Synthesis and Characterization of New Nitric Oxide Donor Compounds Based on Theophylline and Paracetamol

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Starting from two parent drugs - paracetamol and theophylline, new nitric oxide donor derivatives (NO-donors) have been synthesized and evaluated for their toxicity degree and anti-inflammatory activity. The structure of these new compounds were confirmed by ^{1}H-NMR, FT-IR spectroscopy and thermal methods. The synthesized compounds are less toxics and more active in reducing subacute inflammatory oedema as parent compounds. The NO-donors, 7-[2-nitroxyacethyl-oxy-3-(4-acethyl-amino-phenoxy)-propyl]-8-R-1,3-dimethyl-xanthine compounds (R=H, NO_2), are 4 and 2.2 times less toxic than theophylline and of approximately 2.4 and 1.3 times less toxic than paracetamol. In the reduction of the subacute infammatory oedema NO-donors are more active than theophylline and paracetamol, that is a proof of the nitric oxide involvement in the inflammatory process.

Keywords: NO-donors, paracetamol, theophylline, anti-inflammatory activity

In 1992, the free radical, nitric oxide (formula $^{\bullet}N=O$, abbreviated to NO), was awarded the curious accolade of "molecule of the year" and the extensive body of research already built around this small molecule continues to increase unabated, with ~13 000 papers focused on the topic in the past 5 years alone [1]. Despite its structural simplicity, NO has a complex chemistry, having a curious reactivity with many molecules including the superoxide radical ($O_2^{\bullet -}$) and this complex interaction may generate a peroxynitrite anion ($ONOO^-$), which behaves as an important mediator of oxidative stress in many pathological states [2]. NO is synthesized by a number of cell types, where it acts as a highly regulated autocrine and paracrine signalling molecule [3]. Nitric oxide synthesised in endothelial cells that line blood vessels has a wide range of functions that are vital for maintaining a healthy cardiovascular, nervous and immune systems [4]. The most studied actions of NO are in the cardiovascular system where reduced nitric oxide availability is implicated in the initiation and progression of many cardiovascular diseases. Delivery of exogenous NO is an attractive therapeutic option, particularly with a view to slowing progression of atherosclerosis and reducing the risk of thrombosis [5]. Organic nitrates were first used to relieve the symptoms of angina over a century ago, long before the identification of NO as an endogenous messenger and, despite limitations, they remain the most commonly used NO donor drugs in cardiovascular medicine. NO from existing organic nitrates effectively alleviates symptoms of angina through improved blood flow to the ischaemic region of the heart via dilatation of diseased vessels and collateral coronary arteries. Furthermore, NO would be expected to inhibit inflammatory cell activation, preventing infiltration into the plaque and also the propagation of proinflammatory signals. Recently, a range of novel NO donor drugs have been evolved as potential alternatives to conventional nitrates and there have been a plethora of reviews on NO donor drugs, providing detailed descriptions of the different classes and neatly summarizing decades of research [3,5-10]. New compounds, in which NO donor groups are linked to the classical parent molecules have been synthesized. This strategy has as purpose to identify new molecules with an improved pharmacological profile in terms of the increase of the therapeutically efficiency and of the reduction of the side effects.

The aim of this study is the evaluation of new nitric oxide donors, where two parent molecules namely theophylline and paracetamol, are linked by a nitric oxide donor chain.

Paracetamol (acetaminophen) is a common analgesic and antipyretic drug having also weak anti-inflammatory activity. It is one of the most widely used drugs for the symptomatic treatment of affections with small and moderate intensity of pain like headache, dental neuralgia, surgical pain, cefalee, recently being considered for the therapy of neurodegenerative diseases such as Alzheimer's disease that are characterized by oxidant and inflammatory stress [11]. Unlike analgesics such as aspirin or NSAIDs, acetaminophen is not associated with gastrointestinal (GI) tract irritation and has no vaso-constrictive effects, Paracetamol reduces levels of prostaglandin metabolites in urine but does not reduce synthesis of prostaglandins by blood platelets or by the stomach mucosa and is also a weak inhibitor *in vitro* of both cyclooxygenase (COX)–1 and COX-2 [12].

Theophylline is a drug used for the treatment of asthma, due to its bronchodilatatory, anti-inflammatory and immunomodulatory effects. Theophylline relaxes directly smooth muscles of the bronchial and pulmonary blood vessels, so that acts largely as bronhodilatator and relaxing

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the smooth muscle [13]. Main mechanism by which theophylline exercises is the relaxation of smooth muscle by phosphodiesterases inhibiting and antagonistic adenosine, producing such bronhodilatation [14].

The new synthesized compounds could be more active than parent compounds as bronchodilatatory and anti-inflammatory effects and so they should be a new efficient therapeutic strategy more safe in the treatment of the chronic obstructive lung diseases.

Experimental part

Materials and investigation methods

Theophylline and paracetamol were purchased from Fluka. All other chemicals and solvents used for the synthesis of the compounds were purchased from Sigma-Aldrich and Merck and they have analytical purity. The assessment of the NO-donors structures has been made by *elemental analysis*, *spectroscopic and thermal methods*. *Elemental analysis* has been performed using the following methods: Pregl method to determine carbon content; Kjeldhal and Schoneger methods for nitrogen and halogen content, respectively. *FT-IR spectra* were recorded on solid powdered samples in KBr pellets, a FT-IR Bomem MB-104 spectrometer (Canada) with a resolution of 4 cm^{-1}. Sample concentration in the pellets was constant, of 3 mg/500 mg KBr. Grinding by liquid N_2 and drying of samples were performed. Spectra processing was carried out with a Grams/32 program. *^{1}H-NMR spectra* have been recorded by a BRUKER AVANCE DRX spectrometer at 400 MHz in deuterated DMSO. *TG/DTG curves* have been recorded on a Netzsch STA 409 PC/4/H TGA unit, where the Netzsch software permits continuous data acquisition and controls a silicon carbide furnace to achieve the programmed heating rates. Sample weights with 8 mg were used in experimental runs. The TGA tests were performed over a temperature ranging from ambient to 900°C at a heating rate of 10°C /min. All melting points were determined on a Melt-Temp R apparatus equipped with a digital thermometer and are uncorrected.

General procedure for synthesis of nitric oxide donors

General procedure for synthesis of 7-[2-hydroxy-3-(4-acethyl-amino-phenoxy)-propyl]-8-R-1,3-dimethyl-xanthine derivatives (D1, D2)

Syntheses of intermediary (D1, D2) were achieved starting from 4-(2,3-epoxy-propyl)-acetaminophen (**3**) (2.07g, 10 mmol) that was solved in ethanol under heating and then an ethanol solutions of theophylline (**1**) and 8-nitro-theophylline (**2**) (10 mmol) respectively were added. The reaction mixture was heated under reflux for 10 h and then the solvent was removed by distillation under reduced pressure to 1/ 4 from initial volume. The rough products were separated by filtration under vaccum, dried and recrystallized three times from ethanol.

General procedure for synthesis of 7-[2-nitroxyacethyl-oxy-3-(4-acethyl-amino-phenoxy)-propyl]-8-R-1,3-dimethyl-xanthine (65, 77)

Synthesis of nitric oxide donors (65, 77) has been performed in two steps. Firstly the intermediary (D1, D2; been 15 mmol) were stirred at room temperature for 10 h with chloracetic chloride (**4**) (16 mmol, 1.31 ml) in tetrahydrofuran and triethylamine (TEA) as acceptor of protons. After completation of reaction the solvent was removed under vacuum and the reaction mixture was washed with water, filtered and dried, when the 7-[2-chloracethyl-oxy-3-(4-acethyl-amino-phenoxy)-propyl]-8-R-1,3-dimethyl-xanthine intermediary compounds (R=H, NO_2) (**5, 6**) were obtained. In the second step these intermediaries (10 mmol) were solved in dimethylformamide (DMFA) and a solution of silver nitrate (**7**) in DMFA (10 mmol) was added. The reaction mixture was stirred at room temperature, over night, in dark, and then the solvent was removed under vacuum, and the rough product was treated with anhydrous diethyl ether, filtered and dried under vacuum. By recristallization from ethyl alcohol the 7-[2-nitroxyacethyl-oxy-3-(4-acethyl-amino-phenoxy)-propyl]-8-R-1,3-dimethyl-xanthine compounds (65, R=H and 77, R=NO_2) were obtained.

Biological tests

Acute toxicity determination

The first step in pharmacological testing of the new compounds is their toxicity evaluation [15-16]. Groups of six white Swiss strain male mice weighing between 20-25 g and grown in identical laboratory conditions have been used. Compounds to be tested have been intraperitoneal delivered as suspensions (40 mg/mL) in sodium carboxymethylcellulose 0.5 wt%. The death rate has been recorded at intervals of 24 h, 48 h and 72 h.

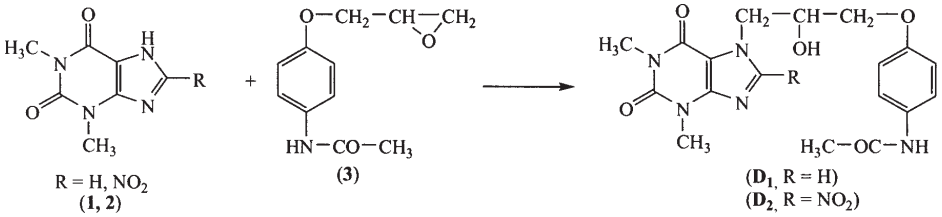
Effect on the subacute oedema inflammation

As a simple test to appreciate the anti-inflammatory effect of intermediary and final compounds, the study of the effect on the subacute oedema inflammation was applied. This was done on rat oedema induced by cotton pellet granuloma. The tests have been performed on groups of six Wistar adult male rats grown in identical laboratory conditions and having a weight varying between 200-250 g. Under ether anaesthesia, the rats have undergone an incision in the scapulohumerale zone and two sterile cotton pellets of 62 mg have been bilaterally introduced. The cotton pellets have been kept 6 days. During this period the studied compounds have been intraperitoneal delivered to the rats in a dose of 1/20 of DL50 (20 mg/0.2 mL/100g rat body) at the intervals of 24 h. In the same conditions a reference lot of rats have been also monitorized, these received the same volume of physiological serum. In the seventh day of the experiment the cotton pellets have been prevaled, kept at 60 °C for 24 h and after that they have been weighed. The smaller the mass of the cotton pellets the more accentuated anti-inflammatory action. Statistical interpretation was done by „One Way Analysis of variance Anova" test corrected with „Anova on Ranks" (Student-Newman-Keuls Method) or with Bonferoni test (which compare all lots with the reference one). The probability error (P) values smaller than $P < 0.05$ have been considered statistically significant (average ± S.E.M., n=6). The effect in the inflammatory subacute oedema was counted by retained inflammation as percent of inflammation produced by test compound in respect to reference test (the retained inflammation of the reference was considered 100%).

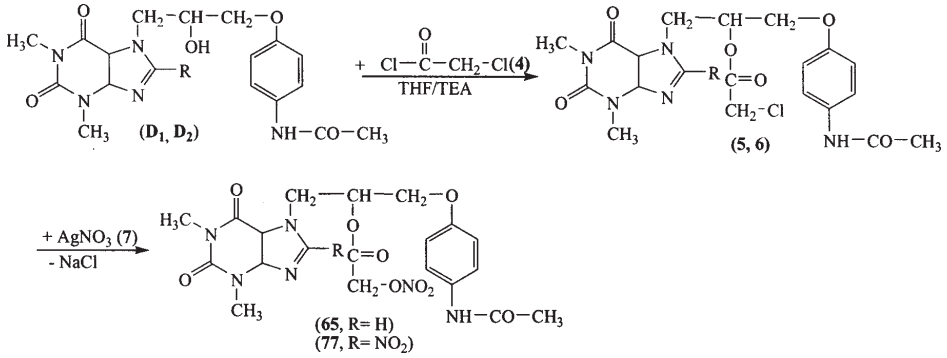
Results and discussions

Chemistry

The synthesis of the NO-donors been has performed in two steps. In the first step by reaction of theophylline (**1**) and 8-nitro-theophyline (**2**) with 4-(2,3-epoxy-propyl)-acetamino-phen (**3**) (scheme 1) the 7-[2-hydroxy-3-(4-acethyl-amino-phenoxy)-propyl]-8-R-1,3-dimethyl-xanthine derivatives (D1, D2) were obtained. In the second step these intermediary derivatives by reaction with chloroacetyl cloride (**4**), in tetrahydrofuran (THF) and tryethylamine (TEA) were turned at corresponding chloroacethyl derivatives (**5, 6**), that by reaction with silver



Scheme 1. Synthesis of the 7-[2-hydroxy-3-(4-acethyl-amino-phenoxy)-propyl]-8-R-1,3-dimethyl-xanthine derivatives (D1, D2)



Scheme 2. Synthesis of the 7-[2-nitroxyacethyl-oxy-3-(4-acethyl-amino-phenoxy)-propyl]-8-R-1,3-dimethyl-xanthine derivatives (65, 77)

nitrate (**7**) in dimethylformamide medium lead to two new nitric oxide donors, 7-[2-nitroxyacethyl-oxy-3-(4-acethyl-amino-phenoxy)-propyl]-8-R-1,3-dimethyl-xanthine (**65**, **77)** (scheme 2).

The structure of the intermediary and nitric oxide donors was confirmed by elemental analysis, spectroscopic and thermal methods. The partial results of the elemental analysis were given in the previous paper [17]. For the NO-donors all values of the percentage of elements amounts have been found in the limit of the experimental errors of ± 0.4 % in respect with calculated values. FT-IR spectra (fig. 1) evidence that both intermediary and NO-donors contain all characteristic bands of theophylline and paracetamol but also apparition of new bands corresponding to the vibration of the aliphatic groups CH, assigned to link of the two molecules. Depending on the radical nature the shift of bands at smaller wavenumbers was found. The main bands found have been given above and they have been assigned according to the literature data [16]. In the ^{1}H-NMR spectra all signals of the parent compounds and those corresponding to the alkyl spacer and CH_2-ONO_2 groups are identified.

Their physico-chemical and spectral characterization is as it follows:

7-[2-hydroxy-3-(4-acethyl-amino-phenoxy)-propyl]-1,3-dimethyl-xanthine (D1): white crystal, yield 90.08%, m.p. 202-203 °C; IR (cm^{-1}, KBr) ν: 3380-3420 cm^{-1} (linked OH); 1310, 1200 cm^{-1} (free OH); 2790 cm^{-1} (>N-CH_3); 2650 cm^{-1} (>C=O); 1660 cm^{-1} (>C=C<$_N$); 1580 cm^{-1} (>C=C<$_{CO}$); 1510 cm^{-1}(>C=N); 1610 cm^{-1} (-NH-CO); 1480 cm^{-1}, 780 cm^{-1}, 700 cm^{-1} (-C_6H_4-); 1380 cm^{-1} (-CO-CH_3); 2900 cm^{-1} (CH_3, -CH_2-); 1350 cm^{-1} (-O-CH_2); ^{1}H-NMR (DMSO-d_6) δ: 2.02 (d, 1H, OH), 2.06 (s, 3H, CH_3-CO), 2.70 (s, 6H, 2CH_3-N<), 3.61 (m, 1H, CH), 3.88 (d, 2H, CH_2-N<), 4.09 (d, 2H, CH_2-O), 6.80 (d, 2H, C_6H_4), 7.59 (d, 2H, C_6H_4), 7.98 (s, 1H, CH); 8.12 (s, 1H, NH).

7-[2-hydroxy-3-(4-acethyl-amino-phenoxy)-propyl]-8-nitro-1,3-dimethyl-xanthine (D2): light yellow crystal, yield 81.01%, m.p. 218 °C; IR (cm^{-1}, KBr) ν: 3300 cm^{-1} (linked OH); 1210 cm^{-1} (free OH); 2790 cm^{-1} (>N-CH_3); 2650 cm^{-1} (>C=O); 1650 cm^{-1} (>C=C<$_N$); 1570 cm^{-1} (>C=C<$_{CO}$); 1500 cm^{-1} (>C=N); 1620 cm^{-1} (-NH-CO); 1480 cm^{-1}, 780 cm^{-1}, 700 cm^{-1} (-C_6H_4-); 1380 cm^{-1} (-CO-CH_3); 2900 cm^{-1} (CH_3, -CH_2-); 1310 cm^{-1} (-O-CH_2); 1560 cm^{-1} (-C-NO_2); ^{1}H-NMR (DMSO-d_6) δ: 2.06 (d, 1H, OH), 2.10 (s, 3H, CH_3-CO), 2.62 (s, 6H, 2CH_3-N<), 3.58 (m, 1H, CH), 3.86 (d, 2H, CH_2-N<), 4.20 (d, 2H, CH_2-O), 6.54 (d, 2H, C_6H_4), 7.41 (d, 2H, C_6H_4), 8.04 (s, 1H, NH).

7-[2-nitroxyacethyl-oxy-3-(4-acethyl-amino-phenoxy)-propyl]-1,3-dimethyl-xanthine (65): yellow crystal, yield 76.28%, m.p. 210-211 °C; IR (cm^{-1}, KBr) ν: 2997, 2948 cm^{-1} (>N-CH_3); 2817 (-CH<); 2850 cm^{-1} (>C=O): 1607 cm^{-1} (>C=C<$_N$); 1549 cm^{-1} (>C=C<$_{CO}$); 1697 cm^{-1}(>C=N); 1653 cm^{-1} (-NH-CO); 1507 cm^{-1}, 760 cm^{-1}, 670 cm^{-1} (-C_6H_4-); 1335 cm^{-1} (-CO-CH_3); 1278 cm^{-1} (-O-NO_2); 2903 cm^{-1} (CH_3, -CH_2-); 1473 cm^{-1} (-O-CH_2); ^{1}H-NMR (DMSO-d_6) δ: 2.01 (s, 3H, CH_3-CO), 3.22 (s, 6H, 2CH_3-N<); 3.42 (m, 1H, CH), 3.20 (d, 2H, CH_2-N<); 3.87 (d, 2H, CH_2-O), 3.91 (s, 2H, CH_2-O), 6.87 (d, 2H, C_6H_4), 7.46 (d, 2H, C_6H_4), 7.97 (s, 1H, CH); 9.77 (s, 1H, NH).

7-[2-nitroxyacethyl-oxy-3-(4-acethyl-amino-phenoxy)-propyl]-8-nitro-1,3-dimethyl-xanthine (77): yellow dark crystal, yield 61.56%, m.p. 240-241 °C; IR (cm^{-1}, KBr) ν: 2927 cm^{-1} (>N-CH_3); 2821 (-CH<); 2892 cm^{-1} (>C=O); 1601 cm^{-1} (>C=C<$_N$); 1542 cm^{-1} (>C=C<$_{CO}$); 1702 cm^{-1}(>C=N); 1542 cm^{-1} (-NH-CO); 1510 cm^{-1}, 848 cm^{-1}, 658 cm^{-1} (-C_6H_4-); 1315 cm^{-1} (-CO-CH_3); 1281 cm^{-1} (-O-NO_2); 2942 cm^{-1} (CH_3, -CH_2-); 1463 cm^{-1} (-O-CH_2); 1363 cm^{-1} (-C-NO_2); ^{1}H-NMR (DMSO-d_6) δ: 2.00 (s, 3H, CH_3-CO); 3.34 (s, 6H, 2CH_3-N<); 3.43 (m, 1H, CH), 3.21 (d, 2H, CH_2-N<); 4.23 (d, 2H, CH_2-O), 4.18 (s, 2H, CH_2-O), 6.86 (d, 2H, C_6H_4), 7.45 (d, 2H, C_6H_4); 8.06 (s, 1H, NH).

The thermal analysis brings new proof on NO-donor compounds formation. From the TG/DTG curves (fig. 2 and table 1) it can be remarked specific temperatures of decomposition for each compounds. Tm - temperature corresponding to the maximum mass loss rate increased from 317 and 342°C for paracetamol and theophylline, respectively to 416 and 426°C for 65 and 77 NO-donor compounds respectively. The entire curve is shifted to high temperature the NO-donor compounds having a particular thermal behaviour in respect with parent molecules. They exhibit an increased thermal stability evidenced by higher temperature and smaller mass loss than those of paracetamol and theophylline.

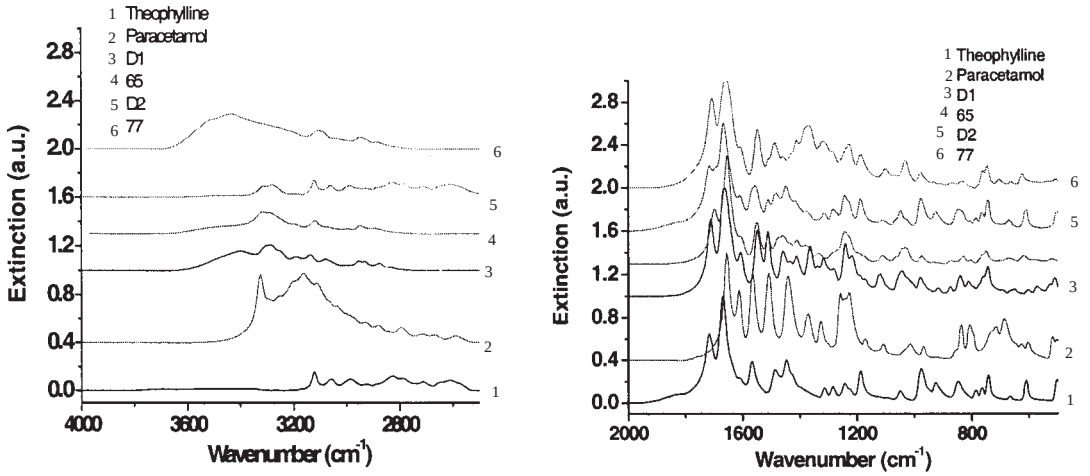
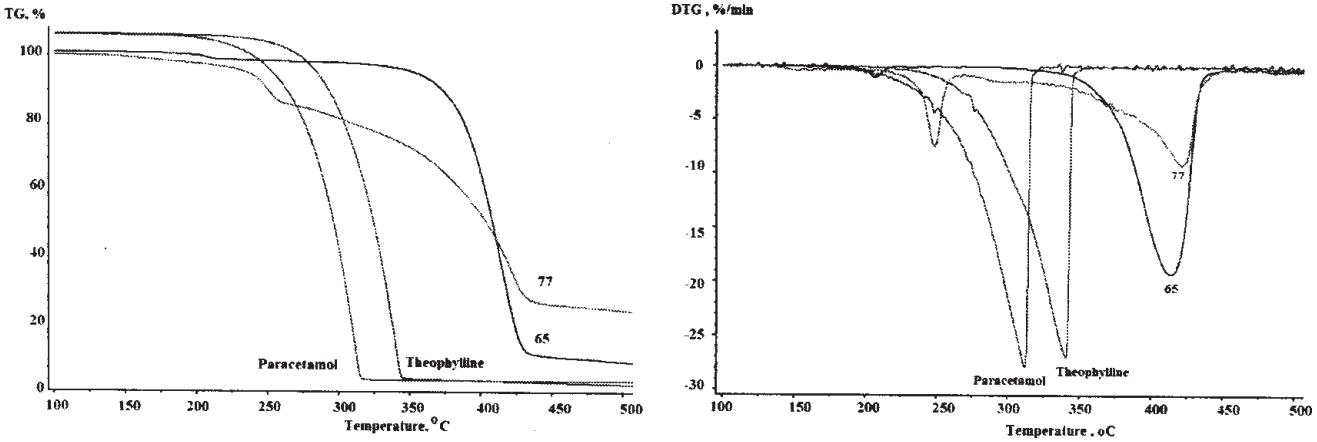


Fig. 1. The FT-IR spectra of the theophylline, paracetamol, intermediary (D1, D2) and nitric oxide donor (65, 77) compounds



a b

Fig. 2. TG (a) and DTG (b) curves of the paracetamol, theophylline and NO-donors (65 and 77)

Compound	Characteristic temperatures (°C)			Mass loss at 400 °C or 450 °C (wt%)
	Ti	Tm	Tf	
Paracetamol	190	317	325	100
Theophylline	230	342	358	100
D_1	229	396	469	64.9
65	320	416	450	88
D_2	210	391	443	62.0
77	215 320	252 426	275 460	17 73

Table 1
THERMOGRAVIMETRIC CHARACTERISTICS OF THE STUDIED COMPOUNDS

Ti- inset temperature; Tm - temperature corresponding to the maximum mass loss rate;

Tf – final temperature.

Biological activity of the studied drugs

Acute toxicity determination

The values of the lethal dose 50 (DL_{50}) are given in table 2 as average of six determinations. From the presented results it can be easily observed that the substitution of the theophylline in the 7 site with 2-hydroxy-3-(4-acetyl-amino)-phenoxy-propyl radical (D1) lead to the reduction of the toxicity increased of the DL_{50} from 200 mg/kg body to 312 mg/kg body up to 1.6 times. The toxicity is much more reduced by the substitution of the xanthine ring in 8 position with nitro radical (D2), the corresponding compound having a DL_{50} of 509 mg/kg body. Comparatively with paracetamol with a DL_{50} 338 mg/kg body, the intermediary derivatives have a comparable toxicity (D1) or lower (D2). The NO-donors, noted as 65 and 77, which are bifunctional compounds are less toxic than parent compounds namely of 4 and 2.2 times less toxic than theophylline and of approximately 2.4 and 1.3 times, respectively less toxic than paracetamol.

Effect on the subacute oedema inflammation

The results of the intermediary compounds and corresponding NO-donors on the subacute inflammatory oedema induced on rat by cotton pellet granuloma are

Compound	DL_{50} (mg/kg body)			
	24 h	48 h	72 h	Average
D1	328	328	280	312
65	819	819	798	812
D2	524	524	480	509
77	454	454	442	450
Theophylline				200
Paracetamol				338

Table 2
DL_{50} VALUES (MG/KG BODY) FOR PARACETAMOL, THEOPHYLLINE, INTERMEDIARY AND CORRESPONDING NO - DONOR COMPOUNDS

Compound	Oral dose (mg/kg body),	Retained inflammation (%) ± S.E.M (n=6)
D1	25.75	58.15± 2.90
65	40.60	23.73± 1.20
D2	28.97	41.80±2.30
77	22.50	18.40±1.10
Theophylline	10.00	61.10±3.20
Paracetamol	16.90	99.54±5.02
Reference	4 mL	100±5.38

Table 3
RETAINED INFLAMMATION UNDER THE ACTION OF THE STUDIED COMPOUNDS

listed in table 3. It can be easily observed that both intermediary (D1, D2) and NO-donors (65, 77) showed higher anti-inflammatory effect in respect both with theophylline and paracetamol. Moreover, the anti-inflammatory effects of the NO-donor compounds are higher than those of the intermediary compounds which are a proof of the NO involvement in the inflammatory process. NO-donors are more active namely with 2.5 times for (65) and 3.3 times (77) than theophylline and of 4.2 times (65) and 5.4 times (77) more active than paracetamol in the reduction of the subacute infammatory oedema induced by cotton pellet granuloma.

Conclusions

Some new nitric oxide donors, derivated from theophylline and paracetamol have been synthesized. The structure of these new compounds was confirmed by ^{1}H-NMR, FT-IR spectroscopy and thermal methods. The pharmacological profile was established, the compounds being evaluated for their toxicity degree and anti-inflammatory activity. The synthesized compounds are less toxic than theophylline and paracetamol. In the reduction of the subacute infammatory oedema NO-donors are more active than theophylline and paracetamol, that is a proof of the nitric oxide involvement in the inflammatory process.

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