Cytotoxic Abietane Diterpenes from *Hyptis martiusii* Benth.

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From roots of *Hyptis martiusii* Benth. two tanshinone diterpenes were isolated, the new 7β-hydroxy-11,14-dioxoabieta-8,12-diene (**1**) in addition to the known 7α-acetoxy-12-hydroxy-11,14-dioxoabieta-8,12-diene (7α-acetoxyroyleanone) (**2**). Structures of **1** and **2** were established by spectroscopic means. The cytotoxic activity against five cancer cell lines was evaluated. Compounds **1** and **2** displayed considerable cytotoxic activity against several cancer cell lines with IC_{50} values in the range of 3.1 to 11.5 μg/ml and 0.9 to 7.6 μg/ml, respectively. The cytotoxic activity seemed to be related to inhibition of DNA synthesis, as revealed by the reduction of 5-bromo-2′-deoxyuridine incorporation and induction of apoptosis, as indicated by the acridine orange/ethidium bromide assay and morphological changes after 24 h of incubation in leukemic cells.

Key words: Hyptis martiusii, Labiatae, Abietane Diterpenes, Cytotoxic Activity

Introduction

Tanshinones are abietane-type diterpenoids characterized by *ortho*- or *para*-benzoquinone chromophores in the aromatic ring C. The occurrence of tanshinones is restricted to species of Labiatae, and they have been repeatedly found in *Salvia* (Jimenez *et al*., 1988; Cardenas and Rodriguez-Hanh, 1995) and *Coleus* species (Mehrotra *et al*., 1989; Kelecon and Santos, 1985). The broad spectrum of their biological activities, *i.e.* antituberculous, antimicrobial, anti-tumor promoting, antibacterial, antioxidant, antiviral, antileishmanial, moluscicide, and significant cytotoxicity against human cancer cell lines (Ulubelen *et al*., 1997; Lee *et al*., 1987; Zhang *et al*., 1990; Tan *et al*., 2002; Chen *et al*., 2002) have attracted the attention of medicinal chemists and clinicians.

As part of our interest in studying biologically active natural substances from the flora of northeastern Brazil, we have recently reported the cytotoxic activity of two abietane diterpenes isolated from the roots of *Hyptis martiusii* Benth. (Labiatae) (Costa-Lotufo *et al*., 2003). In continuation, we report herein the isolation and structural characterization of two tanshinones, the new 7β-hydroxy-11,14-dioxoabieta-8,12-diene (**1**), and the known 7α-acetoxy-12-hydroxy-11,14-dioxoabieta-8,12-diene (7α-acetoxyroyleanone or 7-*O*-acetylhorminon, **2**) (Hensch *et al*., 1975; Bakshi *et al*., 1986). The cytotoxic activity of these compounds against several tumor cell lines as well as their effect on DNA synthesis and apoptosis were also evaluated.

Material and Methods

Plant material

The entire plant was collected in August 1999, at the flowering stage, from plant populations growing wild in "Chapada do Araripe" (Araripe's Plateau), Crato County, Ceará State, Northeastern Brazil. A voucher specimen (# 25046) has been identified by Dr. Afrânio G. Fernandes (botanist) and deposited at the Herbário Prisco Bezerra (EAC), Departamento de Biologia, Universidade Federal do Ceará, Brazil.

Extraction and isolation

Air-dried roots (3.4 kg) of *Hyptis martiusii* were pulverized and extracted with hexane (9.0 l) at room temperature. The solvent was removed under reduced pressure yielding a viscous brown oil

(13.0 g). The marc obtained after hexane extraction was extracted with EtOH (9.0 l) to give a dark brown resinous extract (87.8 g).

The hexane extract (13.0 g) was adsorbed on silica gel (20.0 g) and coarsely fractionated over a silica gel (100.0 g) column by elution with hexane, $CHCl_3$, EtOAc and MeOH, to give four fractions (solvent ratio, solvente volume, mass): A (hexane, 250 ml, 1.7 g); B ($CHCl_3$, 250 ml, 5.6 g); C (EtOAc, 250 ml, 5.7 g); D (MeOH, 200 ml, 0.3 g). Fraction B was rechromatographed over a column of silica gel (50.0 g), further eluted with an isocratic mixture of hexane/EtOAc (9:1) to give 89 fractions (10 ml), which were subsequently combined on the basis of TLC analysis to yield 11 main fractions. Fractions 15–23 gave pure **1** (14.0 mg) and fractions 35–42 gave **2** (6.0 mg).

7β-Hydroxy-11,14-dioxoabieta-8,12-diene (**1**): Yellowish oil. $-[\alpha]_D^{20}$ +20.0° (*c* 0.1, $CHCl_3$). – IR: ν_{max} = 3430, 2958, 1654, 1652, 1458, 1385, 1375, 1342, 1242, 1153 cm^{-1}. – EI-MS: *m/z* (rel. int.) = 316 ([M^+], 6), 298 (81), 283 (100), 269 (34), 255 (22), 241 (34), 228 (28), 213 (36), 189 (31), 161 (28), 109 (25), 91 (38), 69 (44), 55 (72), 43 (69). – ^1H and ^{13}C NMR ($CDCl_3$, 500 and 75 MHz): see Table I.

Determination of cytotoxicity

The cytotoxic potential of both tanshinones was evaluated against five tumor cell lines (National Cancer Institute, Bethesda, MD, USA): B16 (murine skin), HCT-8 (human colon), MCF-7 (human breast), and CEM and HL-60 (human leukemias), using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay as described by Mosmann (1983) after 72 h of incubation. Doxorubicin was used as positive control.

Trypan blue exclusion

Cell viability was determined by the Trypan blue dye exclusion test on HL-60 human leukemia cells (Renzi *et al*., 1993). For the experiments, the cells (3×10^5 cells/ml) were incubated with the two tanshinones at the concentrations of 1, 5 and 10 μg/ml and trypan blue-excluding cells were counted in a Newbauer Chamber, on cells aliquots removed from cultures after 24 h. Viability of untreated and DMSO-treated cells was also assessed and was always greater than 95%. Doxorubicin (0.3 μg/ml) was used as a positive control.

Analysis of morphological changes

Untreated or tanshinones-treated (1, 5 and 10 μg/ml, 24 h) HL-60 cells were examined for morphological changes by optical microscopy. To assay nuclear morphology, cells were harvested, placed on a glass slide using cytospin, fixed with 96% ethanol for 1 h and stained with eosin-hematoxylin. Doxorubicin (0.3 μg/ml) was used as a positive control.

Assessment of apoptosis

Acridine orange/ethidium bromide (AO/EB) staining of HL-60 cells (3×10^5 cells/ml) was done to observe the apoptotic pattern induced by increasing concentrations of the two tanshinones (1, 5 and 10 μg/ml) after 24 h of incubation according to the method described by Cury-Boaventura *et al*. (2004). Doxorubicin (0.3 μg/ml) was used as a positive control. After the incubation, cells were pelleted and resuspended in 25 μl of PBS. Afterwards, each sample was mixed with 1 μl AO/EB solution (1 part of 100 μg/ml of AO in PBS; 1 part of 100 μg/ml EB in PBS) just prior to microscopic examination and quantification. The cell suspension (10 μl) was placed on a microscopic slide, covered with a glass coverslip, and at least 300 cells were examined under a fluorescence miscroscope using a fluorescein filter and a 40× objective lens. The percentages of viable, apoptotic and necrotic cells were then calculated.

Inhibition of DNA synthesis

HL-60 cells (3×10^5 cells/ml) were plated onto 24-well tissue culture (2 ml/well) and treated with the two tanshinones for 24 h at the concentrations of 1, 5 and 10 μg/ml. Doxorubicin (0.3 μg/ml) was used as a positive control. 20 μl of 5-bromo-2′-deoxyuridine (BrdU, 10 mM) were added to each well and incubated for 3 h at 37 °C before the complete period of drug incubation. To assay the amount of BrdU incorporated on cell DNA, cells were harvested, placed on a glass slide using cytospin, and left to dry for 2 h at room temperature. Cells that had incorporated BrdU were labelled by direct peroxidase immunocytochemistry utilizing the chromogen DAB. Slides were counterstained with hematoxylin, mounted, and coverslipped. Evaluation of BrdU incorporation was accomplished by microscopy (Pera *et al*., 1977). 200 cells were

counted per sample to get the percentage of positive cells.

Statistical analysis

For cytotoxicity assays, the IC_{50} values and their 95% confidence intervals (CI 95%) were obtained by nonlinear regression using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA). For cell viability and apoptosis assessment, the differences between experimental groups were compared by ANOVA followed by Student Newman Keuls, and for inhibition of DNA synthesis, the differences were compared by χ^2. The significance level was $p < 0.05$.

Results and Discussion

Compound **1** was isolated as a yellowish oil. The IR spectrum showed the presence of a *para*-benzoquinone moiety (1654, 1652 cm^{-1}), hydroxy (3430 cm^{-1}), and isopropyl groups (1385 and 1375 cm^{-1}). The ^1H NMR spectrum revealed signals relative to an olefinic proton at δ 6.34 (s, H-12), three angular methyl groups at δ 0.90 (H-19), 0.91 (H-18) and 1.35 (H-20), and an oxymethine proton at δ 4.76 (dd, J = 10.1 and 2.5 Hz, H-7). The presence of the isopropyl group attached to the quinone ring, indicative of an abietane-type diterpenoid, was suggested by the presence of the signals at 2.96 (sept, H-15, J = 6.6 Hz), 1.09 (d, H-17, J = 6.6 Hz) and 1.08 (d, H-16, J = 6.6 Hz). This was confirmed by COSY. The H-1β equatorial hydrogen signal at 2.62 appeared at lower field, as a result of the deshielding effect of the carbonyl group at C-11, typical of the tanshinone skeleton (Hayashi and Kakisawa, 1970). The ^{13}C NMR spectrum displayed signals for 20 atoms. The DEPT spectrum, by comparison with the ^{13}C broad band decoupled data, allowed the characterization of five methyl, four methylene, four methine groups and seven non-hydrogen-bearing carbon atoms, two of which were carbonyl groups (Table I). As the ^{13}C NMR chemical shifts of the carbonyl carbon atoms of *ortho*-benzoquinones are more shielded (~ δ 180.2) than those of *para*-benzoquinones (~ δ 187.0) (Levy *et al.*, 1980), and the two carbonyl groups of **1** occurred at δ 190.2 and 188.2 it was obviously a *para*-benzoquinone derivative.

Table I. ^1H (500 MHz) and ^{13}C (75 MHz) NMR spectral data[a] for **1** ($CDCl_3$).

Carbon	HMQC correlations		HMBC correlations	
	δ_C	δ_H	$^2J_{CH}$	$^3J_{CH}$
1	36.3	2.62 (dt, J = 10.1; 2.5) 1.04 (m)		3H-20
2	18.7	1.68 (m) 1.50 (m)		
3	41.1	1.50 (m) 1.17 (m)		
4	33.2	–	H-3	
5	48.9	1.17 (m)		3H-20
6	26.3	2.17 (dd, J = 13.0; 7.5) 1.50 (m)		
7	67.9	4.76 (dd, J = 10.1; 2.5)		H-5
8	141.9	–	H-7	
9	152.5	–		H-7, H-12, 3H-20
10	39.6	–	H-5, 3H-20	
11	188.2	–		H-15
12	132.3			
13	153.1	–	H-15	
14	190.2	6.34 (s)		H-12, H-15
15	26.2	2.96 (sept, J = 6.6)		H-12
16	21.3	1.09 (d, J = 6.6)		
17	21.4	1.08 (d, J = 6.6)		
18	21.7	0.91 (s)		
19	33.3	0.90 (s)		
20	20.0	1.35 (s)		
OH		3.80 (s)		

[a] Assigments were based on DEPT, COSY, HMQC and HMBC experiments. Coupling constants (J) in Hz.

The HMBC spectrum showed long-range correlations between the olefinic proton α to the carbonyl group at δ 6.34 (H-12) with the carbon atoms at δ 141.9 (C-9, $^3J_{C,H}$), 26.2 (C-15, $^3J_{C,H}$) and the carbonyl group at δ 190.2 (C-14, $^3J_{C,H}$). Additionally, correlations between the methine hydrogen atom of the isopropyl system at δ 2.96 (H-15) with the carbon atoms at δ 132.3 (C-12), 153.1 (C-13) and the carbonyl group at δ 190.2 (C-14) allowed the placement of the olefinic hydrogen atom at C-12 and of the isopropyl group at C-13, respectively. The position of the oxymethine hydrogen atom at δ 4.76 on C-7 was established through its correlations with C-8 at δ 141.9 and C-9 at δ 152.5, while the coupling constants involving H-7 (dd, J = 10.1 and 2.5 Hz) indicated that the hydroxy group on C-7 was β-oriented (Table I). Bakshi and co-workers reported the isolation of both 7α- and 7β-hydroxy derivatives of royleanone (12-hydroxy-11,14-dioxoabieta-8,12-diene), otherwise referred as horminon and taxochinon, respectively (Bakshi *et al.*, 1986). They stated that the compounds were identical to those isolated by Brieskorn and Buchberger (1982) and Hensch *et al.* (1975) but the structures they showed in their paper are missing the hydroxy group at C-12, mistakenly driving to the conclusion that they have indeed isolated both **1** and its β-epimer. A patent filling, written in Japanese, for a novel diterpene quinone useful as an aldose reductase inhibitor was found in the literature (Koiwai *et al.*, 1990). Even though the NMR data seem to be identical, the suggested structure has neither a stereochemical definition nor any NMR assignments. Hence, this is the first report of the unambiguous assignments of 7β-hydroxy-11,14-dioxoabieta-8,12-diene, including the relative stereochemistry (Fig. 1).

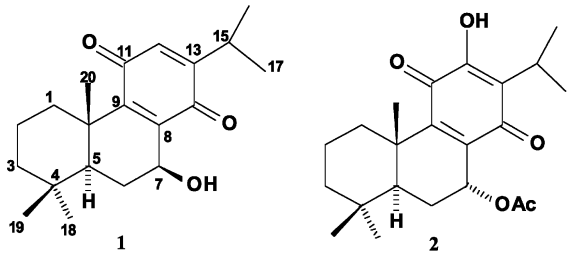

Fig. 1. Structures of the two tanshinones isolated from the roots of *Hyptis martiusii,* the new 7β-hydroxy-11,14-dioxoabieta-8,12-diene (**1**) and the known 7α-acetoxy-12-hydroxy-11,14-dioxoabieta-8,12-diene (7α-acetoxyroyleanone, **2**).

Five tumor cell lines were treated with increasing concentration of both tanshinones (**1** and **2**, 0.39 to 25 μg/ml) for 72 h. In Table II the observed IC_{50} values are shown. It is worthwhile to mention that after 72 h of incubation, compound **2** was slightly more active than compound **1** as observed through the increased IC_{50} values for **1**. The IC_{50} ratio changes from 1.5 on HCT-8 cells to 6.4 on MCF-7 cells. However, after 24 hours the efficacy of **2** against HL-60 is quite smaller than that observed earlier (IC_{50} ratio 24 h/72 h = 14.4), while for compound **1** there is no difference (IC_{50} ratio 24 h/72 h = 1.35), suggesting that the cytotoxicity exhibited by compound **2** is time-dependent. Analysis of cell viability after 24 h of incubation by trypan blue exclusion revealed that compound **1** significantly reduced the number of viable cells at the concentrations of 5 and 10 μg/ml, but only in the samples treated with 10 μg/ml was observed an increasing number of non-viable cells (Fig. 2, $p < 0.05$). Compound **2**, on the other hand, only decreased HL-60 viability at the concentration of

Table II. Cytotoxic activity of the tanshinones **1** and **2** isolated from *Hyptis martiusii* on tumor cell lines. Data are presented as IC_{50} values and 95% confidence interval obtained by non-linear regression for leukemias (CEM and HL-60), colon (HCT-8), breast (MCF-7), and skin (B16) cancer cells from three independent experiments. Doxorubicin was used as positive control.

Compound	Cell line IC_{50} [μg/ml (μM)]				
	CEM	HL-60	HCT-8	MCF-7	B16
Doxorubicin	0.02 (0.03)	0.02 (0.03)	0.04 (0.06)	0.20 (0.34)	0.03 (0.05)
	0.01–0.02	0.01–0.02	0.03–0.05	0.17–0.24	0.02–0.04
1	4.1 (12.9)	4.2 (13.2)	11.5 (36.4)	6.4 (20.4)	3.1 (9.9)
	3.7–4.6	3.7–4.7	10.5–12.6	5.8–7.2	2.8–3.5
2	1.2 (3.4)	1.7 (4.8)	7.6 (21.5)	1.0 (2.8)	0.9 (2.5)
	1.0–1.3	1.5–1.9	7.1–8.0	0.8–1.2	0.7–1.2

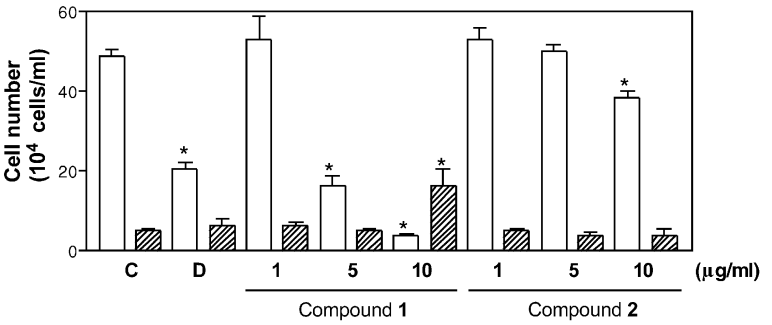

Fig. 2. Effect of the tanshinones **1** and **2** isolated from roots of *Hyptis martiusii* on HL-60 cell viability determined by trypan blue staining after a 24 hour incubation. Open columns show the number of viable cells and hatched columns show the number of non-viable cells. Negative control (C) was performed in the presence of vehicle used for suspension of tested substances. Doxorubicin (0.3 μg/ml) was used as positive control (D). * $p < 0.05$ compared to control by ANOVA followed by Student Newman Keuls. Experiments were performed in triplicate.

10 μg/ml. These data corroborated the MTT analysis. According to Sairafianpour *et al.* (2001) the presence of a D ring in tanshinones is apparently necessary for the high cytotoxicity. Our data showed considerable cytotoxicity for two tanshinones which lack ring D, but exhibit IC_{50} values quite similar to those obtained for previously described cytotoxic tanshinones. Thus, the presence of ring D is apparently not the only structural requirement for cytotoxicity.

Such antiproliferative effects were further investigated in order to assess the mechanism of cytotoxic action presented by the two tanshinones. Morphological examination of promyelocytic HL-

Table III. Determination of the proportion of necrotic and apoptotic HL-60 leukemia cells treated for 24 hours with increasing concentrations of the tanshinone diterpenes **1** and **2** isolated from *Hyptis martiusii*. Doxorubicin was used as positive control. Data represent mean ± SEM obtained from 3 different fields using fluorescence microscopy (× 400).

Compound	Concentration [μg/ml (μM)]	Viable cells (%)	Apoptotic cells (%)	Necrotic cells (%)
Control	–	98.67 ± 0.67	1.33 ± 0.67	0.00 ± 0.00
Doxorubicin	0.3 (0.52)	37.67 ± 2.96[a]	61.33 ± 3.18[a]	1.00 ± 0.77
1	1 (3.2)	70.33 ± 2.85[a]	27.33 ± 3.18[a]	2.33 ± 0.67
	5 (15.8)	51.33 ± 1.86[a]	48.67 ± 1.86[a]	0.00 ± 0.00
	10 (31.6)	9.67 ± 1.20[a]	6.00 ± 0.58	84.33 ± 1.67[a]
2	1 (2.7)	73.00 ± 1.53[a]	25.33 ± 2.73[a]	1.67 ± 1.20
	5 (13.4)	61.00 ± 5.20[a]	39.00 ± 5.20[a]	0.00 ± 0.00
	10 (26.7)	73.00 ± 2.52[a]	27.00 ± 2.52[a]	0.00 ± 0.00

[a] $p < 0.05$, ANOVA followed by Student Newman Keuls.

Compound	Concentration [μg/ml (μM)]	BrdU positivity (%)	T/C[a]
Control	–	66.5	–
Doxorubicin	0.3 (0.52)	36.0	0.54
1	1 (3.2)	59.5	0.89
	5 (15.8)	40.5	0.61[b]
	10 (31.6)	n.d.	n.d.
2	1 (2.8)	66.0	0.99
	5 (14.1)	63.5	0.95
	10 (28.2)	50.5	0.76[b]

Table IV. Inhibition of 5-bromo-2′-deoxyuridine (BrdU) incorporation by the tanshinone diterpenes **1** and **2** isolated from *Hyptis martiusii* on HL-60 human leukemia cells. Doxorubicin was used as positive control. Data are reported as percent of BrdU positivity per 200 cells.

[a] T/C ratio was calculated using the % labeled cells: treated/control.

[b] $p < 0.05$ compared by χ^2 test.

n.d., not determined because most cells are non-viable.

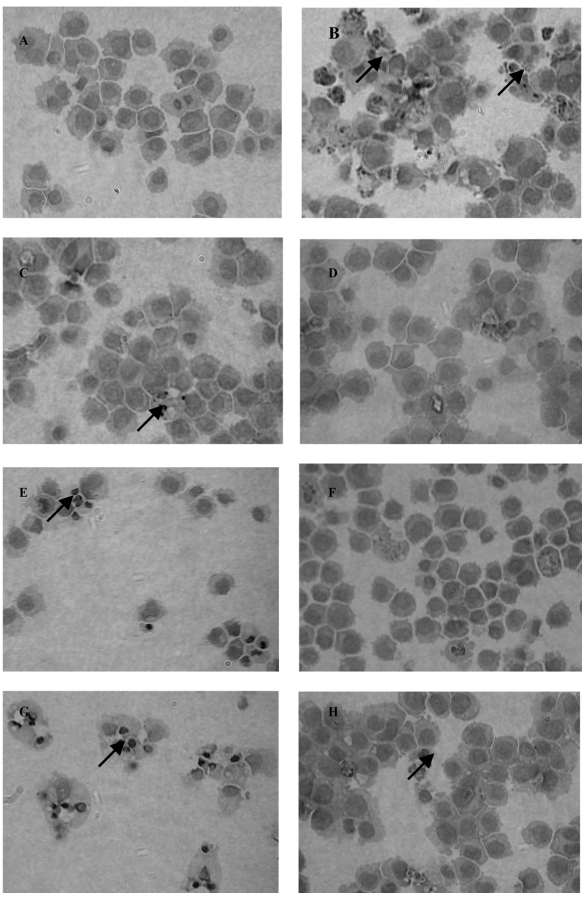

Fig. 3. Microscopic appearance of hematoxylin/eosin-stained HL-60 cells. Cells were untreated (A) or treated with 1 μg/ml (C, D), 5 μg/ml (E, F) and 10 μg/ml (G, H) of compound **1** or **2**, respectively, and analyzed by light microscopy (× 400). Doxorubicin (0.3 μg/ml) was used as positive control (B). Arrows indicate condensed and fragmented nuclei.

60 leukemia-treated and untreated cells revealed severe changes (Fig. 3). Chromatin condensation and fragmentation of the nuclei – a morphology consistent with apoptosis – were observed after 24 h of incubation with compound **1** at 1 μg/ml (Fig. 3C) and compound **2** at 10 μg/ml (Fig. 3H). With increasing concentrations of **1** (5 and 10 μg/ml, Figs. 3E and 3G, respectively), picnosis was quite evident. In the presence of doxorubicin 0.3 μg/ml (Fig. 3B), nuclear fragmentation of high number of cells was observed. The proportion of viable, apoptotic and necrotic HL-60 cells after treatment with compounds **1** and **2** (1, 5 and 10 μg/ml) for 24 h was determined (Table III). Treatment with compound **1** reduced the number of viable cells in a concentration-dependent manner. At concentrations of 1 and 5 μg/ml, an increasing number of apoptotic cells was observed, but at the highest concentration (10 μg/ml), most cells were necrotic, what could be related to the secondary necrosis observed during the apoptotic process. Compound **2** induced a significant reduction of viable cells, but with no correlation with concentration, showing 27.0% of apoptotic cells and no necrotic cells at the concentration of 10.0 μg/ml.

The effect of the isolated compounds on DNA synthesis was also evaluated. The data in Table IV show the inhibition of BrdU incorporation by HL-60 cells after 24 h of incubation with the two tanshinones. Compound **1** was more effective than compound **2**. Compound **1** caused significant inhibition at a concentration of 5 μg/ml, while compound **2** was only active at 10 μg/ml.

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