

## Original article

## Dihydropyrimidin-(2*H*)-ones obtained by ultrasound irradiation: a new class of potential antioxidant agents

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### Abstract

An efficient and simple synthetic protocol for the Biginelli reaction has been developed for the preparation of several new dihydropyrimidinones, under ultrasound irradiation in the presence of  $\text{NH}_4\text{Cl}$ , in good yields and short reaction time. Some of the synthesized compounds were tested *in vitro* for their antioxidant activity. All of the selected compounds showed some antioxidant activity. Analogous compounds **3b** and **4b** exhibited a strong activity against lipid peroxidation induced by  $\text{Fe} + \text{EDTA}$ , while compounds **3b** and **3d** were the most potent in reducing ROS levels.

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**Keywords:** Biginelli; Dihydropyrimidinones; Antioxidant; Reactive oxygen species; Pharmacological

### 1. Introduction

Dihydropyrimidinones (DHPMs) and their derivatives have attracted interest in medicinal chemistry, exhibiting pharmacological and therapeutic properties. The interest has shifted from DHPM calcium channel modulators [1] to other biologically active DHPM derivatives, e.g.  $\alpha_{1a}$  adrenoceptor-selective antagonists, useful for the treatment of benign prostatic hyperplasia [2]. Moreover, the antihypertensive, antiviral, antibacterial and antitumoral activities have been described in [1,3]. In addition, several alkaloids containing the DHPMs core have been isolated from marine sources, which also exhibited interesting biological properties [4]. DHPM derivatives have clearly de-

finer virus-inhibiting properties with respect to type 1 human immunodeficiency [5]. Although, during the last years extensive studies on the pharmacology of DHPMs have been reported in [3–5], the antioxidant activity of this ring system has never appeared in the literature. In the pathologic conditions an overproduction or scavenger diminution of the reactive oxygen species (ROS) can occur. In fact, ROS overproduction has been implicated in the installation and/or progression of a variety of human diseases, including diabetes and various neurodegenerative diseases [6]. Thus, the interest in natural and synthetic antioxidant compounds that could potentially retard the development of these diseases has grown considerably in the scientific community in the last decades.

The aim of this study was to evaluate the antioxidant potential of novel dihydropyrimidin-(2*H*)-ones. The procedure described herein provides an interesting protocol for the synthesis of novel dihydropyrimidin-(2*H*)-ones in good yields and operational simplicity. Importantly, all the dihydropyrimidin-(2*H*)-ones tested were pharmacologically active as antioxidant agents.

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## 2. Chemistry

The simple and direct method for the synthesis of DHPMs reported first by Biginelli in 1893 [7], involves a reaction in which three reactants come together in a single reaction vessel to form a new product that contains portions of all the components, a multicomponent reaction. The classical Biginelli reaction is a condensation reaction between an aldehyde,  $\beta$ -ketoester and urea under strongly acidic conditions. Usually, only low to moderate yields are obtained, in particular when substituted aromatic or aliphatic aldehydes are employed. In order to improve the efficiency of Biginelli reaction, many synthetic strategies involving combinations of Lewis acids and transition metal salts, e.g.  $\text{BF}_3\text{-OEt}_2$  [8], montmorillonite (KSF) [9] polyphosphate esters [10] and reagents like  $\text{InCl}_3$  [11],  $\text{InBr}_3$ ,  $\text{LiBr}$  [12],  $\text{TMSCl/NaI}$  [13],  $\text{LaCl}_3\cdot 7\text{H}_2\text{O}$  [14],  $\text{CeCl}_3\cdot 7\text{H}_2\text{O}$  [15],  $\text{Mn(OAc)}_3\cdot 2\text{H}_2\text{O}$  [16], ammonium chloride ( $\text{NH}_4\text{Cl}$ ) [17], which give better yields of DHPMs.

## 3. Result and discussion

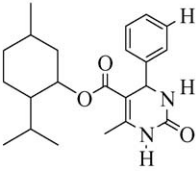
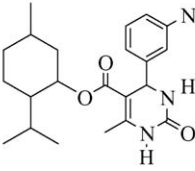
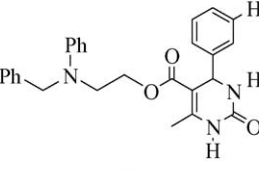
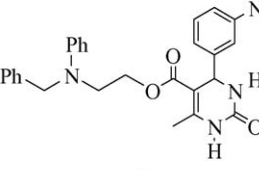
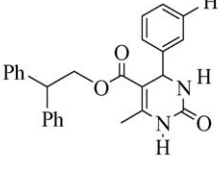
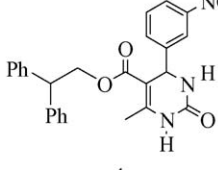
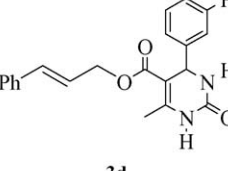
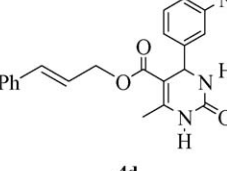
A survey of literature shows that many organic reactions have been accelerated by ultrasound irradiation. Compared

with traditional methods, this technique is more convenient and easily controlled [18].

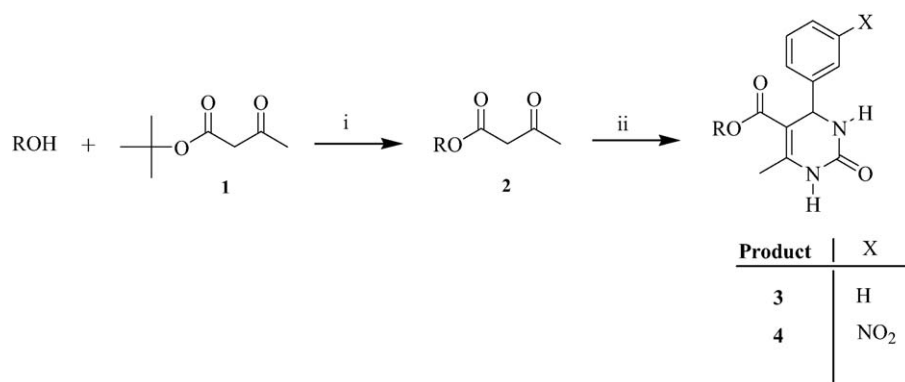
Recently, we have developed a general method for the synthesis of 4-halo-3,5-dimethyl pyrazoles under ultrasound irradiation [19]. Stefani and Gatti [20] reported an efficient protocol for the synthesis of DHPMs by microwave irradiation in solvent free conditions. As part of our study searching on heterocyclic chemistry, we herein report the convenient synthesis of novel DHPMs by the one pot condensation of  $\beta$ -ketoesters **2** with appropriated aldehyde and urea, using ammonium chloride ( $\text{NH}_4\text{Cl}$ ) as a mediator of the reaction. In the present study, the Biginelli-type cyclocondensation of a number of the four  $\beta$ -ketoesters **2** have been studied. The choice of ammonium chloride ( $\text{NH}_4\text{Cl}$ ) was dictated by the inexpensive cost and the ability to promote the Biginelli reaction [17]. In our studies, we found that the methanol solvent reaction appropriated for these reactions gave the best results and in the absence of ammonium chloride no cyclocondensation occurred.

The cyclocondensation of  $\beta$ -ketoester **2** with aromatic aldehyde, urea, ammonium chloride ( $\text{NH}_4\text{Cl}$  99.998%, *Merck Index* **12**, 537), in 15 ml of absolute methanol was irradiated in a water bath of an ultrasonic cleaner with a frequency of 40 kHz and a nominal power of 130 W, at 60 °C for the period as indicated in Table 1. The reaction flask was located in the

Table 1  
The novel DHPMs 3–4

Dihydropyrimidinones 3	Time (h)	Yields (%) <sup>a</sup>	Dihydropyrimidinones 4	Time (h)	Yields (%) <sup>a</sup>
 3a	5	65	 4a	3.5	65
 3b	2.5	80	 4b	3	90
 3c	3.5	75	 4c	3.5	73
 3d	3.5	75	 4d	3.5	70

<sup>a</sup> Yields of isolated products



Scheme 1. Reagents and conditions

i = DMAP, toluene, reflux/48–72 hours.

ii = urea, MeOH, aldehyde, NH<sub>4</sub>Cl, ultrasound irradiation/3–5 hours.

maximum energy area in the cleaner and addition or removal of water was used to control the temperature of the water bath. The progress of the reaction was monitored by TLC. The mixture was sonicated until the condensation has been completed. Dihydropyrimidin-(2H)-ones **3–4** were obtained after removal of the solvent and purified by column chromatography (silica gel; eluting with ethyl acetate and hexane 3:7) (Scheme 1).

All starting  $\beta$ -ketoesters **2**, were synthesized via transesterification reaction of *tert*-butyl acetoacetate (Aldrich) with the corresponding alcohols [21].

The scope and generality of this process is demonstrated by a series of dihydropyrimidin-(2H)-ones **3–4** obtained, the results are presented in Table 1.

### 3.1. Antioxidant evaluation

Dihydropyrimidin-(2H)-one **3d** protected against lipid peroxidation only at 400  $\mu$ M (Fig. 1), whereas compound **4d** was effective at concentrations higher than 100  $\mu$ M (Fig. 2). Thus, dihydropyrimidin-(2H)-one bearing a nitro group in the aromatic system (**4d**) presented the best result against lipid peroxidation when compared to **3d**, an analogous without any substituent in the aromatic system. In addition, compound **4d** at

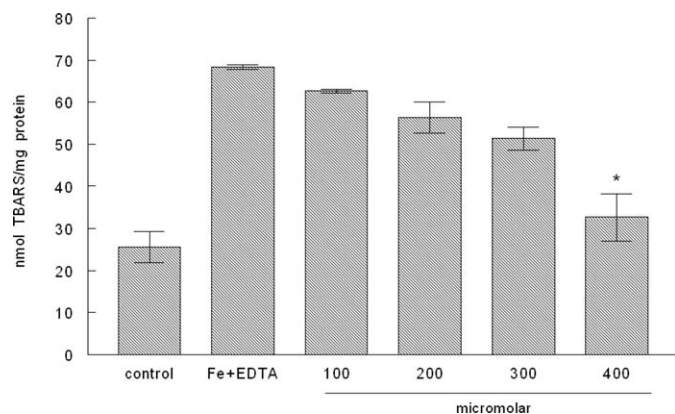


Fig. 1. Effect of compound **3d** on TBARS levels in liver mice. Data are expressed as mean  $\pm$  S.E.M. of three animals per group. (\*) denoted  $P < 0.05$  as compared to the induced group (one-way Anova/Duncan).

400  $\mu$ M decreased lipid peroxidation to the control level while the analogous **3d** reduced lipid peroxidation only 50%.

Analogous compounds **3b** and **4b** inhibited lipid peroxidation in the similar manner. In fact, compounds **3b** and **4b** displayed the antioxidant effect at concentrations higher than 100  $\mu$ M and presented the maximal effect at 200  $\mu$ M, suggesting that the substituent in the aromatic system was not responsible for the antioxidant effect (Figs. 3 and 4).

Dihydropyrimidin-(2H)-one **3a** reduced lipid peroxidation at concentrations higher than 100  $\mu$ M ( $P < 0.05$  by Duncan's multiple range test) and the maximal effect was attained at 300  $\mu$ M (Fig. 5). These results indicated that analogues compounds **3b** and **4b** exhibited the best antioxidant activity. Thus, we can conclude that the presence of an amino group at the side chain in the ester group has an important influence in the antioxidant activity of these compounds.

Compounds **3a**, **3b**, **3d** and **4b**, **4d**, at both concentrations tested, decreased ROS (Table 2). Dihydropyrimidin-(2H)-one **3b** and **3d** were the most efficient compounds in the fluorimetric assay, suggesting that nitro group in the aromatic ring influences the efficacy in decreasing ROS levels.

The thiol-peroxidase like activity can explain, at least in part, the in vitro antioxidant properties of several compounds

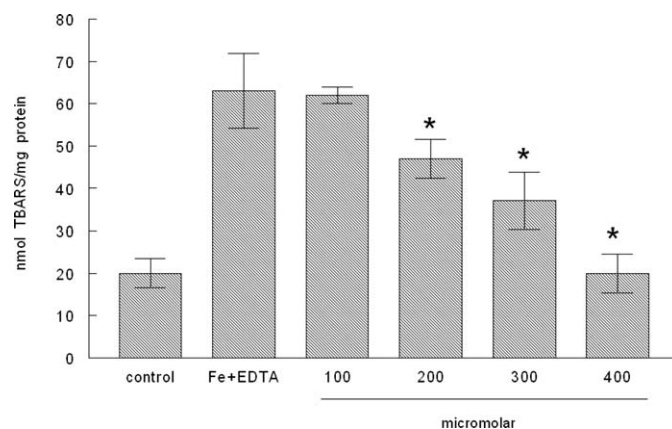


Fig. 2. Effect of compound **4d** on TBARS levels in liver mice. Data are expressed as mean  $\pm$  S.E.M. of three animals per group. (\*) denoted  $P < 0.05$  as compared to the induced group (one-way Anova/Duncan).

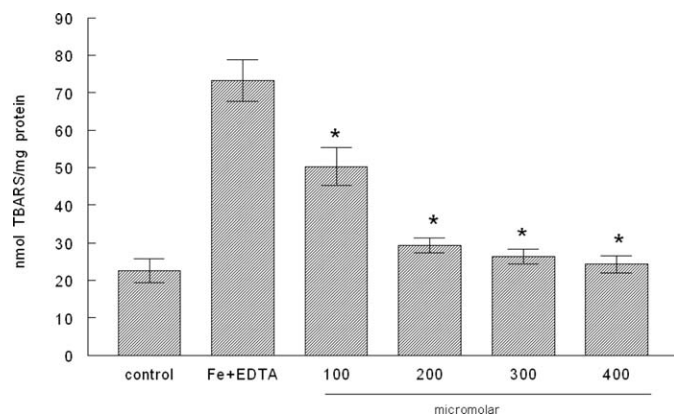


Fig. 3. Effect of compound **3b** on TBARS levels in liver mice. Data are expressed as mean  $\pm$  S.E.M. of three animals per group. (\*) denoted  $P < 0.05$  as compared to the induced group (one-way Anova/Duncan).

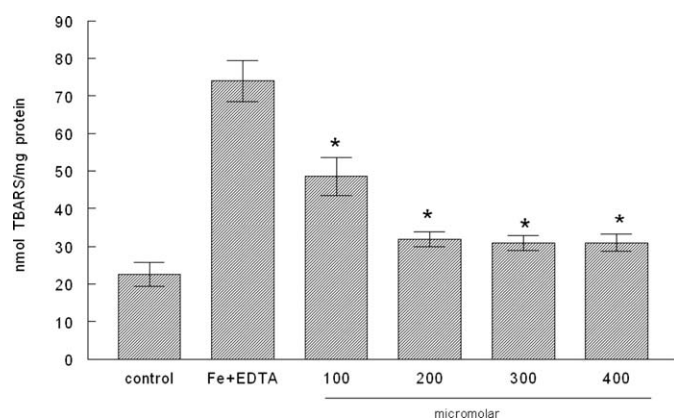


Fig. 4. Effect of compound **4b** on TBARS levels in liver mice. Data are expressed as mean  $\pm$  S.E.M. of three animals per group. (\*) denoted  $P < 0.05$  as compared to the induced group (one-way Anova/Duncan).

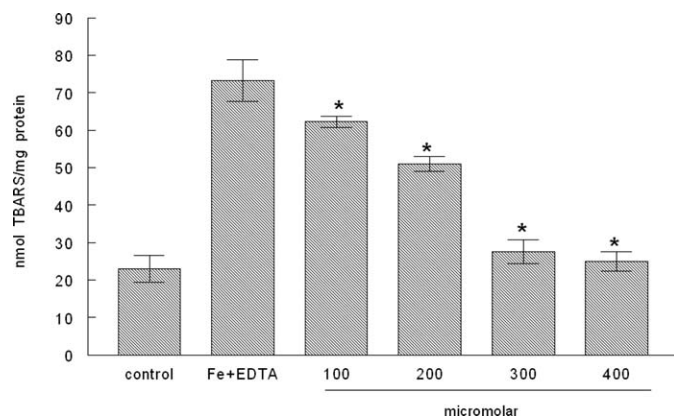


Fig. 5. Effect of compound **3a** on TBARS levels in liver mice. Data are expressed as mean  $\pm$  S.E.M. of three animals per group. (\*) denoted  $P < 0.05$  as compared to the induced group (one-way Anova/Duncan).

[22]. Since dihydropyrimidin-(2H)-ones tested did not present glutathione-peroxidase like activity (data not shown) the antioxidant activity of these compounds could not be related to this activity.

In conclusion, we have reported a convenient protocol, for the synthesis of novel dihydropyrimidin-(2H)-ones by conden-

Table 2

Effect of dihydropyrimidin-(2H)-ones on ROS levels in rat liver

Compounds ( $\mu$ M)	15 (min) <sup>a</sup>	30 (min) <sup>a</sup>
Control	64.14 $\pm$ 19.3	77.82 $\pm$ 19.1
<b>3a</b> - 100	43.74 $\pm$ 4.0*	64.41 $\pm$ 2.7
400	35.54 $\pm$ 3.9*	49.80 $\pm$ 3.1*
<b>3b</b> - 100	26.20 $\pm$ 0.1*	41.92 $\pm$ 0.4*
400	17.55 $\pm$ 9.0*	26.23 $\pm$ 12.3*
<b>3d</b> - 100	26.60 $\pm$ 5.6*	43.66 $\pm$ 7.3*
400	11.86 $\pm$ 2.1*	19.23 $\pm$ 4.5*
<b>4b</b> - 100	47.91 $\pm$ 8.1	69.43 $\pm$ 7.1
400	28.54 $\pm$ 1.1*	43.11 $\pm$ 0.7*
<b>4d</b> - 100	39.84 $\pm$ 13.0*	57.50 $\pm$ 10.8
400	17.13 $\pm$ 0.8*	24.91 $\pm$ 0.9*

\* Denoted  $P < 0.05$  as compared to the control tube (one-way Anova/Duncan).

<sup>a</sup> Data of ROS levels are presented as fluorescence intensity emission (UAF). Data are expressed as mean  $\pm$  S.E.M. of three different experiments.

sation of  $\beta$ -dicarbonyl compound, aldehyde and urea, using  $\text{NH}_4\text{Cl}$  as mediator of the reaction. The method reported here is not only simple to operate but also efficient. All of the selected compounds showed some antioxidant activity. Analogues compounds **3b** and **4b** exhibited a strong activity against lipid peroxidation induced by Fe + EDTA, while compounds **3b** and **3d** were the most potent in reducing ROS levels.

We believe that our work will find an important application in the synthesis of dihydropyrimidin-(2H)-ones compounds to fulfill the needs of academia as well as pharmaceutical industries.

## 4. Experimental

Melting points were determined on a glass disk and are uncorrected. The  $^1\text{H}$  (300 MHz) and  $^{13}\text{C}$  (75 MHz) NMR spectra were acquired on a Bruker DPX 300 spectrometer equipped with a 5 mm broadband probe with z-gradients and a SGI computer in DMSO with TMS as internal standard. Coupling constants ( $J$ ) are quoted to the nearest 0.1 Hz. Chemical shifts ( $\delta$ -scale) are quoted in parts per million and the following abbreviations are used: s = singlet; d = doublet; t = triplet; m = multiplet; br = broad. Near-IR spectra were obtained on a Bohmen MB-100 spectrometer. Standard flash chromatography procedures were followed using 32–63 mm silica gel. Sonication was performed in Bransonic® tabletop cleaners, Model-3510, overall size 16"  $\times$  12"  $\times$  14.5", tank size 11.5"  $\times$  6"  $\times$  6", weight 12 lbs, volume 1.5 gal., frequency of 40 kHz and a nominal power 130 W.

### 4.1. Chemistry

#### 4.1.1. General procedure: synthesis of DHPMs 3–4

A 50 ml round bottomed flask equipped with a reflux condenser, a septum and nitrogen purge was flushed with  $\text{N}_2$  is charged with  $\beta$ -ketoester **2** (4 mmol), aromatic aldehyde (4 mmol), urea  $\geq 99.5\%$  (Merck Index **12**, 10005/6 mmol, 0.36 g), ammonium chloride 99.99% ( $\text{NH}_4\text{Cl}$  Merck Index **13**, 537/10.0 mmol, 0.53 g). The mixture was dissolved in absolute methanol (15 ml), irradiated in a water bath of an ultrasonic cleaner at 60  $^\circ\text{C}$ , for a period as indicated in Table 1

(2.5–5 h). After consumption of the starting material (TLC monitoring) the solvent was removed under reduced pressure and the organic phase extracted with ethyl acetate ( $2 \times 10$  ml). The organic extract was dried ( $\text{MgSO}_4$ ) and the solvent removed under reduced pressure. The DHPMs **3–4** were purified by flash column chromatography (silica gel, hexane/ethyl acetate 7:3).

All solvents and reagents were obtained from Aldrich and used without further purification, except the  $\beta$ -ketoesters **2**, that were prepared according to literature [21]. Sonication was performed in a Bransonic® tabletop cleaners, Model-3510, frequency of 40 kHz and a nominal power of 130 W.

**(3a)**  $\text{C}_{22}\text{H}_{30}\text{N}_2\text{O}_3$ , mw = 370.2, m.p. 191 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 0.43–2.0 (menthyl), 2.38 (s, 3H,  $\text{CH}_3$ ), 3.72 (m, 1H, OCH), 5.37 (s, 1H, H4), 6.45, 8.04 (2NH), 7.23–7.52 (m, 5H, Ph);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 14.2–67.0 (12C), 100.7 (C5), 126.0–146.5 (6C, Ph), 147.0 (C6), 153.6 (C=O, C-2), 165.2 (OC=O). IR (KBr): 3241, 2943, 2868, 1493, 1458, 1383, 1155, 1140, 915, 873, 863, 629, 567,  $\text{cm}^{-1}$ .

**(3b)**  $\text{C}_{27}\text{H}_{27}\text{N}_3\text{O}_3$ , mw = 441.2, m.p. 134–135 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 2.30 (s, 3H,  $\text{CH}_3$ ), 3.50 (m, 2H,  $\text{CH}_2$ ), 4.26 (m, 2H,  $\text{OCH}_2$ ), 4.43 (s, 2H,  $\text{CH}_2\text{Ph}$ ), 5.30 (s, 1H, H4), 7.15–7.32 (m, 15H, 3Ph), 5.68, 8.04 (s, 1H, 2NH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 18.7 ( $\text{CH}_3$ ), 49.7 ( $\text{CH}_2$ ), 55.4 ( $\text{CH}_2\text{Ph}$ ), 56.5 ( $\text{OCH}_2$ ), 61.2 (CH), 100.7 (C5), 116–147.7 (18C, 3Ph), 148.0 (C6), 154.0 (C=O, C-2), 165.7 (OC=O). IR (KBr): 3246, 3113, 2943, 1384, 1358, 1317, 983, 752, 726, 514,  $\text{cm}^{-1}$ .

**(3c)**  $\text{C}_{26}\text{H}_{24}\text{N}_2\text{O}_3$ , mw = 412.2; m.p. 186 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 2.19 (s, 3H,  $\text{CH}_3$ ), 4.46 (t, 1H, CH), 4.59 (m, 2H,  $\text{CH}_2$ ), 5.17 (s, 1H, H4), 7.08–7.30 (m, 15H, 3Ph), 5.91, 8.59 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 19.9 ( $\text{CH}_3$ ), 51.3 (CH), 57.4 (CH), 67.9 ( $\text{OCH}_2$ ), 102.1 (C5), 127.8–142.2 (18C, 3Ph), 144.7 (C6), 155.2 (C=O, C-2), 166.9 (OC=O). IR (KBr): 3027, 2943, 2258, 1649, 1599, 1452, 1382, 1319, 1289, 1088, 1031, 987, 726, 514, 454,  $\text{cm}^{-1}$ .

**(3d)**  $\text{C}_{21}\text{H}_{20}\text{N}_2\text{O}_3$ , mw = 348.1, m.p. 166 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 2.30 (s, 3H,  $\text{CH}_3$ ), 4.65 (m, 2H,  $\text{CH}_2$ ), 5.7 (s, 1H, NH), 6.10 (t, 1H, CH), 6.40 (d, 1H, CH), 7.24–7.34 (m, 10H, 2Ph), 8.1 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 19.3 ( $\text{CH}_3$ ), 56.2 (CH), 64.9 ( $\text{CH}_2$ ), 101.5 (C5), 123.7–144.0 (12C, 2Ph), 147.3 (C6), 153.5 (C=O, C-2), 165.7 (OC=O). MS (70 eV, EI)  $m/z$  (%) = 348 ( $\text{M}^+$ , 5), 231 (25), 117 (100). IR (KBr): 3237, 3111, 2934, 2352, 1717, 1650, 1598, 1493, 1460, 1384, 1323, 1152, 943, 761, 572, 458,  $\text{cm}^{-1}$ .

**(4a)**  $\text{C}_{22}\text{H}_{29}\text{N}_3\text{O}_5$ , mw = 415.2, m.p. 188–189 °C;  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$ : 0.40–2.22 (menthyl), 1.97 (s, 3H,  $\text{CH}_3$ ), 3.35 (m, 1H, OCH), 5.27 (s, 1H, H4), 7.87, 8.04 (2NH), 7.63–8.14 (m, 5H, Ar);  $^{13}\text{C}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$ : 14.0–68.0 (12C), 100.4 (C5), 126.1–146.0 (6C, Ph), 148.0 (C6), 153.5 (C=O, C-2), 165.2 (OC=O). IR (KBr): 3220, 2941, 2865, 1491, 1459, 1386, 1150, 1143, 901, 874, 869, 621, 560,  $\text{cm}^{-1}$ .

**(4b)**  $\text{C}_{27}\text{H}_{26}\text{N}_4\text{O}_5$ , mw = 486.2, m.p. 116–117 °C;  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$ : 1.91 (s, 3H,  $\text{CH}_3$ ), 3.50 (m, 2H,  $\text{CH}_2$ ), 4.26 (m, 2H,  $\text{OCH}_2$ ), 4.43 (s, 2H,  $\text{CH}_2\text{Ph}$ ), 5.30 (s, 1H,  $\text{CHPh}$ ), 5.68 (s, 1H, NH), 7.15–7.32 (m, 15H, 3Ar), 8.04 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$ : 18.5 ( $\text{CH}_3$ ), 49.9, 53.8, 55.4 (3 $\text{CH}_2$ ),

61.5 (CH), 98.4 (C5), 112.0–147.0 (18C, 3Ar), 148.2 (C6), 154.3 (C=O, C-2), 165.7 (OC=O). IR (KBr): 3243, 3111, 2939, 1381, 1355, 1314, 981, 749, 724, 511,  $\text{cm}^{-1}$ .

**(4c)**  $\text{C}_{26}\text{H}_{23}\text{N}_3\text{O}_5$ , mw = 457.2; m.p. 175 °C;  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$ : 1.92 (s, 3H,  $\text{CH}_3$ ), 2.97 ( $\text{CH}_2$ ), 4.07 (t, 1H, CH), 4.87 (m, 2H,  $\text{CH}_2$ ), 4.90 (s, 1H, CH), 7.30 (s, 1H, NH), 6.92–7.88 (m, 14H, 3Ph), 9.21 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$ : 17.8 ( $\text{CH}_3$ ), 48.6, 53.2 (2CH), 65.7 ( $\text{OCH}_2$ ), 97.8 (C5), 120.0–150.3 (18C, 3Ar), 144.7 (C6), 151.8 (C=O, C-2), 164.9 (OC=O). IR (KBr): 3400, 2361, 1684, 1493, 1424, 1384, 1298, 1272, 1095, 1048, 997, 872, 789, 767, 728, 621, 586, 548,  $\text{cm}^{-1}$ .

**(4d)**  $\text{C}_{21}\text{H}_{19}\text{N}_3\text{O}_5$ , mw = 393.1, m.p. 216–217 °C;  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$ : 2.32 (s, 3H,  $\text{CH}_3$ ), 4.67 (m, 2H,  $\text{CH}_2$ ), 5.37 (s, 1H, CH), 6.22–6.27 (m, 2H, 2CH), 7.26–7.75 (m, 9H, 2Ar), 7.90 (s, 1H, NH), 8.10 (s, 1H, NH), 9.47 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$ : 18.0 ( $\text{CH}_3$ ), 53.5 (CH), 63.8 ( $\text{CH}_2$ ), 98.0 (C5), 120.0–146.0 (12C, 2Ar), 150.2 (C6), 159.8 (C=O, C-2), 164.8 (OC=O). IR (KBr): 3239, 3113, 2932, 2360, 2339, 1713, 1597, 1464, 1436, 1383, 1324, 1284, 1152, 1114, 1100, 963, 787, 761, 739, 572,  $\text{cm}^{-1}$ .

## 4.2. Antioxidant activity

### 4.2.1. Animals

Male adult albino Wistar rats (150–200 g) from our own breeding colony were used. The animals were kept in separate animal rooms, on a 12 h light/dark cycle, at a room temperature of 22 °C, and with free access to food and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, School of Medicine, Veterinary, and Animal Science of the University of Sao Paulo, Brazil.

### 4.2.2. Lipid peroxidation

$\text{FeCl}_2$  and EDTA are used as classical inductor of lipid peroxidation (Braugher et al., 1988). Animals were decapitated and liver was rapidly homogenized in 50 mM Tris-Cl, pH 7.5 (1/10, w/v) and centrifuged at  $4000 \times g$  for 10 min. An aliquot of liver homogenized (200  $\mu\text{l}$ ) was incubated at 37 °C in the presence of 50  $\mu\text{M}$   $\text{FeCl}_2$  and 100  $\mu\text{M}$  EDTA and DHPMs **3a**, **b**, **d** and **4b**, **d** at different concentrations (100–400  $\mu\text{M}$ ) for 1 hour. TBAR'S was determined as described by Ohkawa et al. [23].

### 4.2.3. ROS measurement

To estimate the level of liver oxidation, samples of liver were diluted (1:10) in Tris-Cl 10 mM (pH 7.4) and incubated with 10  $\mu\text{l}$  of dichlorofluorescein (DCF, 1 mM) in the presence or the absence of pro-oxidant (1 mM sodium azide) and compounds **3a**, **3b**, **3d**, **4b** and **4d** (100 and 400  $\mu\text{M}$ ). The ROS levels were determined by a spectrofluorimetric method, using 2',7'-dichlorofluorescein diacetate (DCHF-DA) assay. The oxidation of DCHF-DA to fluorescent DCF is measured for the detection of intracellular ROS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation).

#### 4.2.4. Thiol-peroxidase activity

The catalytic effects of DHPMs on the reduction of H<sub>2</sub>O<sub>2</sub> by reduced glutathione were assessed using the rate of GSH oxidation. Free –SH groups were determined according to Ellman [24]. DHPMs **3a**, **b**, **d** and **4b**, **d** (10–400 μM) were incubated in the medium containing GSH (1.0 mM) with and without H<sub>2</sub>O<sub>2</sub> (0.2 mM). At 0, 30, 60 and 120 min, aliquots of the reaction mixture (200 μl) were checked for the amount of GSH.

#### 4.2.5. Statistical analysis

Statistical significance was assessed by analysis of variance (Anova), followed by Duncan's test when appropriate. A value of  $P < 0.05$  was considered to be significant.

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