Mentha pulegium crude extracts induce thiol oxidation and potentiate hemolysis when associated to t-butyl hydroperoxide in human's erythrocytes

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ABSTRACT

Mentha pulegium (Lamiaceae) tea has been used as a traditional medicine; however, the modulatory effect of *M. pulegium* extracts on damage to human erythrocytes associated to t-butyl hydroperoxide (t-BHP) exposure remains to be investigated. Accordingly, we perform this study in order to test the hypothesis that aqueous and ethanolic extracts of *M. pulegium* could modulate the hemolysis associated to t-BHP exposure, non-protein thiol (NPSH) oxidation and lipid peroxidation (measured as thiobarbituric acid reactive substances - TBARS) in human erythrocytes. Samples were co-incubated with t-BHP (4 mmol/L) and/or aqueous or ethanolic extracts (10-1000 mg/mL) during 120 min to further analysis. We found that both extracts, when associated to t-BHP, potentiate NPSH oxidation and hemolysis. Moreover, both extracts significantly prevents against t-BHP-induced TBARS production. A significant correlation among hemolysis and NPSH levels was found. Taking together, our data points that the association of *M. pulegium* extracts with t-BHP culminates in toxic effect to exposed erythrocytes, besides its protective effect against t-BHP-induced TBARS production. So, we infer that the use of this extract may exert negative effect during painful crisis in sickle cell anemia. However, more studies are still necessary to better investigate/understand the mechanism(s) involved in the toxic effect resultant from this association.

Key words: *Mentha pulegium*, Lamiaceae, hemolysis, lipid peroxidation, NPSH oxidation, erythrocytes.

INTRODUCTION

The interest in research regarding pharmacological activity of plants has increased in last decades. Of particular importance, it has been recently reported that the consumption of medicinal plant extracts may exert positive effects on different models of human disorders (Martins et al. 2012, Courtes et al. 2015, Salgueiro et al. 2016). Accordingly, consumption of plant extracts supplies active compounds that may have different beneficial functions in the body (Doblado et al. 2005, Oboh 2005, 2006). Interestingly, literature data have indicated that the biological properties of different diets can be lost after isolation of specific compounds, indicating that part of their pharmacological properties can be related to a combination of different classes of such compounds (Pietrovski et al. 2006, Klimaczewski et al. 2014). As a consequence, researches with crude

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extracts (*i.e.* in the form that are primarily consumed by people) are necessary/useful to better address the pharmacological/toxicological of plants.

Mentha pulegium (Lamiaceae) tea has been used as a traditional medicine for the treatment of fever, pain and dysentery (Vertuani et al. 2004). Of note, *M. pulegium* was found to contain flavonoids that may account for the high antioxidant activity observed for the polar extracts of this aromatic herb (Zaidi et al. 1998, Justesen and Knuthsen 2001). Additionally, *M. pulegium* was reported to present anti-genotoxic effect and antiacetylcholinesterase activity (Romero-Jimenez et al. 2005, Mata et al. 2007). However, to the best of our knowledge, the modulatory effect of aqueous extract (AE) and ethanolic extract (EE) from *M. pulegium* on damage to human erythrocytes associated to t-butyl hydroperoxide (t-BHP) exposure remains to be investigated.

Accordingly, erythrocytes are a convenient model to understand oxidative damage to membranes, once they are particularly sensitive. Accordingly, they have been used as a model for the investigation of free-radical induced oxidative stress due to its high amounts of polyunsaturated fatty acids, oxygen and heme (Konyalioglu and Karamenderes 2005, Nikolaidis and Jamurtas 2009). Additionally, the use of complementary/alternative models are necessary in order to preliminary determine the potential safe or risk of a great number of extracts and/or its phytoconstituents. Considering the exposed, this study was design to test the hypothesis that AE and EE of *M. pulegium* could modulate the hemolysis associated to t-BHP exposure, NPSH oxidation and lipid peroxidation in human erythrocytes.

MATERIALS AND METHODS

PLANT MATERIAL, EXTRACT PREPARATION AND QUANTIFICATION OF COMPOUNDS BY HPLC-DAD

M. pulegium was obtained from local commercial sources. Accordingly, three independent batches (SN/132; SN/133 and SN/134) were randomly purchased and used in this study. Extracts were prepared and characterized by HPLC accordingly previously described (Tamborena et al. 2015). In short, ethanolic extract (EE) was prepared by using dried plant (0.9 g) that was macerated with 10 mL of ethanol and maintained in this solvent for further 7 days. On the 7th day, the resulting solvent was filtered and evaporated to dryness under reduced pressure. Thereafter, the resulting pellet was re-suspended in 6.6 mM phosphate buffer, pH 7.4, containing 150 mM NaCl at the same volume used to the extraction. In turn, the aqueous extract (AE) of *M. pulegium* was prepared by mimicking its popular use, except that phosphate buffer saline (6.6 mM; pH 7.4) was used in the preparation in order to avoid effect of osmolality in our experimental conditions. Briefly, 0.9 g of the dried plant was prepared by infusion (10 min) using 10 mL of 6.6 mM phosphate buffer, pH 7.4, containing 150 mM NaCl. The aqueous extract was prepared just before use.

Thereafter, both AE and EE extracts from *M. pulegium* were furter analysed, for quantification of compounds by HPLC-DAD, as previously described (Tamborena et al. 2015). Accordingly, high performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (YL9100) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu YL9110 reciprocating pumps connected to an YL9101 degasser with an YL9150 integrator, and YL9160 diode array detector. The phenolic compound profiles were determined according to the following procedure: The extracts were analyzed using a reversed phase carried out under gradient conditions using Synergi Fusion-RP 80A column (4.6 mm x 250 mm). The mobile phase was composed of water (pH=3): acetonitrile (5:95, v/v) in a gradient mode, until 35 min, in which the mobile phase was 100% acetonitrile. At 38 min water (pH=3): acetonitrile (5:95, v/v) was used again, in isocratic mode, as a mobile phase, until 50 min. A flow rate of 0.8

ml/min was used, 20 µL of sample were injected and the wavelengths were: 220 nm for gallic acid, benzoic acid, syringic and vanilic acid, and rutin; 320 nm for caffeic acid, cumaric acid, and ferulic acid; and 368 nm quercetin. Phenolic compounds were identified and quantified by comparing the retention time and UV-visible spectral data to known previously injected standards. The chromatography peaks were confirmed by comparing the retention time with those of reference standards and by DAD spectra. Calibration curve for standard compounds were performed as described previously (Tamborena et al. 2015). All chromatography operations were carried out at ambient temperature and in triplicate, revealing the presence of the rutin and benzoic acid (being rutin in greater amount as compared to benzoic acid) in both extrtacts (more details are provided in Tamborena et al. 2015); whereas gallic acid, syringic acid, vanilic acid, caffeic acid, ferulic acid, cumaric acid and quercetin were not found on these extracts.

PREPARATION OF ERYTHROCYTES SUSPENSION AND EXPOSURE

Erythrocytes were prepared and used according previously described (Santos et al. 2009). Samples were co-exposed to t-BHP (4 mM) and/or *M. pulegium* extracts (10 – 1000 µg/mL) for 120 min at 37 °C. Thereafter, erythrocytes were washed three times with phosphate buffer saline (6.6 mM, pH 7.4, containing 150 mM NaCl) and subsequent biochemical assays were performed as described below. The protocol was reviewed and approved by the appropriate institutional review board from Guidelines of the Committee of UNIPAMPA (127.779).

BIOCHEMICAL ASSAYS

The hemolysis test was performed accordingly to Schiar et al. 2007. The levels of non-protein thiols (NPSH) were determined accordingly to Ellmann (1959); while TBARS were determined accordingly to Ohkawa et al. 1979.

STATISTICAL ANALYSIS

Statistical significance was assessed by one way analysis of variance (ANOVA), followed by Tukey's multiple range test, when appropriate. All results were expressed as mean ± SEM and the differences were considered significant when $P<0.05$. Pearson correlation between variables was also carried out.

RESULTS

The effect of *M. pulegium* on hemolysis is depicted in Table I. Accordingly, *M. pulegium* AE (at 100 µg/mL – at saline ranging from 0.7 to 0.9%) and EE (at 10 µg/mL – at saline ranging from 0.65 to 0.75%; and at 100 µg/mL – at saline ranging from 0.65 to 0.9%) significantly induced hemolysis under basal (without t-BHP) conditions. However, in *M. pulegium* treated cells the degree of hemolysis associated to t-BHP exposure was significantly higher (approximately 100%) than that found in t-BHP treated cells, irrespective of *M. pulegium* and/or saline concentrations. In turn, t-BHP significantly induced hemolysis *per se* only at saline ranging from 0.65 to 0.75%.

The effect of *M. pulegium* on basal and/or t-BHP-induced NPSH oxidation is depicted in Figure 1. Accordingly, *M. pulegium* AE (Figure 1a; at 10 and 100 µg/mL) and EE (Figure 1b; at 10-1000 µg/mL) significantly induced NPSH oxidation in cells exposed to t-BHP. In contrast, *M. pulegium* AE (Figure 1a; at 1000 µg/mL) significantly increases the NPSH content under basal conditions. Finally, neither EE (Figure 1b) nor t-BHP (Figure 1) significantly changed basal NPSH levels *per se*.

The effect of *M. pulegium* on basal and/or t-BHP-induced TBARS production is depicted in Figure 2. Accordingly, both *M. pulegium* AE (Figure 2a) and EE (Figure 2b) significantly prevent the t-BHP-induced TBARS production, being without effect on basal TBARS levels (Figure 2).

Finally, Table II summarizes the correlations among NPSH content and/or TBARS on hemolysis.

TABLE I
Effect of *M. pulegium* AE and EE on basal and/or t-BHP associated hemolysis.

Saline concentration	mg/mL of Dried Plant	Without t-BHP		With t-BHP	
		Aqueous Extract	Ethanolic Extract	Aqueous Extract	Ethanolic Extract
0.4%	0	38,62 ± 3,581	32,51 ± 4,122	99,55 ± 4,389[#]	95,46 ± 0,738[#]
	10	56,68 ± 3,294*	65,12 ± 3,206*	98,89 ± 0,443	98,53 ± 0,435
	100	48,37 ± 3,019	53,14 ± 1,808*	99,62 ± 0,090	99,54 ± 0,002
	1000	59,48 ± 4,487*	62,96 ± 1,429*	99,67 ± 0,375	98,74 ± 0,046
0.65%	0	11,71 ± 2,763	11,51 ± 2,763	27,44 ± 3,045#	27,24 ± 4,244[#]
	10	25,97 ± 4,792	30,26 ± 2,148*	98,66 ± 0,660*	97,91 ± 0,341*
	100	24,18 ± 4,569	35,17 ± 2,211*	99,67 ± 0,077*	99,54 ± 0,002*
	1000	25,43 ± 6,028	17,27 ± 0,616	99,32 ± 0,395*	99,05 ± 0,0347*
0.7%	0	8,210 ± 2,474	8,224 ± 3,600	21,35 ± 3,192[#]	21,33 ± 3,598[#]
	10	19,11 ± 4,055	24,77 ± 2,705*	98,55 ± 0,595*	97,91 ± 0,341*
	100	21,59 ± 4,834*	34,04 ± 2,471*	99,69 ± 0,100*	99,85 ± 0,009*
	1000	16,22 ± 2,737	14,85 ± 1,032	99,03 ± 0,378*	99,21 ± 0,028*
0.75%	0	7,440 ± 2,634	7,403 ± 3,957	18,77 ± 2,747#	18,97 ± 3,616[#]
	10	18,25 ± 4,064	23,46 ± 2,233*	98,56 ± 0,619*	97,91 ± 0,341*
	100	20,86 ± 4,789*	33,36 ± 2,836*	99,69 ± 0,100*	100,0 ± 0,307*
	1000	14,42 ± 2,663	15,12 ± 0,743	99,31 ± 0,446*	99,86 ± 0,303*
0.8%	0	10,311 ± 2,382	10,62 ± 5,781	16,12 ± 2,311	16,45 ± 1,337
	10	16,81 ± 3,617	21,47 ± 0,862	98,50 ± 0,604*	98,28 ± 0,619*
	100	19,67 ± 4,777*	33,36 ± 2,836*	99,67 ± 0,077*	100,2 ± 0,399*
	1000	12,23 ± 2,282	14,96 ± 0,739	99,16 ± 0,428*	99,54 ± 0,300*
0.9%	0	9,568 ± 2,510	9,45 ± 5,436	14,69 ± 4,650	14,34 ± 0,888
	10	13,71 ± 2,751	20,28 ± 0,815	98,44 ± 0,617*	99,04 ± 0,649*
	100	18,11 ± 4,454*	38,47 ± 4,605*	99,53 ± 0,162*	99,69 ± 0,308*
	1000	10,99 ± 1,940	15,22 ± 0,565	99,13 ± 0,414*	99,70 ± 0,301*

The values are expressed % of control (samples exposed to saline 0% were considered with 100% of hemolysis); (n=6). * indicates differences respective control (without *M. pulegium* extracts) by one-way ANOVA following by Tukey's test. # indicates differences among control (without plant extracts) and t-BHP by one-way ANOVA following by Tukey's test.

In short, we found that NPSH content was negatively and significantly associated with hemolysis, at all saline concentrations and both in AE and EE of *M. pulegium*. In turn, TBARS was found to be practically not correlated to hemolysis, except at saline 0.4% (both AE and EE) and at saline 0.9% (only EE) in which TBARS levels were positively and significantly associated with hemolysis (Table II).

DISCUSSION

We found here that the exposure to t-BHP increased TBARS levels and makes erythrocytes more prone to hemolysis when exposed to low saline (0,4% - 0,75%). Importantly, t-BHP does not induce hemolysis *per se* at saline ranging from 0.8% to 0.9%, and does not induced NPSH oxidation. Accordingly, we suggest that the t-BHP-induced damage to lipids (evaluated here as TBARS) weakens the membrane making cells more susceptible to hemolysis at lower saline, which are in accordance to literature (Trotta et al. 1983, Itri et al. 2014). In turn, we found here that t-BHP does not increase NPSH oxidation and methaemoglobin content (data not shown) under our experimental
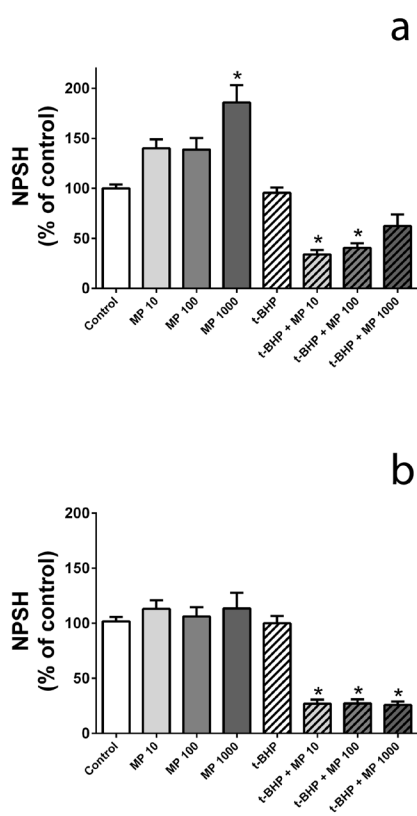
Figure 1 – Effect of *M. pulegium* AE (**a**) and EE (**b**) on basal and/or t-BHP-induced NPSH oxidation in erythrocytes. * indicates statistical difference (P<0.05) from respective control (*i.e.* from basal or from t-BHP); n=5; MP = *Mentha pulegium.*

Figure 2 – Effect of *M. pulegium* AE (**a**) and EE (**b**) on basal and/or t-BHP-induced TBARS production. * indicates statistical difference (p<0.05) from respective control (*i.e.* from basal or from t-BHP). # indicates statistical difference (P<0.05) from basal; n=5; MP = *Mentha pulegium.*

conditions, contrasting to previous data (Trotta et al. 1983, Adesanoye et al. 2013, Portela et al. 2017).

Our data also confirms the hypothesis that both AE and EE extracts of *M. pulegium* could modulate the oxidative changes associated to t-BHP exposure in human erythrocytes. Our data also supports the notion that hemolysis is significantly correlated to NPSH content and (at some extent) to TBARS. Anyway, we cannot exclude the possibility that other(s) mechanism(s) is(are) also involved in hemolysis. Importantly, we suggest that the putative incorporation of phenolic compounds from extracts in the membranes of erythrocytes can also contribute to hemolysis (both in non-exposed
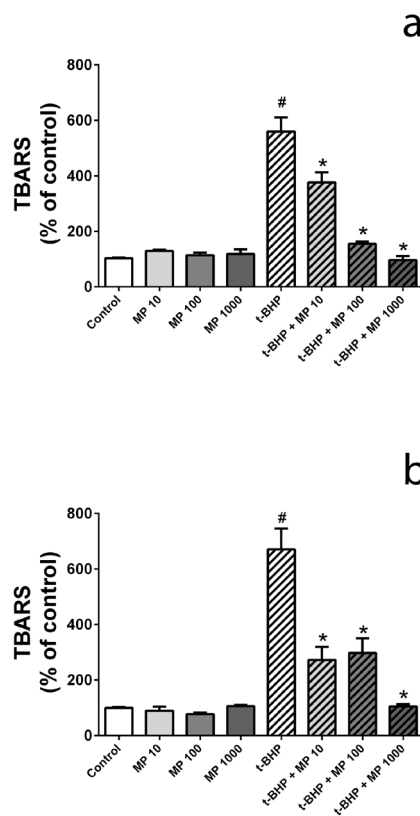
and exposed erythrocytes), which is in accordance to literature (Bors et al. 2012, Duchnowicz et al. 2012). On the other hand, it cannot be excluded that incorporation of phenolic compounds contained in extracts can also positively influence the red blood cells (Bors et al. 2012, Wloch et al. 2016). Of note, differences in the chemical structure of phenolic are critical to determine the type of interactions that occurs between these compounds and membrane surface (Oteiza et al. 2005, Hendrich 2006, Bors et al. 2012, Duchnowicz et al. 2012).

In line with this, rutin (also called quercetin-3-O-rutinoside; one of the major metabolites found in *M. pulegium* extracts - see Tamborena et al.

TABLE II
Pearson correlations between saline concentration and NPSH or TBARS content.

Saline concentration	0.4%	0.65%	0.7%	0.75%	0.8%	0.9%
Aqueous Extract						
NPSH content	-0.4112*	-0.6601*	-0.6808*	-0.6834*	-0.6925*	-0.6621*
	P=0,0084	P< 0,0001	P< 0,0001	P< 0,0001	P< 0,0001	P< 0,0001
TBARS	0.5218*	0.0837	0.0754	0.0572	0.0468	0.1880
	P=0,0006	P=0,6076	P=0,6435	P=0,7257	P=0,7743	P=0,2453
Ethanolic Extract						
NPSH content	-0.4588*	-0.6909*	-0.6914*	-0.6929*	-0.7035*	-0.5400*
	P=0,0029	P< 0,0001	P< 0,0001	P< 0,0001	P< 0,0001	P= 0,0003
TBARS	0.5142*	0.0251	0.0075	0.0030	0.0115	0.4912*
	P=0,0007	P=0,8778	P=0,9630	P=0,9851	P=0,9439	P=0,0013

2015) was previously found to modify membrane-dependent processes, such as the lipid peroxidation (Saija et al. 1995). In the same study, authors suggest that this effect could be related not only to their structural characteristics but also to their ability to interact with and also penetrate the lipid bilayers (Saija et al. 1995), reinforcing our assumption that *M. pulegium* extracts could induce hemolysis due to membrane destabilization/disorganization (due to interaction among phospholipids and phytochemicals), at the same time that prevent TBARS production due to its antioxidant activity. Similarly, it was previously found that quercetin (a phenolic compound structuraly related to rutin) leads to a significant changes in size and shape of the treated erythrocytes (Pawlikowska-Pawlega et al. 2003, Bors et al. 2012). Of particular importance, the effect of quercetin was due to its incorporation into erythrocyte membrane and also due to its interaction with cytoskeleton proteins. Indeed, it was previously shown that cytoskeletal proteins and integral membrane proteins are responsible for maintaining of erythrocytes morphology (Sato et al. 1986, Bors et al. 2012).

Both AE and EE from *M. pulegium* were found also to produce protective effect against t-BHP-induced TBARS production, which may be attributed to its antioxidant potential (Jain et al. 2012, Brahmi et al. 2017). Accordingly, it is reasonable to conclude that both extracts from this plant protects erythrocytes from lipid damage cased by t-BHP. On the other hand, our results allow us to also suggest that both extracts enhances t-BHP-induced hemolysis by forming a complex(es) with cell components released during damage mediated by t-BHP, which are believed to destabilizes the cell membrane, accordingly to previously described (Huy et al. 2002, 2004, Lisovskaya et al. 2009).

Importantly, the potentiation in the t-BHP-induced hemolysis, by both extracts from *M. pulegium*, allows us to suggest that this plant may act as a cytotoxic agent in pathologies associated with oxidative stress and increased blood cells damaged (e.g., intravascular hemolysis and others). As a consequence, the implications of these findings may be double-faced. Indeed, the use of this extract may have/exert negative/adverse effect during painful crisis in sickle cell anemia, similar to caffeine (Wang et al. 2009). In contrast, the effect of this plant on membrane of blood cells may be employed to increase the efficacy of antitumor

therapy, accordingly to previous report (Elsyana et al. 2016, Saghir et al. 2016, Tabrizi et al. 2016).

The potentiation in NPSH and in the hemolysis in erythrocytes treated with plant extracts and t-BHP deserves, doubtless, further research to investigate the involved mechanism(s). Likewise, the increase in NPSH content in erythrocytes treated with AE of this plant needs to be further addressed. Importantly, with respect to increase in NPSH content, it was previously found that erythrocytes exposed to *Bauhinia forficata* and to *Ilex paraguariensis* aqueous extracts, under *in vitro* conditions, were found to have an increase in NPSH content (Salgueiro et al. 2013, Portela et al. 2017), which is similar to results present here.

In conclusion, we found here that association of AE or EE from *M. pulegium* with t-BHP makes erythrocytes more prone to hemolysis, which was significantly correlated to NPSH oxidation, besides protective effect against t-BHP-induced TBARS.

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