

Keywords: Chios mastiha; antioxidant activity; SH-SY5Y cells; ROS; Glucocorticoid receptor, PPARα; neuroprotection

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1. Introduction

Chios mastiha or Chios mastic gum, is a natural aromatic resin derived from the trunk and branches of the *Pistacia lentiscus* L. *var. Chia* (Mastic Tree). Even though *P. lentiscus* is distributed throughout the Mediterranean, the Chia variety is exclusively cultivated on the Greek island Chios and particularly in the southern part of the island, due to the climatic and soil conditions that prevail there. Chios mastic has been harvested for over 2500 years. Mastiha is widely used as a food flavoring additive and beverage and it has many applications in producing natural chewing gum, skin-, dental-, and cosmetic- products. Most importantly, Chios mastic has been known since ancient times due to its healing properties in gastrointestinal and inflammatory disorders [1]. In 2015 Chios mastiha was awarded by the European Medicine Agency as a natural medicine for skin inflammation and dyspeptic disorders [1, 2]. Moreover, anti-cancer [3-5] and cardiovascular protective activities [6, 7], of Chios mastiha, have been proposed. These actions are attributed to the anti-bacterial, anti-fungal activities [8] anti-inflammatory [9-12] antilipemic, antioxidant and apoptotic [7, 13, 14] properties of Chios mastiha and its essential oil.

Chios mastiha's therapeutic actions on gastrointestinal and inflammatory disorders, namely inflammatory bowel diseases, are associated with mastiha's protective anti-inflammatory effects on intestinal epithelial cells [11, 15]. Anti-inflammatory actions of Chios mastiha are also beneficial for other cell types [9, 10, 16], including cardiovascular cells, where the antioxidant activities of Chios mastiha play also a crucial role. Thus, it has been shown that treatment with mastiha improves the total antioxidant status of patients with nonalcoholic fatty liver disease NAFLD [17] and the antioxidant activity of oxidized low density lipoprotein (LDL) in peripheral blood mononuclear cell (PBMC) cells, suppressing the main causative factor of atherosclerosis [6]. Increased reactive oxygen species (ROS) levels are responsible for many pathological conditions primarily for neurodegenerative diseases [18] and cancer. In this context, the apoptotic and antiproliferative properties of Chios mastic gum in neuroblastoma SH-SY5Y and SK-N-BE cells [19] have been reported. Maintenance of mitochondria functionality, due to their fundamental involvement in ROS and energy production is crucial for both cancer and neurodegenerative diseases [20]. Thus, further investigation of the neuroprotective actions of Chios mastiha and its anticancer activity on neuroblastoma cells, with emphasis on Chios mastiha effect on preservation of mitochondrial functionality could be of great importance.

Chemical composition of mastiha is associated with its biological activities. To this point, chemical analyses of mastiha, applying gas spectrometry, mass spectrometry and NMR analysis, have revealed that the sticky texture of the resin is attributed to a component named β-polymyrcene, which constitutes approximately 25%-30% of the total composition. The rest, 65-70% of mastiha is enriched in terpenoids [21-23]. Furthermore, terpenoids such as masticadienonic, isomasticadienonic acidic, moronate, oleanolate and oleanonate, have been identified in acidic fraction of mastiha resin. Moreover, triterpenic compounds like oleanolic aldehyde, 28-norolean-17-en-3-one, β-amyrone, isomasticadienolic aldehyde, tirucallol, and dammaradienone, lupeol, 24Z-masticadienonic acid methyl ester, 24Z-isomasticadienonic acid methyl ester keto-oleanolic aldehyde, oleanolic aldehyde, have been detected in neutral fractions or medium polar fractions of mastiha resin [12, 14]. In addition, other phenolic and flavonoid compounds such as gallic acid, caffeic acid, α-terpinolene, linalool, benzoic acid, phenylacetic acid, and tyrosol detected, although in smaller amounts [12, 13, 21, 24, 25].

The structural similarity of triterpenoids with the steroid hormone glucocorticoid that act through their cognate receptors, the glucocorticoid receptor [26-28], indicate that triterpenoids could interact with glucocorticoids signaling, affecting many glucocorticoids-mediated biological actions, including energy metabolism, inflammation, and apoptosis. In this

context, we have recently shown that Chios mastic essential oil as well as different polarity fractions from Chios Mastic tree leaves and resin, enriched in triterpenoids, could exert anti-inflammatory actions and regulation of apoptosis [12, 14, 29] affecting both glucocorticoid receptor activity and protein levels.
In this study LC-QTOF-MS/MS analysis of three different polarity fractions, apolar (a), medium polar (mp) and polar (p) from mastic resin, diluted in DMSO, was applied aiming to identify additional unidentified mastiha's components of chemical origin other than terpenoids and phenolic compounds. In addition, the possible neuroprotective actions of mastiha's fractions were evaluated by investigating their possible antioxidant activities, their effect on mitochondrial functionality, and their interference with steroid receptors signaling in human neuroblastoma SH-SY5Y cells. In consequence, assessment of mastiha fractions' effect on the H_2O_2-induced reduction in SH-SY5Y cell viability, depolarization of mitochondrial membrane, ROS production, and regulation of GR and antioxidants factors level, applying MTT assay, confocal microscopy, and Western blot was performed. To the best of our knowledge this is the first time that new compounds such as palmitic, stearic, and myristic acids in Chios mastiha's different polarity fractions were detected. Moreover, this study revealed the presence of 37 compounds identified through suspect and non-target screening in the above mastiha fractions composition. In addition, the antioxidant activity of Chios mastiha fractions in SH-SY5Y cells was reported, highlighting their potential application as neuroprotective agents.

2. Results

2.1 Chemical characterization of the three different polarity fractions of Chios mastiha

Chemical analysis of mastiha revealed its enrichment in terpenoids compounds. Although extensive studies have focused on terpenoids compounds identification, mastiha resin composition involves also additional unidentified compounds that remain to be characterized. To this effort, HPLC-QTOF-MS/MS analysis was performed in different polarity mastiha fractions. The analysis revealed a plethora of masses m/z (Table 1, 2 and Supplementary data, Table S1, S2, S3). The identification of the suspect compounds was carried out according Karadimou et al. [30].
Twenty-seven compounds were identified through suspect screening (Table 1) and are presented along with their literature reported biological activities in Supplementary data, Table S1. Non-target identification (Supplementary data, Table S3) was performed using the SCIEX Natural Products Library with a Library Match Score above 50.0, according to Mitsikaris et al. [31]. Nine compounds were identified through non-target screening (Supplementary data, Table S2). Tentatively identified compounds involve fatty acids (Table 1), whose presence in Chios mastic is reported for the first time. Specifically, the enrichment of fatty acids, such as palmitic acid, stearic, myristic, arachidonic and pentadecanoic acid was observed in the ap fraction (Table 1). The exclusive enrichment of the coniferaldehyde compound in the ap was also tentatively identified. Moreover, to the best of our knowledge, the enrichment of medium polar and polar fraction in compounds such as betulinic acid, euscapic acid (triterpenoid with antioxidant activity), flavidin, leuteolin glucoside, α-linolenic acid, ricinoleic acid (fatty acid), crepenynic acid, gamma linolenic acid was revealed for the first time (Table 1). Furthermore, nine non-target compounds were detected in Chios mastiha different polarity fractions (Table 2). Interestingly, unequivocal molecular formulas were attributed to approximately 250 detected features (Supplementary Data, Table S3), highlighting mastic resin rich composition.

Table 1: Suspect compounds by HPLC-QTOF-MS/MS analysis in Chios mastiha different polarity mastiha fractions (ap, mp and p). Normalized concentrations (μg/L) and their relative enrichment are presented.

Compounds	Quantitative measurement (μg/L)			Relative enrichment		
	apolar, ap	medium polar, mp	polar, p	apolar, ap	medium polar, mp	polar, p
Coniferaldehyde	103,9	-	-	-	-	-
Palmitic acid	9174,3	1768,3	1102,9	8,3	1,6	1,0
Stearic acid	13951,4	2437,4	1620,9	8,6	1,5	1,0
Myristic acid	395,7	69,3	79,8	5,7	1,0	1,2
Arachidonic acid	25,9	7,0	0,6	43,2	11,7	1,0
Pentadecanoic acid	246,2	48,9	38,8	6,3	1,3	1,0
Betulinic acid	2119,7	11462,9	11725,7	1,0	5,4	5,5
Euscaphic Acid	40,4	199,6	482,0	1,0	4,9	11,9
Flavidin	30,7	265,9	148,1	1,0	8,7	4,8
Luteolin glucoside	0,4	2,2	2,3	1,0	5,5	5,8
α-Linolenic acid	23,0	55,7	103,6	1,0	2,4	4,5
Hesperidin	4,9	6,0	5,3	1,0	1,2	1,1
Oleic acid	2502,3	1443,1	1131,4	2,2	1,3	1,0
Linoleic acid	267,3	248,3	320	1,1	1,0	1,3

Caprylic acid	54,1	35,4	31,7	1,7	1,1	1,0
Nebraskanic acid	47,5	50,0	65,8	1,0	1,1	1,4
Octyl formate	122,2	54,7	40,6	3,0	1,3	1,0
6,7-dihydro-7-hydroxylinalool	64,1	51,3	53,7	1,2	1,0	1,04
α-irone	10,3	18,5	13,5	1,0	1,8	1,3
Oleanonic acid	29571,4	32200,0	25114,3	1,2	1,3	1,0
Resveratrol	5,7	6,4	6,7	1,0	1,1	1,2
Isoliquiritigenin di-glucoside	3,1	0,1	3,1	31,0	1,0	31
Methoxycinnamic acid	10,3	18,5	13,5	1,0	1,8	1,3
Masticadecanoic acid	29571,4	32200,0	25114,3	1,2	1,3	1,0
Gamma linolenic acid	23,0	55,7	103,6	1,0	2,4	4,5
Oleanolic acid	601,1	2920	2885,7	1,0	4,9	4,8
Ricinoleic acid	23,0	55,7	103,6	1,0	2,4	4,5

Crepenynic acid	23,0	55,7	103,6	1,0	2,4	4,5

143

Table 2: Non-target compounds in different polarity mastiha fractions as revealed by non-target analysis using the SCIEX Natural Products Library and their chemical classification. 144 145

146

Non-target compounds	Non-target compounds chemical classification
Ethyl 2-acetyl heptanoate	straight-chain fatty acid
Sesterstatin	sesterterpenoid
Cyclohexanecarboxylic acid	organic compound
1,2-Hydroxylauric acid	medium-chain fatty acid
Dodecanedioic acid	saturated aliphatic dicarboxylic acid
Tisocalcitate	organic compound (vitamin D derivative)
Trivalerin	(ester of valeric acid) straight-chain saturated fatty acid
Frangulin B	anthraquinone
4,4',6,6'-Tetra-tert-butyl-2,2'-biphenol	aromatic hydrocarbon

147

2.2 Different polarity fractions from Chios mastiha induced resistance to H_2O_2-triggered reduction in cell viability of SH-SY5Y cells 148 149

150

To assess the antioxidant activity and the potential neuroprotective effects of the different polarity fractions from resin of *Pistacia lentiscus* L. *var. Chia*, their possible defensive actions on H_2O_2-induced reduction in cell viability of SH-SY5Y cells, by applying MTT assay, were investigated. Firstly, we focused on setting up the experimental conditions that lead to approximately 30% H_2O_2 -induced reduction in cell viability of SH-SY5Y cells. Moderate (20-30%), and not severe reduction in cell viability by H_2O_2, could allow us to observe any possible protective effects, achieved upon pretreatment with potential antioxidant factors. Thus, effects of 50 μM to 500 μM H_2O_2 treatment for 6 h, and 1 mM or 2 mM H_2O_2 treatment for 3 h on SH-SY5Y cell viability (Figure 1A) were examined. As shown in Figure 1A incubation of SH-SY5Y cells with 500 μM H_2O_2 for 6 h caused approximately 20% reduction in cell viability, whereas incubation of the cells with 1mM H_2O_2 for 3h caused more than 60% reduction in cell viability. Thus, incubation of the cells with 700 μM H_2O_2 for 6 h was considered as optimum condition to study the possible antioxidant activity of different polarity fractions from mastiha resin. Then, MTT assay was applied in SH-SY5Y cells pretreated with the different polarity fractions from Chios mastiha, at concentrations of 10 and 20 μg/ml of apolar (ap) fraction, 5 and 10 μg/ml of medium polar (mp) and 10 and 20 μg/ml of polar (p) fraction for 24 h and subsequently subjected to 700 μM H_2O_2 treatment for additional 6 h. As shown in Figure 1B, upon 24 h incubation, 10 and 20 μg/ml of apolar fraction (ap), 5 and 10μg/ml of medium polar fraction (mp), and 10 and 20μg/ml 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169

of polar fraction (p) did not cause any statistically significant reduction in cell viability of SH-SY5Y cells. Moreover, as was expected, incubation of SH-SY5Y cells with 700 μM H_2O_2 for 6 h caused approximately 30% statistically significant reduction in cell viability compared to control cells. Interestingly, statistically significant resistance to H_2O_2-induced reduction in cell viability was observed in cells pre-incubated with 10 μg/ml or 20 μg/ml of apolar fraction (ap) for 24 h and then subjected to 700 μM H_2O_2 treatment (for 6h). Resistance to H_2O_2-induced reduction in cells viability was also observed, although to a lower extent, by pretreatment of the cells with 5 μg/ml of medium polar fraction (mp) and 10 μg/ml of polar fraction (p).

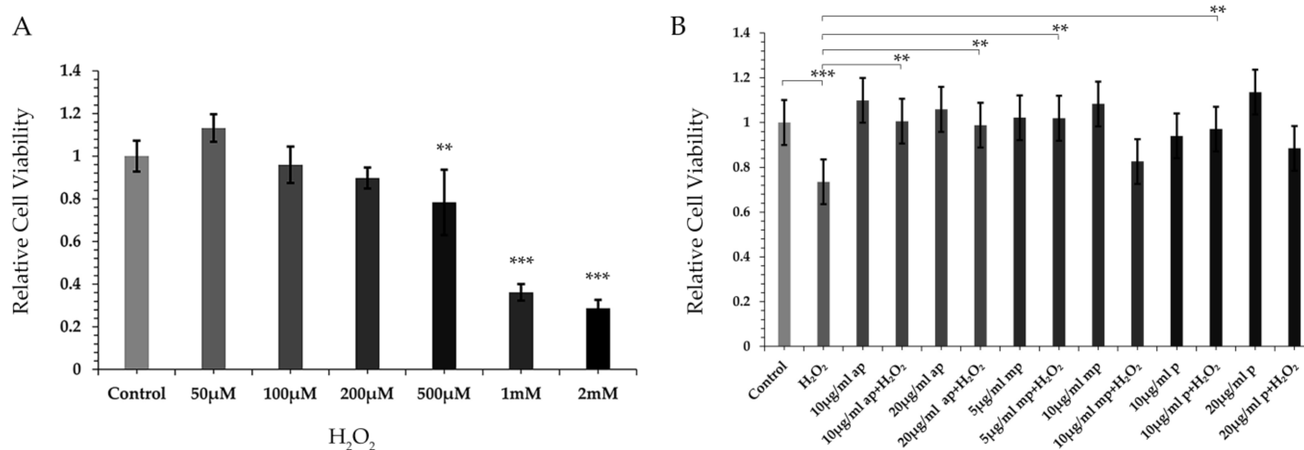

Figure 1: Resistance to H_2O_2-induced reduction in neuroblastoma SH-SY5Y cells viability, by different polarity mastiha fractions. A) Relative cell viability, assessed by MTT assay, of SH-SY5Y cells treated with 50-500 μM H_2O_2 for 6 h and 1 mM or 2 mM H_2O_2 for 3 h. Viability of vehicle-treated (1/1000 v/v DMSO) control cells was set as 1. B) Relative cell viability of SH-SY5Y cells pretreated or not with apolar (ap), medium polar (mp) and polar (p) mastiha fractions for 24 h and/or not subsequently subjected to 700 μM H_2O_2 for 6 h. Control vehicle-treated (1/1000 v/v DMSO) cells viability was set as 1. Relative cell viability is expressed as the viability of the cells pretreated with various concentrations of the ap, mp, and p fractions compared to the viability of the control cells. Data were analyzed by 1-way ANOVA (Figure 1A) and 2-way ANOVA (Figure 1B) respectively and are expressed as mean ± SD (n = 4, n=5), * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$.

2.3 Protective effect of the different polarity mastiha fractions on H_2O_2-induced ROS production in SH-SY5Y cells

Potential antioxidant activity of the different polarity mastiha fractions was estimated by measuring ROS production in H_2O_2-treated cells. ROS production was evaluated by assessing DCF staining in SH-SY5Y cells pretreated or not with mastiha fractions and subsequently subjected to H_2O_2-induced ROS production. Confocal microscopy images of the DCF dye, an indicator of ROS production, were taken in live SH-SY5Y cells. As shown in Figure 2, treatment of SH-SY5Y cells with 700 μM H_2O_2 for 6 h caused statistically significant 2.5 folds increase in DCF staining compared to control cells. Interestingly, pretreatment of SH-SY5Y cells with mastiha fractions, for 24 h, at the indicated concentrations, provide them with resistance to H_2O_2-induced ROS production, leading to statistically significant decrease in DCF staining compared to that in H_2O_2-treated cells. In accordance with the results from MTT assay, the ap fraction showed the highest antioxidant activity.

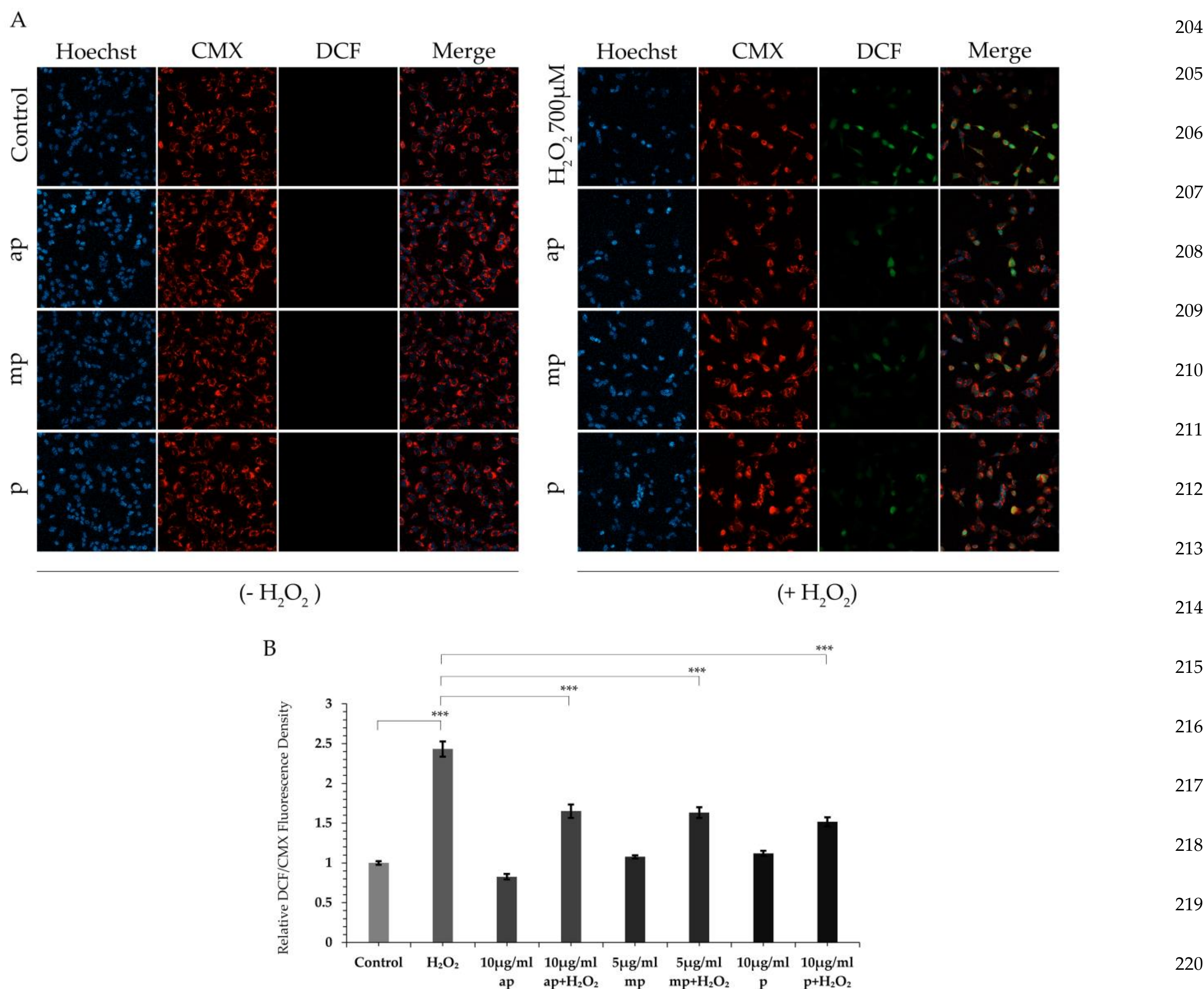

Figure 2: Reduced ROS production in SH-SY5Y cells pretreated with mastiha fractions and subsequently subjected to H_2O_2-induced oxidative stress. A) Representative confocal microscopy single images of SH-SY5Y cells stained with Hoechst-33342 (nuclear staining), MitoTracker CMXRos (living mitochondria, Red), DCF (ROS production dye, Green) upon pretreatment with 10μg/ml ap, 5μg/ml mp and 10μg/ml p mastiha fractions for 24 h and/or not subsequently subjected to 700 μM H_2O_2 for 6 h. Representative images were taken with Zeis LSM-800 confocal microscope in 40 X objective B) Quantification analysis of DCF staining per CMX staining of single cells. The ratio of DCF/CMX staining in control cells was set as 1. Data were analyzed by 2-way ANOVA (Figure 2) and relative DCF fluorescence density is expressed as mean ± SD (n =60-80), * $p < 0.05$, *** $p < 0.001$.

2.4 Maintenance of the mitochondrial functionality by the different polarity mastiha fractions upon conditions of H_2O_2-induced oxidative stress.

The protective effect of mastiha fractions on H_2O_2-induced oxidative stress and reduction in cell viability of SH-SY5Y neuroblastoma cells lead us to evaluate the possible mastiha's fractions protective effect on mitochondrial functionality. Thus, mitochondrial membrane depolarization was assessed in SH-SY5Y cells pretreated or not with mastiha fractions and subsequently subjected to H_2O_2 treatment, using the JC-1 dye. JC-1 monomer/aggregate

(green/red) staining was assessed applying confocal microscopy analysis. As shown in Figure 3, H_2O_2 treatment of the cells caused increased depolarization of the mitochondrial membrane as indicated by the increased ratio of the green to red staining compared to control vehicle treated cells. Incubation of SH-SY5Y cells with the apolar, medium polar, and polar mastiha fractions at concentrations of 10 μg/ml, 5 μg/ml, and 10 μg/ml, respectively, did not cause any mitochondrial depolarization, in accordance with results from MTT assay. On the contrary, green/red JC1 staining was lower in the presence of apolar and polar fractions, compared to control vehicle-treated cells, indicating potential beneficial effect of apolar and polar fractions on the preservation of the mitochondrial membrane potential. Interestingly, preincubation of H_2O_2-treated SH-SY5Y cells with the different polarity mastiha fractions led to maintenance of the mitochondrial membrane potential even in the presence of the oxidizing agent.

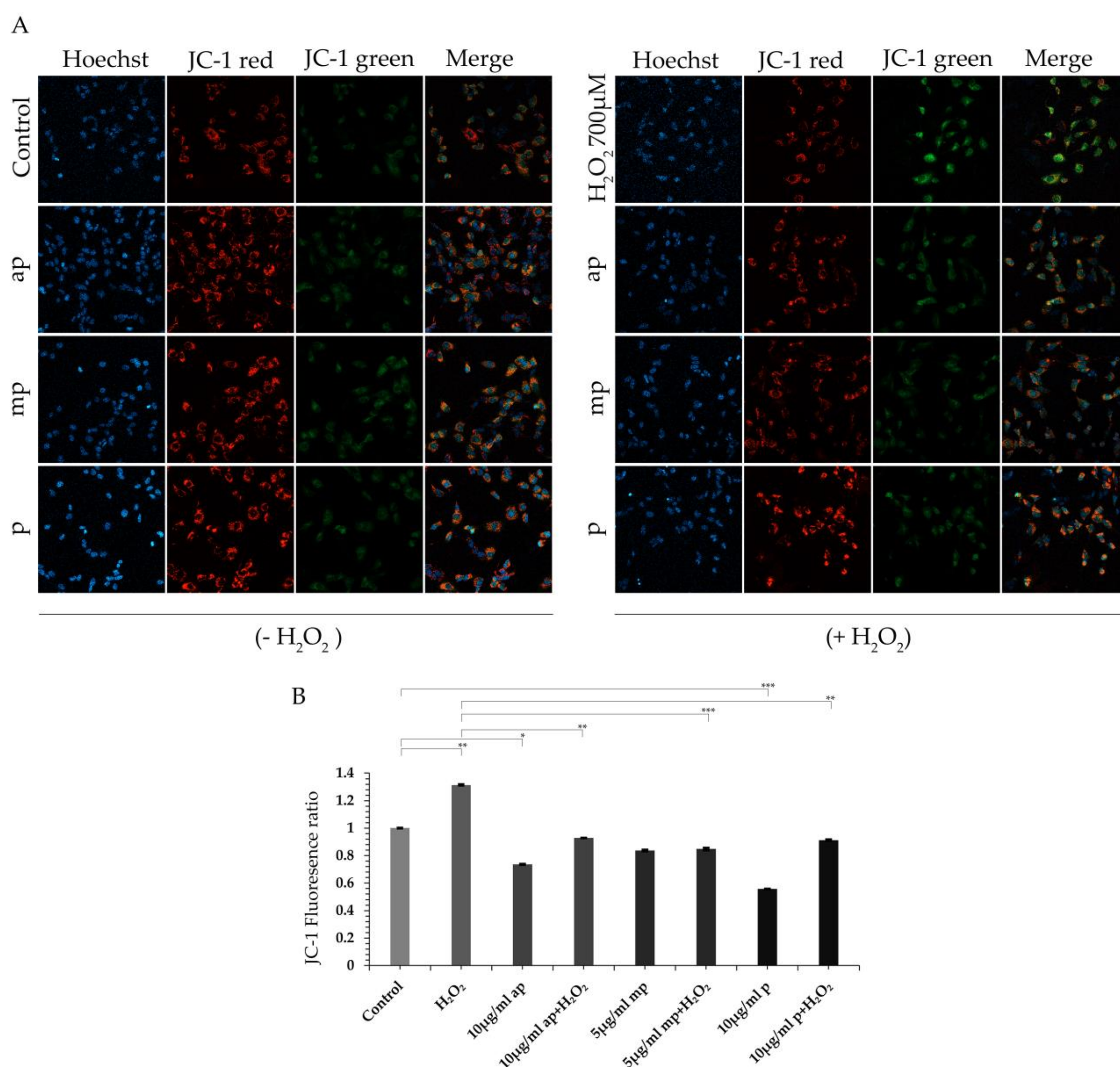

Figure 3: Antioxidant activities of different polarity mastiha fractions contribute to the maintenance of the mitochondrial membrane potential in H_2O_2-treated SH-SY5Y cells. A) Representative images of JC-1 staining (JC-1 dimers-red aggregates; JC-1 monomers-green fluorescence) and Hoechst-33342 staining (nuclear staining) in human neuroblastoma SH-SY5Y cells, pretreated or not with 10 μg/ml ap, 5 μg/ml mp and 10 μg/ml p mastiha fractions for 24 h and then, subjected or not to 700

μM H_2O_2 for 6 h. Vehicle-treated control cells were incubated with 1/1000 v/v DMSO. B) Quantification of green to red staining. Data were analyzed by 2-way ANOVA and JC-1 fluorescence relative ratio (JC-1 monomers fluorescence density to relative JC-1 dimers fluorescence density per single cell) is expressed as mean ± SD (n =35-50), * $p < 0.05$, ** $p<0.01$, *** $p < 0.001$. JC-1 fluorescence ratio of control cells was set as 1.

2.5 Regulation of GR, PPARα και Bcl-2 protein levels by the apolar fraction

Taking into consideration the fatty acids detection in mastiha's fraction and the crucial role of PPARα, a fatty acid activating factor, in the regulation of many cellular fractions including lipid metabolism, antioxidant and anti-inflammatory actions, the effect of mastiha resin fractions on PPARα protein level was assessed. Western blot analysis of PPARα in SH-SY5Y cell extracts pretreated with the ap, mp and p mastiha fractions and subsequently subjected to H_2O_2 treatment revealed a dose dependent increase (1.3 to 2.6 folds increase) in PPARα protein levels. Considerable induction in PPARα levels was also observed by 700 μM H_2O_2 treatment. Similar dose dependent effect, by the ap and mp fractions, was also observed on GR protein levels, whose activity is proposed to be regulated by triterpenes and constitute an activator of PPARα expression (36, 37). Evaluation of mastiha resin fractions on the antiapoptotic Bcl-2 molecule showed a moderate increase (20%) in the Bcl-2 protein levels by the ap fraction and approximately 20% decrease by the mp fraction.

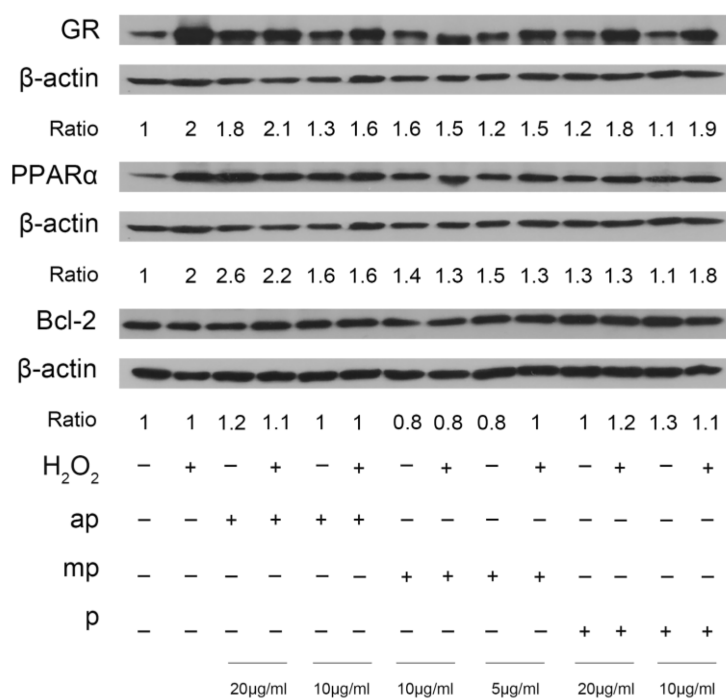

Figure 4: Regulation of the GR, PPARα and Bcl-2 protein levels by the three different polarity fractions (ap, mp, p) of Chios mastic gum. SH-SY5Y cells were treated with 20 μg/ml and 10 μg/ml ap and p mastiha fractions and 10 μg/ml and 5 μg/ml with mp fraction for 24 h and subsequently treated with 700μM H_2O_2 for 6 h. Vehicle cells were incubated with 1/1000 v/v DMSO. Western blot analysis results were expressed as the ratios of the GR, PPARα and Bcl-2 bands intensity normalized against the respective band intensity of the β-actin. Relative protein levels in control cells were set as 1.

3. Discussion

Chios mastiha, the first natural gum, secreted from the *Pistacia lentiscus* L. *var. Chia*, is widely used in Mediterranean cuisine, perfumery, dentistry and cosmetics, because of its distinctive smell and taste. Most importantly, Chios mastiha is also used in traditional medicine from ancient times, mainly because of its therapeutic effects on gastrointestinal disorders (1). Nowadays, many biological activities of Chios mastiha have been uncovered, including anti-microbial, anti-inflammatory, anti-cancer and anti-lipidemic actions (2, 8, 25), which are attributed to mastiha's chemical composition. Specifically, the anti-inflammatory, anti-microbial and apoptotic activities of mastiha are attributed to its enrichment in triterpenoids and phenol compounds (6, 7, 38-43).

In this study we attempted to assess in depth the chemical composition of mastiha as regards the presence of other chemical groups such as fatty acids and unidentified compounds in three different polarity fractions (ap, mp and p). Thus, HPLC-QTOF-MS/MS analysis was applied. Results from the analysis revealed enrichment of apolar and medium polar fraction with fatty acids such as palmitic, stearic, arachidonic acid, and pentadecanoic, compounds that are known to be involved in cellular signaling, affecting, among others, lipid metabolism and inflammatory responses (44-48). To our knowledge, this is the first time that the enrichment of mastiha in such fatty acids is uncovered, shedding light on the identification of the molecular mechanisms of many mastiha's activities such as anti-inflammatory and regulation of fatty acid metabolism. Moreover, the presence and enrichment of polar fraction with acid such as betulinic, euscaphic, linolenic acid, ricinoleic and creoenynic acid was revealed, unwrapping the presence of both saturated but also non saturated fatty acids in mastiha resin. Furthermore, the presence of coniferaldehyde, an organic compound with many biological activities, such as anti-inflammatory, antioxidant, cytoprotective (49-54), but also neuroprotective via Nrf2 activation (55) was detected in apolar fraction. Interestingly, a variety of unidentified compounds were also detected in mastiha's fractions, that remains to be identified. The plethora of identified and non-identified molecules in mastiha's composition unveiled its unique chemical composition which is closely related to its plethora of biological activities.

To further establish a link between mastiha's composition and its biological activities, focusing on the investigation of the antioxidant activities of mastiha resin, the antioxidant, neuroprotective activities of the three different polarity mastiha fractions, apolar, medium polar, and polar, was evaluated in SH-SY5Y cells. Emphasis was given to the elucidation of the effect of mastiha fractions on the mitochondrial origin ROS production, the regulation of factors involved in these processes and the association of the chemical composition of the fractions with their biological activity.

Evaluation of the potential cytoprotective actions of mastic resin fractions on the H_2O_2-induced oxidative stress in SH-SY5Y cells, have shown that the apolar mastiha fraction exerted the highest antioxidant activity, exhibiting statistically significant resistance in H_2O_2-induced reduction in cell viability of SH-SY5Y cells, which was accompanied by resistance in H_2O_2-induced increase in ROS production and mitochondrial membrane depolarization. Enrichment of apolar fraction with triterpenoids may be responsible for the antioxidant activity of apolar fraction of mastiha resin. The terpenoids-related neuroprotective, antioxidant activities of mastiha resin are supported by previous observations demonstrating that triterpenes of mastiha are responsible for decrease in LDL oxidation (13), reduction in several oxidative stress biomarkers (15) and enhancement of the antioxidant status in NAFLD obese patients (17). Moreover, results from the chemical analysis of this study showing enrichment of apolar fraction with fatty acids, such as arachidonic, palmitic, stearic acid and pentadecanoic acid, which constitute signaling molecules with known antioxidant activities (43, 47, 54, 56-61), indicate that the fatty acids mastiha

composition may also contribute to this action. In addition, enhanced antioxidant activities of apolar fraction may be associated with its enrichment with the potential neuroprotective factor coniferaldehyde (55) that is first demonstrated as a mastic compound, in this study.

Resistance to H_2O_2-induced decrease in cell viability, increase in ROS production and mitochondrial membrane depolarization, in SH-SY5Y cells, was also observed by the medium polar fraction, which was also enriched in triterpenes such as lupeol, oleanolic aldehyde, and triterpenoid methyl esters such as 24Z-masticadienonic acid methyl ester and 24Z-isomasticadienonic acid methyl ester, as we have previously shown (12). The considerable antioxidant activities of medium polar fraction may be attributed to lupeol that has been proposed as an antioxidant and anti-inflammatory agent with potential impact on oxidative stress resistance in Alzheimer's disease (42, 62).

Structural similarities of triterpenoids with glucocorticoids (27) prompted us to evaluate whether the antioxidant activity of mastiha's fractions is related with the interference of their components with glucocorticoid signaling. We observed that ap fraction caused increase in GR protein level, which was accompanied by increase in PPARα that constitutes a GR target (36, 37). This action further supports the potent antioxidant activity of apolar and medium polar mastiha's fraction, since PPARα is consider as an antioxidant factor, inducing among others catalase expression, and its expression is known to be triggered in the presence of antioxidant factors (63-66). Apolar (ap) fraction-induced increase in PPARα protein level may be associated with the enrichment of the fraction with palmitic and stearic fatty acids, which are known as PPARα agonists and induce PPARα gene expression (44, 67, 68). Arachidonic acid, an apolar fraction identified compound, which is known to interact with GR signaling (69), may also contribute to the ap-induced anti-inflammatory actions (12). Polar (p) mastiha fraction also showed antioxidant activity, via reversal of the H_2O_2-induced increase in ROS production and mitochondrial membrane depolarization in SH-SY5Y cells. Since polar fraction is enriched in phenolic compounds, its antioxidant activity may be associated with these compounds, well-known for its antioxidant activity (70).

To conclude, in this study the characterization of many fatty acids as mastiha's compounds was uncovered. Moreover, the antioxidant activity of three different polarity mastiha fractions, apolar, medium polar and polar, was detected in human neuroblastoma SH-SY5Y cells. Apolar and medium polar fractions, enriched in triterpenes, fatty acids and coniferaldehyde caused the highest antioxidant activity as documented by the enhanced suppression of the H_2O_2-induced reduction in cell viability, increased in the PPARα protein level, ROS production and depolarization of the mitochondrial membrane potential of SH-SY5Y cells. Antioxidant activity was also observed by the polar fraction which can be attributed to its enrichment in phenolic compounds. Our results indicate neuroprotective actions of mastiha fractions, highlighting their potential application as neuroprotective agents in neurodegenerative diseases.

4. Materials and Methods

4.1 Chemicals

Dulbecco's Modified Eagle Medium (DMEM), Trypsin, Fetal Bovine Serum (FBS), MitoTracker Red CMX Ros (CMX), the 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (6-carboxy H2DCFDA), and the (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) JC-1, were obtained from Thermo Fisher Scientific (GmbH, Basel, Switzerland). The Molecular protein weight marker was purchased from ProteinTech (Rosemont, IL, USA, North America). Cocktail protease inhibitors were purchased from

Roche (Mannheim, Germany). All other solvents and chemicals including H_2O_2 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.2 Chios mastiha fractionation and chemical characterization

Chios mastiha three different polarity fractions were obtained as previously described [12, 14]. Details on the methodology applied are presented in the Supplementary Materials (Supplementary Data 1). The methodology applied for the chemical characterization of the three different polarity fractions of Chios Mastiha is followed. Chromatographic analysis was performed on an ExionAC LC system (SCIEX, MA) equipped with a controller, two pumps, a degasser, and an auto-sampler. The X500R Q-TOF mass spectrometer (SCIEX, Framingham, MA) was equipped with an electrospray ionization (ESI) turboVTM source and operated in negative ionization mode. TOF-MS and TOF-MS/MS data were acquired using a data-dependent acquisition ESI mode. For the separation of the analytes, a Fortis C18 column (100 mm length, 2.1 mm i.d, 2.6 μm particle size) purchased from Fortis (Cheshire, United Kingdom) was used, thermostated at 40°C. The mobile phase consisted of: (A) 0.1% (v/v) formic acid in water and (B) 0.1%(v/v) formic acid in methanol. The flow rate was initially set at 0.2 mL min^{-1}, increased to 0.4 mL min^{-1} at 15 min, and decreased to 0.2 mL min^{-1} at 16 min. The elution program was as follows: 0–4 min, 99%–61%A; 4–12 min, 61%–5% A; 12–15 min, 5% A; 15–16 min, 5%–99% A; 16–20 min, 99% A. The QTOF-MS system was equipped with an ESIinterface, operating in negative mode with the following settings: spray voltage, 4500 V; heater gas temperature, 55°C; declustering potential, 80 V. The MS/MS spectra were obtained at a collision energy of 45 eV and a collision energy spread of 15 eV. External calibration was performed before analysis with a cluster solution provided by SCIEX, and additionally, the calibration solution was injected at the beginning of each run for internal calibration and once per five samples during batch acquisition. Mass spectra were recorded in the m/z range from 50 to 1000 at an accumulation time of 0.25 s. MS/MS experiments were conducted in data-dependent acquisition mode at an accumulation time of 0.08 s for the 10 most abundant precursor ions per full scan. Sample acquisition was monitored by SCIEX OSsoftware. Extraction ion chromatograms were generated using SCIEXOS software. Non-target screening was carried out using Analytics SCIEX OS software and the SCIEX Natural Products Library.

4.3 Cell culture

SH-SY5Y cells were used in the study. SH-SY5Y is a human neuroblastoma cell line widely used as an in vitro model for a variety of neurobiological analyses [32]. SH-SY5Y cells were obtained from the American type culture collection (ATCC) and were cultured in DMEM 1g/L glucose, with phenol red, 10% v/v FBS, 2 mM L-glutamine and 100 units/mL penicillin/streptomycin at 37°C and 5% CO_2. For live cell fluorescence microscopy analysis, cells were maintained in low glucose (1g/L), without phenol red DMEM, supplemented with 2 mM L-glutamine and 100 units/mL penicillin/streptomycin.

4.4 Antibodies

For Western blot analysis specific antibodies against Bcl-2, PPARα, GR, and β-actin, were used. Monoclonal antibodies against GR, PPARα, and β-actin were obtained from Santa Cruz Biotechnology (Europe Inc., Heidelberg, Germany) and ProteinTech (Rosemont, IL, USA, North America), respectively. Polyclonal antibodies against Bcl-2, were purchased from Cell Signaling Technology (Leiden, Netherlands).

4.5 Cell Viability Assay-MTT

To investigate the effects of different polarity fractions, apolar (ap), medium polar (mp) and polar (p) from mastiha resin on cell viability of SH-SY5Y cells, in the presence or

absence of H_2O_2, MTT assay was applied as previously described [33]. Briefly, 8 x 10^3 cells were plated in 96-well plate and cultured in low glucose (1g/L) DMEM, supplemented with 10% v/v FBS, 2 mM L-glutamine and 100 units/mL penicillin/streptomycin for 24 h. Then, cells were treated with 10 and 20 μg/ml of apolar and polar mastiha fractions and with 5 and 10 μg/ml of medium polar fraction for 24 h. Incubation with 700 μM H_2O_2 for 6 h was followed. Control, vehicle-treated cells were incubated with 1/1000 v/v DMSO for 24 h as well. Then, MTT reagent was added at a final concentration 0.5 μg/ml for 3 h at 37°C. Finally, the produced formazan crystals were dissolved in 100% isopropanol, upon shaking and the absorbance was measured at 570 nm and 690 nm, as a reference, in a multimode plate reader (EnSpire, PerkinElmer, Beaconsfield, UK). Relative cell viability was expressed as the viability of the cells treated with the indicated concentrations of the respective different polarity mastiha fractions (apolar, medium polar and polar) and/or H_2O_2, compared to the cell viability of the vehicle-untreated (control) cells. The viability of control cells was set as 1.

4.6 Electrophoresis and Western blot analysis

For Western blot analysis 25 x 10^4 SH-SY5Y cells were growth in 6-well plates in low glucose (1g/L glucose) DMEM supplemented with 10% v/v FBS, 2 mM L-glutamine and 100 units/mL penicillin/streptomycin for 48 h. Then, cells were incubated with 20 μg/ml and 10μg/ml of apolar and polar mastiha resin fraction, and with 10μg/ml and 5μg/ml for medium polar fraction, for 24 h. Subsequently, SH-SY5Y cells were further incubated with 700 μM H_2O_2 for 6 h, or 1/1000 v/v DMSO (control vehicle-treated cells). Then, cells were washed with PBS (4°C) and lysed in lysis buffer (20 mM Tris-HCl pH:7.5, 250 mM NaCl, 0.5 % v/v Triton-X, 3 mM EDTA, supplemented also with DTT, PMSF and cocktail of protease inhibitors). Bradford assay was applied for protein determination [12]. Then, cell extracts were electrophoresed in discontinuous SDS-PAGE and Western blotted with specific antibodies against Bcl-2, GR, PPARα and β-actin as previously described [34]. Enhanced chemiluminescence was used for the detection of the protein bands. Quantification of proteins band density was performed by the ImageJ program (v1.52). β-actin bands intensity was used for the normalization of the results. Relative protein levels were expressed as band intensity normalized against the respective band's intensity of β-actin. Relative protein levels in control vehicle-treated cells were set as 1 [29].

4.7 Intracellular ROS measurement

To evaluate the antioxidant activity of different polarity fractions from Chios mastiha, total ROS production measurement was held in SH-SY5Y cells pretreated with apolar (ap), medium polar (mp), polar (p) fractions and then subjected to H_2O_2-induced oxidative stress. 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, (6-carboxy H2DCFDA) dye (Thermo Fisher Scientific) was used for ROS detection. The cell-permeant H2DCFDA is a nonfluorescent indicator for reactive oxygen species (ROS) in cells. Upon cleavage of the acetate groups by intracellular esterases and oxidation, the nonfluorescent H2DCFDA is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF). Thus, for ROS production measurement, 1 x 10^5 SH-SY5Y cells were plated on 60 x 15 mm culture dishes and cultured in low glucose (1g/L) DMEM, supplemented with 10% v/v FBS, 2 mM L-glutamine and 100 units/mL penicillin/streptomycin for 48 h. Then, cells were incubated with 10 μg/ml of ap, and p fractions or with 5 μg/ml of mp fraction for 24 h. Vehicle (1/1000 v/v DMSO)-treated cells were used as control. Subsequently, cells were subjected to H_2O_2 (700 μM) or vehicle (1/1000 H_2O) treatment for 6 h. Then, cell culture medium was replaced with low glucose (1g/L glucose), without phenol red DMEM, supplemented with 2 mM L-glutamine and 100 units/mL penicillin/streptomycin, containing 10 μM H2DCFDA dye, 200 nM CMX and 1 μg/ml Hoecsht-33342 at 37°C for 30 min. Finally, cells were washed in

DMEM without phenol red and subjected to live cell imaging by confocal microscopy analysis. Fluorescence excitation of the oxidized DCF-DA dye was at 488 nm, and fluorescence emission was measured at 530–550 nm using a Zeiss LSM 800 confocal microscope [35].

4.8 Analysis of mitochondrial membrane potential

To assess the ability of mastic resin fractions to reverse the H_2O_2-induced mitochondrial membrane depolarization of SH-SY5Y cells, mitochondrial membrane potential changes were measured using (5,5′,6,6′-tetrachloro-1,1′,3,3′- tetraethylbenzimidazolylcarbocyanine iodide) JC-1 dye. JC-1 is a lipophilic, cationic dye, which can enter the mitochondria where it accumulates and forms reversible red fluorescent J-aggregates. In apoptotic cells the JC-1 dye also enters the mitochondria but to a lesser degree since the inside of the mitochondria is less negative. Under such circumstances, JC-1 does not reach the appropriate concentration to trigger the formation of J aggregates, thus retain as a green fluorescence dye. Based on that, assessment of the green/red fluorescence ratio depicts the state of the mitochondrial membrane polarization [36]. For JC-1 assay, 1 x 10^5 SH-SY5Y cells were plated on 60 x 15 mm culture dishes and cultured in low glucose DMEM (1g/L glucose), supplemented with 10% v/v FBS, 2 mM L-glutamine and 100 units/mL penicillin/streptomycin for 48 h. Then, cells were treated with 10 μg/ml of apolar (ap), and polar (p) fraction or with 5 μg/ml of medium polar (mp) fraction for 24 h or with 1/1000 v/v DMSO (control cells) and subsequently cultured in the presence or absence of 700 μM H_2O_2 for 6 h. Finally, cells were incubated with 1 μg/ml Hoechst-33342 and 2 μM JC-1 in without phenol red-DMEM cultured medium, at 37°C for 30 min. Then, DMEM was replaced with fresh medium, and cells were subjected to confocal microscopy live imaging analysis. Green JC-1 fluorescence was measured at the excitation of 488 nm and the emission was measured at 494–535 nm. The red fluorescent J-aggregates were measured at the excitation of 560 nm and the emission wavelength was at 560–590 nm, using a Zeiss LSM 800 confocal microscope [35].

4.8 Statistical analysis

Results are expressed as mean ± SD. Data were analyzed by one-way analysis of variance (one-way ANOVA) or two-way ANOVA followed by Tukeys's post-hoc test using StatPlus LE 7.3.0 Software (AnalystSoft, Brandon, FL, USA). Differences were considered significant at a two tailed P value < 0.05.

Supplementary Materials: Protocol of Chios mastiha fractionation. Table S1: Suspect compounds identified in different polarity fractions from Chios mastiha by HPLC-QTOF-MS/MS analysis, their chemical classification and biological activities. Table S2: Non-target screening results, identified by HPLC-QTOF-MS/MS analysis, in different polarity fractions from mastiha, their chemical classification, and biological activities. Table S3: Unequivocal molecular formulas and hit scores of non-target compounds identified in apolar, medium polar and polar fractions from Chios mastiha assigned by SCIEX OS software and the SCIEX Natural Products Library.

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619