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Ex vivo and *in vivo* antioxidant activity of β-hydroxy-β-arylalkanoic acids

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Abstract: The interplay between oxidative stress and inflammation is implicated in many chronic diseases including Alzheimer`s disease, cardiovascular diseases, diabetes and cancer. Thirteen β-hydroxy-β-arylalkanoic acids were previously synthesized and evaluated for their anti-inflammatory activity. The aim of this study was to asses *ex vivo* antioxidant activity of synthesized acids, as well as ibuprofen and to identify the compounds with the most promising results for further investigation on their capacity to counteract *in vivo* oxidative stress triggered by inflammation. The antioxidant potential of tested compounds was evaluated by determining the concentrations of total antioxidative status, total oxidative status, prooxidant antioxidant balance and the total sulfhydryl groups. Z score statistics were used to calculate the summary scores for antioxidative activity, prooxidative activity and oxy score. The tested compounds and ibuprofen demonstrated mild prooxidative activity *ex vivo*. Seven acids with substituents on one benzene ring exhibited better results than ibuprofen and were selected for *in vivo* testing. *In vivo* results demonstrated better antioxidant protection compared to *ex vivo* results. Compound g which contains nitro group on the benzene ring demonstrated the lowest oxy score, and four compounds exhibited better results than ibuprofen.

Keywords: Inflammation, oxidative stress, oxy score, anti-inflammatory compounds.

INTRODUCTION

Living organisms produce pro-oxidants such as reactive oxygen and nitrogen species, as normal byproducts of many metabolic pathways. Organisms have evolved intricate mechanisms of combating these reactive species, including the production of enzymatic and non-enzymatic antioxidants. However, when the production of pro-oxidants exceeds the amount of antioxidants, oxidative stress occurs. Pro-oxidants can damage lipid structures, proteins and DNA molecules (Rifai *et al.,* 2018). Inflammation is a defensive reaction of organism to various endogenous or exogenous stimuli such as injury, pathogens or chemicals (Medzhitov 2010). Oxidative stress and inflammation are closely linked interdependent processes (Biswas 2015). Persistent inflammation and oxidative stress, can lead to the development of chronic diseases like diabetes (Yaribeygi *et al*, 2020; Tsalamandris *et al.*, 2019), atherosclerosis (Steven *et al.*, 2019), neurodegenerative diseases (Tansey *et al.*, 2022) or cancer (Rifai *et al.*, 2018, Federico *et al.*, 2007, Zhao *et al.*, 2021). Oxidized proteins can trigger the release of inflammatory signals, and subsequently, the inflammatory cascade starts with producing prostaglandins from arachidonic acid. The production of prostaglandins some of which are inflammatory mediators is catalyzed by cyclooxygenase (COX), which exists in two isoforms, COX-1 (considered to be physiological) and COX-2 (considered to be mostly pathological). Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of inflammation and can either inhibit both COX-1 and COX-2 (nonselective NSAIDs), or selectively inhibit COX-2 (Rang *et al.,* 2019)

On the other hand, during inflammatory processes, reactive oxygen species (ROS) are produced, causing oxidative stress. Many studies demonstrate a direct relationship between oxidative stress and COX-2 expression (Wu *et al.,* 2010; Onodera *et al.,* 2015; Hamburger and McCay 2009). The expression of COX-2 is triggered by ROS through activation of the transcription factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPK), which directly bind to the promoter region of COX-2 or stimulate its expression via regulation of other transcription factors. NF-κB and MAPK are also activated by interleukin 1β (IL-1β) and tumor necrosis factor α (TNFα) mediated phosphorylation or by free radicals, which can lead to the amplification of the signal and production of COX-2 (Onodera *et al.,* 2015, Barbieri *et al.,* 2003, Chen *et al.,* 2013). Arachidonic acid may also be metabolized via the lipoxygenase (LOX) pathway to leukotrienes, in addition to being converted via COX. This pathway culminates in increased formation of lipid peroxides, which require more glutathione for conversion

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to leukotrienes (Devlin 2011). NSAIDs can inhibit the formation of free radicals or scavenge them. There is some evidence that the scavenging activity of ibuprofen can be beneficial during the management of rheumatic diseases (Hamburger and McCay 2009) as well as in the prevention of Parkinson`s and Alzheimer`s diseases (Swiatkiewiz et al., 2012, Dumont and Flint Beal 2011).

β-Hydroxy-β-arylalkanoic acids (BHAs), which structurally resemble the NSAID ibuprofen were previously designed, synthesized and examined for their anti-inflammatory activity (Dilber *et al.,* 2011, Savić *et al.,* 2017). In this work the same compounds were investigated for their antioxidant activity in *ex vivo* and in *in vivo* model.

MATERIALS AND METHODS

The synthesis and the results of rat paw oedema test indicating anti-inflammatory activity of the investigated compounds used in this study have already been published (Dilber *et al.,* 2011, Savić *et al.,* 2017).

Ex vivo antioxidant tests were performed using a serum pool of healthy individuals. 450μL of serum was treated with 50μL of appropriate concentration of the test compounds (series of dilution from 3μmol/mL to 40 μmol/mL in dymethylsulfoxide, DMSO, Sigma-Aldrich cat. No. 276855), or with combination of a test compound and 0.25 mmol/L of tert-butyl hydroperoxide (TBH, (Acros Organics, cat. No. 75-91-2), an exogenous pro-oxidant which induces oxidative stress in the sample). One sample was treated with ibuprofen (Cayman chemicals, cat. No. 70280) and the other with combination of ibuprofen and TBH. To ensure the uniformity of the biomatrix, the total volume of the test compound/ibuprofen and pro-oxidant was identical to the volume of tested antioxidant solution, because maximum allowed dilution was set to be 1:10 ratio. The incubation lasted for 2h at 37°C after the addition of each test compound, or a combination of the test compound and pro-oxidant and after that antioxidant potency of tested samples was evaluated.

Blood for *in vivo* tests was collected during the rat paw edema test, 3 h after onset of inflammation, caused by carrageenan (Savić *et al.,* 2017). The animals were pretreated with tested compounds or ibuprofen, at equimolar doses of 96.96 μmol/kg. Blood was collected from the vena portae using a syringe containing heparin, so that plasma samples could be prepared. Control group was not pretreated with any compounds. This experiment was approved by the Ethical Committee for Animal Studies of the Faculty of Pharmacy, Belgrade, Serbia No. 323-06-5023/2013-05.

The antioxidant activity of the test compounds was evaluated by measuring the following parameters: total antioxidant status (TAS) and total concentration of sulfhydryl groups (SHG) as protective parameters; total oxidative status (TOS) and prooxidant-antioxidant balance (PAB) as damaging parameters. The concentrations of all parameters were measured using spectrophotometer analyzer Ilab 300Plus (Instrumentation Laboratory, Milan, Italy), except PAB, which was measured using an ELISA reader at 450nm (Pharmacia LKB, Wien, Austria).

Measurement of total antioxidative status

TAS was measured according to Erel's method (Erel 2004). The method consists in the oxidation of 2,2-azino-bis-3-ethyl-benzo-thiazoline-6-sulfonic acid (ABTS, Sigma-Aldrich, cat. No A1888) by hydrogen peroxide present in acidic medium, giving the reaction mixture the emerald color. The antioxidants present in the sample cause the reagent discoloration, which is proportional to their concentration. The reaction was calibrated with trolox (analog of Vitamin E, Sigma-Aldrich cat. No 648471). TAS value in the results is given as μmoL trolox equivalent/L (Kotur-Stevuljević *et al.,* 2015).

Measurement of total concentration of sulfhydryl groups

The total concentration of sulfhydryl groups in the serum was determined by Ellman's method (Janković *et al.,* 2020) modification in our laboratory. This spectrophotometric method is based on the reaction of 2,2'-dinitro-5,5'-dithio-benzoic acid (DTNB) with aliphatic thiol compounds in alkaline medium (pH 9.0). This reaction produces 1 mol of p-nitrophenol anion per mol of thiol. It requires rapid read-out of the generated anion. The total concentration of SH groups can be determined via molar extinction coefficient of p-nitrophenol at 412 nm (Kotur-Stevuljević et *al.,* 2015).

Measurement of total oxidative status

The TOS values were determined by a modification of the automated colorimetric method for measuring total oxidant status (Erel 2005). The ferrous ion-*o*-dianisidine complex is oxidized to ferric ion by oxidants present in sample. The number of oxidant molecules present in the sample is directly proportional to the color intensity of a reaction mixture. The assay was calibrated with aqueous hydrogen peroxide solution. The results are expressed as μmoL H_2O_2 equivalent/L (Kotur-Stevuljević *et al.,* 2015).

Measurement of pro-oxidative-antioxidative balance

The PAB values represent simultaneously the total content of prooxidants and the antioxidant capacity of the organism. This colorimetric method is based on the reaction of 3,3'-5,5'-tetrametylbenzidine (TMB, Sigma-Aldrich, cat. No 860336) with hydrogen peroxide and antioxidants simultaneously. Firstly mentioned reaction is enzymatically catalyzed by peroxidase, while the reaction of antioxidants in serum and TMB is a non-enzymatically catalyzed reaction. The standard solution was prepared by mixing different proportions of 1 mmol/L of H_2O_2 with 6

mmol/L uric acid. The values of PAB are expressed in arbitrary units, corresponding to the percentage of H_2O_2 in the standard solution (Alamdari *et al.*, 2007).

Antioxidative, pro-oxidative and oxidative scores

The primary results were used to calculate Z scores from the individual parameters using formula (Xi-μ)/σ, where μ and σ are the population mean and standard deviation, respectively. Population values are derived from the values of the native serum pool analyzed during previous period. The antioxidant score (indicating protective capacity) was calculated as the mean of the Z scores of the measured antioxidant parameters TAS and SHG. The pro-oxidative score (indicating the damaging potential) was calculated as the mean of the Z scores of the measured pro-oxidative parameters TOS and PAB. The oxidative score (OS) was calculated as the difference between the pro-oxidative and antioxidative scores (Veglia *et al.*, 2009, Miljković *et al.*, 2018, Socrier *et al.*, 2019).

STATISTICAL ANALYSIS

Data distribution was determined by Shapiro-Wilk test. Initially, five different dilutions of each BHA were used to determine if there was some dose dependence with the BHAs reactivity in biological samples. Since no consistent change in redox status parameters was observed, results are presented as the average value of all five concentration levels. Results for all parameters are presented as medians (25^{th} - 75^{th} percentile). For the inter-group comparison, the nonparametric Mann-Whitney U test was used for two independent samples and the Wilcoxon's signed-rank test was used for related samples. The correlation coefficient between variables was determined using Spearman's test. For statistical analysis of the obtained data, the SPSS for Windows 18.0 software package (SPSS, INC. Chicago, Illinois USA) was used.

RESULTS

Because the results obtained for TAS, TOS, PAB and glutathione (GSH) (provided in the supplement) were complicated to follow and interpret simultaneously, antioxidant scores were calculated. The values of the oxidative score of the synthesized compound and ibuprofen in *ex vivo* medium are presented in table 1.

The tested BHAs and ibuprofen showed mild prooxidant activity, but some of them were better than ibuprofen, as evident by the statistically lower OS value compared with ibuprofen. The OS value of the serum incubated with DMSO showed that this solvent did not lead to the formation of free radicals which qualifies it as a suitable solvent. Based on their structure, the tested BHAs can be classified into two groups: 1) unsubstituted derivatives of butanoic acid and diphenylpropanoic acid (a-f) and 2) substituted derivatives of diphenylpropanoic acid (g-m). Substitution of benzene ring contributes to antioxidant activity. OS value increased after pretreatment with all compounds including ibuprofen. After the addition of TBH, OS values further increased indicating that the tested compounds could not maintain antioxidant protection after the addition of the pro-oxidant. However, compared to ibuprofen, all seven substituted derivatives (g-m) had lower OS values. When evaluated in the presence of a pro-oxidant, compound h gave the lowest OS value (7.67 (7.50-7.82)).

Corresponding to the primary oxidative stress parameters TAS, TOS, PAB and SHG (table in the supplemental data), correlations between parameters of phenylpropanoic acid derivatives (g-m) were analyzed using Sperman`s nonparametric analysis (a measure of the strength and direction of the relationship that exists between two variables). There was only statistically significant negative relationship between TAS and PAB values of compound h, as it is shown in table 2.

Comparison of SHG values of phenylpropanoic acid derivatives, ibuprofen and serum (fig. 1) showed that compounds appear to "consume" SHG, and differences between SHG values of compounds and serum were statistically significant (p<0.05, p- according to Man-Whitney U test).

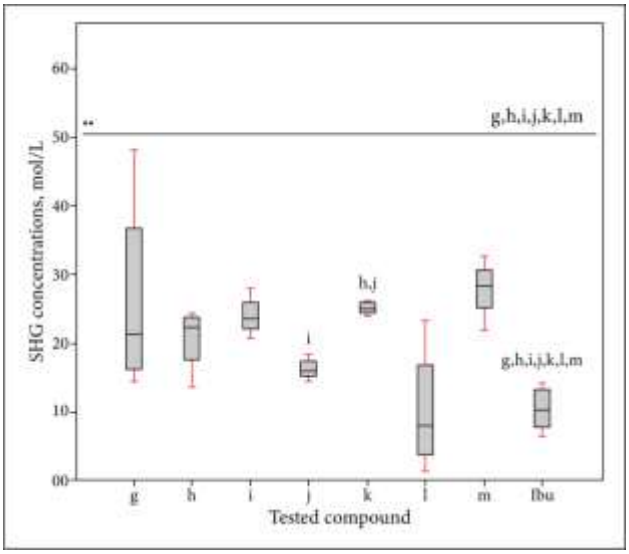

Fig. 1: SHG concentration [mmol/L] of BHAs and ibuprofen in samples without TBH incubation, *ex vivo* medium

Fig. 2 shows OS values of all compounds in both *in vivo* and *ex vivo* medium, with and without TBH incubation.

Since substituted acids (g-m) showed lower pro-oxidative activity, they were selected for *in vivo* experiments in an which inflammatory reaction is induced in animal`s paw, and oxidative stress occurs as disorder secondary to

inflammation burden. The total oxidative score for each tested compound *in vivo* are lower than *ex vivo* (table 1, table 3 and fig. 2).

DISCUSSION

In this study we evaluated the antioxidant activity of thirteen BHAs (a-m) and ibuprofen *ex vivo* in a biological environment (human serum pool) both in its natural state and under conditions of exogenously induced oxidative stress. Seven BHAs and ibuprofen were selected for *in vivo* testing (g-m), 3h after the onset of inflammation. There was no correlation between *ex vivo* and *in vivo* activities, suggesting different mechanisms involved in the two conditions.

In the *ex vivo* experiments, substituted derivatives of 3-hydroxy-3,3- dyphenylpropanoic acid (g-m) provided better protection compared to the unsubstituted derivatives of either butanoic or dyphenylpropanoic acid (a-f). Thus it can be concluded that substituting of one benzene ring generally contributes to antioxidant activity. Compounds g-m showed significant protective effects against TBH pro-oxidant and "buffer" OS levels to those found under the basal conditions, without exogenous pro-oxidant. Furthermore, compound h with a trifluoromethyl group in the para position, had even lower OS value in the TBH sample compared to other derivatives. The possible mechanism of antioxidant activity of these compounds is αβ dehydration, which is influenced by both electronic and steric effects of present groups. The nitro group in compound g is in the para position and has a strong negative resonance effect, decreasing the electron density of the benzene ring. The same effect nitro group has on oxygen atom of tertiary alcohol group, which reduces the possibility of its protonation and dehydration. In contrast molecules containing groups with -I effect (Cl and CF_3) are more likely to be protonated due to electronic density of oxygen in the tertiary alcohol group is less affected. This is because the -I effect depends on the position of the groups, and its effect is weaker as groups are more distant from each other (Sandomenico *et al.*, 2017, Patel *et al.*, 2019, Salman *et al.*, 2017). The low OS score value of h is due to fluorine atom in acid structure, particularly in form of trifluoromethyl group, which was shown to have a beneficial effect in providing antioxidant protection (Sandomenico *et al.*, 2017, Patel *et al.*, 2019, Salman et al., 2017). Fluorine is electron withdrawing element and powerful antioxidative agent because it can easily receive electrons from free radicals, thus stabilizing their structure and preventing the spreading of free radical chain reaction that can damage biomolecules (Berczyński *et al.*, 2020, S. de Oliveira *et al.*, 2014). The introduction of fluorine in the acid structure could help maintaining activity of two important antioxidant enzymes glutathione-peroxidase system and activity of glutathione S-transferase, which can be inhibited by pro-oxidants (Magni *et al.*, 2012). In opposite to nitro group, groups with -I effect, such as Cl and CF_3, have steric effect which could not be neglected and which is proven by the obtained values of OS (table 2). In compounds with these groups present in para position (h and i), the steric effect is less pronounced and the reaction of dehydration is facilitated. The opposite situation is in compounds k and l with the same groups but in meta position, the steric effect is present and jointed with electronic effect, so reaction of dehydration is slowed down. Derivatives with an electroneutral methyl group (j and m) have dominant steric effect preventing protonation of the oxygen atom in the tertiary alcohol group, so is dehydration. In the group of compounds a-m, all compounds, except m show better antioxidant protection than ibuprofen. As previously described, there is a connection between production of free radicals and cytokines and inflammatory reaction. Two of the most important signaling pathways in oxidative stress, induced by anti-inflammatory reaction are NF-κB and MAPK, and they can be amplified due to phosphorylation of control points inside these pathways (Zeeshan *et al.*, 2019). This altered phosphorylation is the consequence of oxidative stress development, which occurs due to the enormous production of ROS from macrophages. Substances with trifluoromethylphenyl group, like compound h, have protective role and prevent the formation of oxidative stress. Oh *et al.*, (2018) showed that pretreatment with trifluoromethylphenyl group-rich molecules substantially inhibited activation of MAPK thus prevents the production of inflammation induced by oxidative stress. Further evidence for the protective effect of this group is the fact that pretreatment of macrophages with trifluoromethylphenyl group-substituted molecules inhibits NADPH oxidase and further free radical production.

In *in vivo* tests, compound g with nitro group in para position provided the best antioxidant activity (OS=-1.60). This result collides with the fact that nitro group has a pronounced negative inductive and resonance effects favoring pro-oxidative activity. The explanation for obtained favorable antioxidant result may lie in the fact that this compound also showed the highest anti-inflammatory activity among compounds g-m (ED_{50}=47.76 μmol/kg similar to ibuprofen ED_{50}=43.97 μmol/kg) (Savić *et al.*, 2017), so it can be assumed that it also can protect organism from oxidative stress induced by inflammation. Based on aforementioned, chemical properties of g which has convincing antioxidant capability, it might be assumed that the mechanism of antioxidant protection is inhibition of activation of NF-κB or MAPK which needs to be explored furthermore. Better antioxidant activity of g, i, j, and m compared to ibuprofen is an important advantage. Results in table 3 (*in vivo* experiment) showed that compounds i, j and m produce better antioxidant protection compared to compounds h, k and l.

Table 1: Oxidative score values of BHAs and ibuprofen, with and without TBH, *ex vivo* medium

Compound	OS value without TBH	OS value with TBH
a	9.19 (8.51-9.29)$^{f, h, i, k, l, ss, sdsd, stst}$	10,72 (10.27-11.46)$^{gg, hh, ii, kk, ll, ss, sdsd, stst}$
b	9.01(8.64-9.52)$^{h, i, k, l, ss, sdsd, stst}$	10.47 (10.23-11.39)$^{gg, hh, ii, kk, ll, ss, sdsd, stst}$
c	8.80 (8.66-9.28)$^{h, i, k, l, ss, sdsd, stst}$	10.12 (9.35-10.81)$^{gg, hh, ii, kk, ss, sdsd, stst}$
d	8.55 (8.32-8.78)$^{f, h, i, k, m, x, ss, sdsd, stst}$	10.80 (10.54-11.31)$^{gg, hh, ii, kk, ss, sdsd, stst}$
e	8.38(8.14-9.22)$^{k, l, x, ss, sdsd, stst}$	10.98 (9.94-11.41)$^{gg, hh, ii, kk, ll, ss, sdsd, stst}$
f	9.48 (9.03-9.85)$^{g, h, i, k, l, ss, sdsd, stst}$	10.60 (10.07-10.97)$^{gg, hh, ii, kk, ll, ss, sdsd, stst}$
g	8.54 (7.04-9.21)$^{ff, ss, sdsd, stst}$	9.04 (7.05-9.29)$^{aa, bb, cc, dd, ee, ff, jj, kk, ll, mm, ss, sdsd, stst}$
h	7.67(7.22-8.28)$^{aa,bb, cc, dd, ff, jj, mm, xx,ss, sdsd, stst}$	7.67 (7.50-7.82)$^{aa, bb, cc, dd, ee, ff, jj, kk, ll, mm, xx, ss, sdsd, stst}$
i	7.97 (7.01-8.43)$^{aa, bb, cc, dd, ee, ff, jj, ll, ss, sdsd, stst}$	8,12 (7.62-8.42)$^{ee, ff, jj, ll, xx, ss, sdsd, stst}$
j	7.60 (7.16-8.04)$^{hh, ii, kk, ll, ss, sdsd, stst}$	10.11 (9.94-10.86)$^{gg, hh, ii, kk, ll, xx, ss, sdsd, stst}$
k	7.61 (7.42-7.91)$^{aa, bb, cc, dd, ee, ff, jj, mm, xx, ss, sdsd, stst}$	8.66 (7.93-8.96)$^{aa, bb, cc, dd, ee, ff, hh, jj, mm, xx, ss, sdsd, stst}$
l	9.20 (8.74-9.42)$^{aa, bb, cc, ff, jj, mm, xx, ss, sdsd, stst}$	8.87 (8.61-9.68)$^{aa, bb, cc, dd, ee, ff, hh, jj, ll, mm, xx, ss, sdsd, stst}$
m	9.44 (9.03-9.85)$^{hh, ii, kk, ll, xx, ss, sdsd, stst}$	10.71 (10.35-11.00)$^{gg, hh, ii, kk, xx, ss, sdsd, stst}$
x	10.28 (9.52-10.45)$^{dd, ee, hh, ii, kk, ll, ss, sdsd, stst}$	11.53 (10.02-11.94)$^{aa, gg, hh, ii, jj, kk, ll, ss, sdsd, stst}$
s	0.05 (-0.64-0.67)	2.59 (0.77-4.42)
sd	0.15 (-0.74-0.75)	/
st	-2.95 (- 4.29 – -1.68)	/

Letters a-m in superscript reffer to tested compound names, X - ibuprofen; S - serum; SD - serum + DMSO; ST - serum + trolox
*according to Kruskal-Wallis Test; one letter in superscript $p<0.05$, two letters in superscript: $p<0.01$.

Table 2: Correlations of parameters of compound h without and with TBH incubation, *ex vivo* medium

Samples without TBH incubation		Samples with TBH incubation	
	TAS		TAS
PAB	$\rho = -0.95$ $p = 0.05$	PAB	$\rho = -1.00$ $p = 0.01$

ρ- Spearman's nonparametric correlations coefficient

Table 3: Oxidative score values of BHAs and ibuprofen, *in vivo* medium

Compound	OS values	Significant difference with listed compounds ($p<0.01$)
g	-1.60 (-0.56-0.69)	l, i, ibuprofen, control
h	0.10 (-0.42-0.62)	l, i
i	-0.86 (-1.23-0.58)	j, l, g, h, k,ibuprofen, control
j	-0.29 (-0.65-0.41)	l, i, ibuprofen
k	0. 12 (-0.88-0.46)	l, i
l	0. 77 (-0.16-1.22)	j, g, h, i, k, m, control
m	-0.71 (-0.95- -0.16)	l, ibuprofen, control
Ibuprofen	0.40 (0.01-1.21)	j, g, i, m
Control group	0.36 (-0.20-0.30)	g, i, l, m

Letters in superscript reffer to compound names

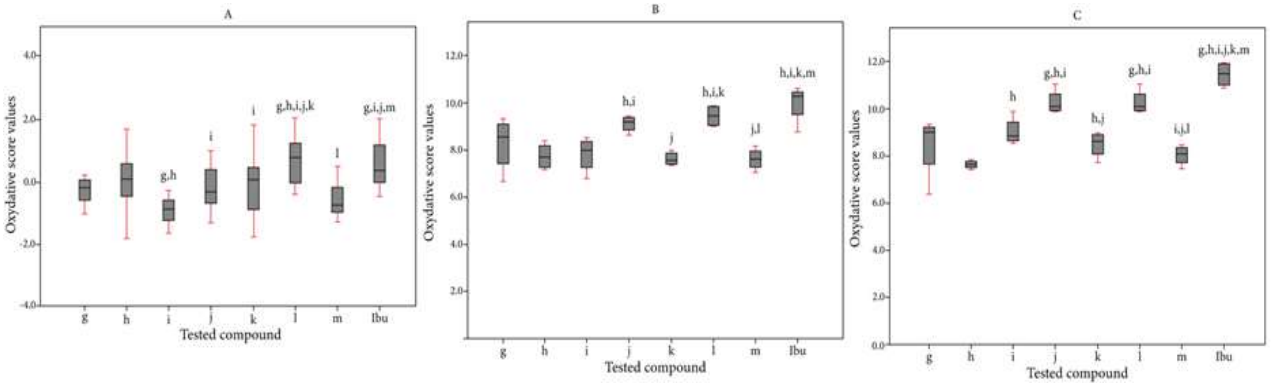

Fig. 2: Oxidative score values (OS) of BHAs and ibuprofen.
A-*in vivo* medium; B-*ex vivo* medium without TBH; C-*ex vivo* medium with TBH

Supplementary table

β-hydroxy-β-arylalkanoic acid derivatives without TBH incubation	TAS [mmol/L]	TOS [mmol/L]	PAB [HK]	SHG [mmol/L]
g	1782.5 1773.5-1787.5	7.65 7.10-8.15	171.1 156.3-182.2	0.215 0.161-0.366
h	1822.5 1802.0-1836.5	8.30 7.90-8.70	155.5 149.3-164.7	0.224 0.176-0.237
i	1811.5 1779.0-1828.0	6.95 6.50-8.00	164.8 152.4-166.8	0.234 0.220-0.258
j	1774.5 1755.0-1793.5	6.60 6.05-7.30	175.1 173.8-178.6	0.160 0.152-0.174
k	1838.5 1814.0-1863.0	7.20 6.60-7.90	166.4 164.1-167.1	0.249 0.243-0.257
l	1781.0 1776.5-1793.5	6.05 5.65-6.90	180.9 170.9-185.5	0.082 0.038-0.168
m	1837.9 1830.1-1850.4	6.89 6.36-7.40	178.9 168.0-185.2	0.281 0.248-0.300
β-hydroxy-β-arylalkanoic acid derivatives with TBH incubation	TAS [mmol/L]	TOS [mmol/L]	PAB [HK]	SHG [mmol/L]
g	1812.5 1783.0-1828.5	16.7 16.5-16.9	166.9 161.7-172.0	0.212 0.199-0.412
h	1840.0 1817.5-1851.5	7.7 7.3-8.0	159.1 153.8-161.3	0.213 0.188-0.242
i	1852.0 1821.5-1877.0	13.3 12.3-14.6	178.7 175.1-183.2	0.191 0.150-0.240
j	1770.5 1761.5-1783.0	18.5 18.4-19.2	177.1 174.1-179.3	0.165 0.130-0.208
k	1935.5 1906.0-1961.0	13.4 13.1-13.6	182.0 176.4-191.8	0.173 0.168-0.220
l	1777.5 1643.5-1713.5	17.8 17.5-18.6	190.7 176.3-192.8	0.087 0.041-0.137
m	1870.1 1861.8-1884.9	14.2 13.9-15.8	182.9 170.4-189.6	0.249 0.220-0.351

These results cannot be explained by electrochemical properties, but they are in correlation with their anti-inflammatory activity and support the assumption that these compounds might have impact on NF-κB or MAPK. This can be seen in particular in the structure of compound i which contains chlorine atom in the para position that reduces the electron density in the molecule due to its inductive effect. This compound should have high OS value, but on the contrary it is quite low. Interestingly, compounds j and i (ED_{50} around 85μmol/kg) showed lower anti-inflammatory activity than ibuprofen, but better antioxidant activity. It can be concluded that compounds a-m are preferentially anti-inflammatory compounds and may respond better to oxidative stress in inflamed tissue (even better than ibuprofen), than to oxidative stress induced by TBH. In the case of COX blockade by NSAIDs, a decrease in glutathione levels may occur as it is consumed a consequence of its consumption in the synthesis of peptido-leukotriens via the lipoxygenase pathway (Batu and Erol 2007, Haeggstrom 2018). This study supported these findings by lower SHG values of samples compared to native serum. In this way, it is proven that tested compounds do not act as LOX inhibitors.

CONCLUSION

Inflammation and oxidative stress are closely linked pathophysiological processes that can lead to and exacerbate each other. The results obtained *ex vivo* and *in vivo* in this study for antioxidant activity of BHAs are not in a correlation because of the different antioxidant mechanisms. In the *ex vivo* experiment, when oxidative stress is originated with pro-oxidant TBH, the antioxidant protection of these compounds is lower compared to the *in vivo* results, and the ranking of the OS values of the derivatives of 3-hydroxy-3,3-dyphenylpropanoic acid (g-m) is different. These compounds showed good antioxidant protection *in vivo* when oxidative stress is triggered by inflammatory process. The antioxidant activity correlates with their anti-inflammatory activity, so that the compound with the nitro group in para position on one of the benzene rings (g) shows the best antioxidant activity (lowest OS value). It should be emphasized that

four compounds showed statistically higher antioxidant activity compared to ibuprofen. It can be concluded that compounds g-m respond better to oxidative stress caused by inflammation (even better than ibuprofen), than to oxidative stress induced by exogenously added pro-oxidant.

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