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Original article

Synthesis and *in vitro* and *in vivo* biological evaluation of substituted nitroquinoxalin-2-ones and 2,3-diones as novel trichomonacidal agents

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Dedicated to our dear friend and colleague Prof. José Elguero (IQM, CSIC, Madrid) for his outstanding contribution to heterocyclic chemistry, on the occasion of his 80th birthday.

Keywords:
Antiprotozoal drugs
Nitroheterocycles
Trichomonas vaginalis
Quinoxaline
Lipinski's rule

ABSTRACT

Two series of ten novel 7-nitroquinoxalin-2-ones and ten 6-nitroquinoxaline-2,3-diones with diverse substituents at positions 1 and 4 were synthesized and evaluated against the sexually transmitted parasite *Trichomonas vaginalis*. Furthermore, diverse molecular and drug-likeness properties were analyzed to predict the oral bioavailability following the Lipinski's "rule of five". 7-Nitroquinoxalin-2-one derivatives displayed moderate to high *in vitro* activity while the efficiency of most nitroquinoxaline-2,3-diones was rather low; both kinds of compounds did not show cytotoxic effects in mammalian cells. 7-Nitro-4-(3-piperidinopropyl)quinoxalin-2-one **9** achieved the highest trichomonacidal activity (IC_{50} = 18.26 μM) and was subsequently assayed *in vivo* in a murine model of trichomonosis. A 46.13% and a 50.70% reduction of pathogenic injuries were observed in the experimental groups treated orally during 7 days with 50 mg/kg and 100 mg/kg doses. The results obtained in the biological assays against *T. vaginalis* indicate that compounds with ω-(dialkylamino)alkyl substituents and a keto group at positions 4 and 2 of quinoxaline ring, respectively, provide interesting structural cores to develop novel prototypes to enhance the nitroquinoxalinones activity as trichomonacidal agents with interesting ADME properties according to virtual screening analysis.

1. Introduction

Trichomonas vaginalis is a microaerophilic flagellate responsible of one of the most important sexually transmitted infections (STI) with an incidence of more than 276 million cases per year in individuals between the ages of 15–49 [1]. Trichomonosis is associated with adverse reproductive health outcomes [2], pelvic inflammatory disease [3] and development of prostate [4,5] and cervical neoplasia [6,7]. Furthermore, *T. vaginalis* infection increases risk of transmission and acquisition of human immunodeficiency virus (HIV-1) [8]. Therefore, prevention and control of this STI is one of the main Millennium Development Goals towards to prevent and reduce HIV infections [9]. Although nearly one million people is infected by a sexually transmitted pathogen every day [9], trichomonosis continues being one of the "neglected diseases of poverty" [10] and the STI with the lowest public health attention in relation to other bacterial or viral STI [11]. Metronidazole and tinidazole (5-nitroimidazole derivatives) are the only drugs approved by the U.S. Food and Drug Administration (FDA) for the treatment of trichomonosis [12] since the early 60s. Metronidazole presents a good pharmacokinetic profile [13] and high percentage of cure rates have been reported [14]; however, the development of resistance cases [15] and iatrogenic side effects [16] make necessary the search for

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novel chemical candidates to increase the therapeutic arsenal.

On the other hand, quinoxaline derivatives are interesting molecules with diverse pharmacological properties. In the field of chemotherapeutic agents, for example, reports on their anti-chagasic [17,18], antimalarial [19] and anticancer [20] properties have recently been published. More specifically, quinoxalin-2-ones and quinoxaline-2,3-diones have shown to display antiviral [21–24], antimicrobial [24–26] and anticancer [23,24,27] activities. In connection with this article, we have previously found that some simple and fused nitroquinoxalinones show activity against *T. vaginalis* [28–31], *Trypanosoma* sp. [31–35] and *Plasmodium* sp. [31,36], as well as towards other protozoan parasites [31].

In the present study we have synthesized and sequentially evaluated *in vitro* and, eventually, *in vivo* against *T. vaginalis* two series of quinoxaline derivatives: a) 4-(ω-aminoalkyl)- and 4-(ω-hydroxyalkyl)-7-nitroquinoxalin-2-ones **6**–**10** and **12**–**16**, based on some previously studied 4-[5-(dialkylamino)pentyl]quinoxalin-2-ones [30,31] and designed to explore the effect on trichomonacidal activity of the alkyl chain length and its ω-substituent, and b) 1-alkyl- and 1-(ω-halogenoalkyl)-6-nitroquinoxaline-2,3-diones **26**–**35**, directed to study the effect of introduction of a second CO group in the previously studied [28,29] quinoxalin-2-one analogs. In addition, compounds with high *in vitro* trichomonacidal activity have been screened to determine unspecific cytotoxicity against mammalian cells and selectivity indexes (SI, see below). All the synthesized molecules have been subjected to absorption, distribution, metabolism and excretion (ADME) molecular properties prediction by Molinspiration online calculation software and MolSoft program, to determine their bioavailability following the Lipinski's "rule of five" [37].

2. Results and discussion

2.1. Chemistry

Compounds **5**–**16** were prepared (Scheme 1) from 2-chloro-2′-halogenoacetanilides **1** and **2** and the corresponding primary amines. The reaction of these anilides with secondary amines to yield 2-dialkylamino-2′-halogenoacetanilides, as well as the intramolecular cyclization of the latter to quinoxalinium salts through a nucleophilic aromatic substitution have previously been reported by us [38]. Now, since primary amines are involved in the process, we have studied, as a representative model, the reaction of compounds **1** and **2** with methylamine at room temperature. A HPLC-MS study of these reactions has shown that 2,2′-dichloro derivative **2** reacts through intermediate **3** to afford 1,4-dimethylquinoxalinone **11** (85% yield); the reaction of 2-chloro-2′-fluoro derivative **1** with methylamine to yield 4-methylquinoxalinone **5** (77% yield) is more complex, and the two possible intermediates **3** and **4** could be detected in the reaction mixture. From a preparative point of view, 4-substituted (**6**–**10**; 52–66% yield from **1**) and 1,4-disubstituted (**12**–**16**; 61–86% yield from **2**) quinoxalin-2-ones were conveniently prepared by reaction of anilides **1** and **2** with the corresponding primary amines in ethanol at room temperature. In the preparation of 4-(3-hydroxypropyl) derivative **10**, fused quinoxalinone **17**, arising from the intramolecular cyclization of the former, could also be isolated.

Previously studied 4-[5-(dialkylamino)pentyl]quinoxalin-2-ones were prepared [31] from the corresponding 4-(5-bromopentyl) derivatives (compounds **20** and **24**, Scheme 2), which were in turn obtained by ring opening [31,38] of quinoxaline-1-spiro-1′-piperidinium salts. Thus, the one-pot procedure reported here for the preparation of 4-[ω-(substituted)alkyl]quinoxalin-2-ones starting from chloroacetanilides **1** and **2** and amines is much more simple, general and convenient than the previously used for compounds of this class.

On the other hand, it has been reported that 3,4-dihydroquinoxalin-2-ones are sensitive to atmospheric oxygen in solution, on chromatographic supports or on heating [38–40]; on this basis, we have found that the previously described compounds **5**, **11**, **18**–**25** [31,38] are easily converted into the corresponding quinoxaline-2,3-diones **26**–**35** (90–98% yield) by oxidation with potassium dichromate in acidic medium (Scheme 2).

2.2. Computational analysis: study of bioavailability parameters

In order to determine oral bioavailability of the synthetic molecules, computational studies related to drug-like physicochemical parameters were carried out. A high number of new synthetic compounds with significant activity failed during human clinical trials due to ADME and toxicity problems [41]. In the present study, different physicochemical descriptors, associated with compound's absorption and membrane permeability, have been calculated according to Lipinski's "rule of five" [37]. Several researchers have correlated molecular weight, lipophilicity (log *P*), hydrogen bond acceptor and donor properties, polar surface area and/or rotatable bonds with oral absorption and membrane permeability of compounds [37,42–44]. According to these criteria, all the quinoxalinone derivatives studied in the current research have achieved good bioavailability properties as shown in Table 1. The percentage of absorption (%Abs) calculated according to Zhao et al. [45] was in general good; values lower than 80% were only found for 4-(3-hydroxypropyl)quinoxalin-2-ones **10** and **16**, the tricyclic compound **17** and quinoxaline-2,3-diones **26**–**35**. On the other hand, 4-[ω-(dialkylamino)alkyl]-1-methylquinoxalin-2-ones **12**–**15** displayed the lowest Topological Polar Surface Area (TPSA = 72.609). TPSA is a useful molecular descriptor of drug transport properties through membranes [42]. Low TPSA values are correlated with high intestinal absorption [46], and thus the mentioned compounds **12**–**15** achieved the highest value (83.95%) of predicted absorption. The number of rotatable bonds, related to molecular flexibility [44] and furthermore, a good predictor of oral bioavailability of drugs [47], was also calculated.

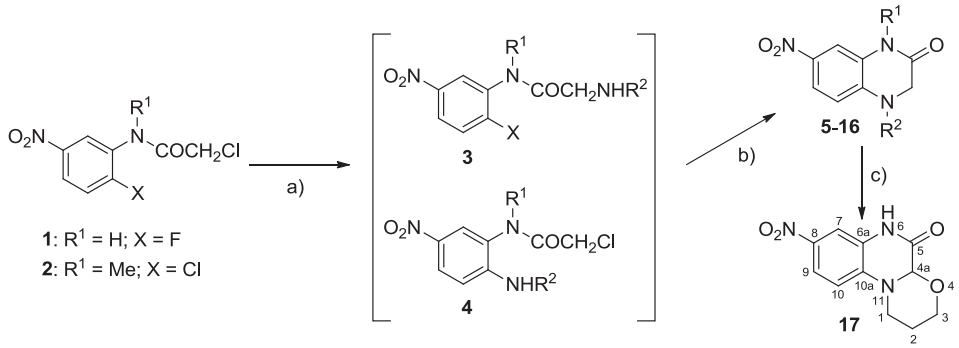
2.3. Biology

2.3.1. In vitro activity

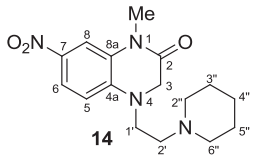
In the current study, 21 new quinoxaline derivatives belonging to two different series have been synthesized and evaluated *in vitro* as trichomonacidal agents by a fluorimetric assay using resazurin as redox dye [48]. All compounds were screened against the *T. vaginalis* isolate JH31A#4 at six different concentrations from 300 μM to 9.37 μM, using the two-fold serial dilution technique (Table 2 and Supplementary material, Table S1).

Most compounds of 7-nitroquinoxalin-2-one series, especially 4-[ω-(dialkylamino)alkyl] derivatives **6**–**9** and **12**–**14** (IC_{50} values $\leq$ 50 μM), showed a moderate to good trichomonacidal activity (Table 2). Compound **9**, with a 3-piperidinopropyl substituent at N-4 of the quinoxalinone moiety, showed the lowest IC_{50} value (18.26 μM) against the parasite.

Activities against *T. vaginalis* of related 4-[5-(dialkylamino)pentyl]quinoxalin-2-ones have been published [30,31]; however, a direct comparison with the results obtained in the current article is not possible owing to differences in the way of studying trichomonacidal activity (IC_{50} and SI values not determined, μg/mL vs μM concentrations, etc.). However, for the previously reported 5-(dialkylamino)pentyl analogs, derivatives containing a methyl substituent at position 1 of quinoxalinone ring are more active [45–92% of *in vitro* growth inhibition after 24 h of treatment at 10 μg/mL



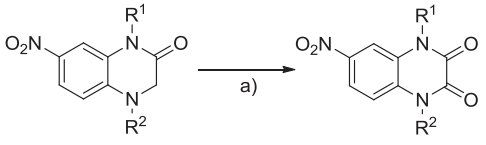
R^1 = H	R^1 = Me	R^2
5	**11**	Me
6*	**12**	$[CH_2]_2NMe_2$
7*	**13***	$[CH_2]_3NEt_2$
8*	**14**	$[CH_2]_2Pip$
9*	**15***	$[CH_2]_3Pip$
10	**16**	$[CH_2]_3OH$



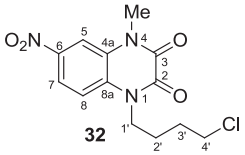
*Compounds isolated and characterized as the corresponding hydrochlorides

Reagents and conditions: a) R^2NH_2, ethanol, rt, 5-7 days; b) spontaneous under conditions a), 52-86%; c) from **10**, spontaneous under conditions a), 27% (Pip: piperidino).

Scheme 1. Synthesis of 1-substituted and 1,4-disubstituted 7-nitroquinoxalin-2-ones **5**–**16** and 8-nitrooxazino[3,2-*a*]quinoxalin-5-one **17**.



R^1 = H	R^1 = Me	R^2
5, **26**	**11**, **31**	Me
18, **27**	**22**, **32**	$[CH_2]_4Cl$
19, **28**	**23**, **33**	$[CH_2]_5Cl$
20, **29**	**24**, **34**	$[CH_2]_5Br$
21, **30**	**25**, **35**	$[CH_2]_6Cl$



Reagents and conditions: a) $K_2Cr_2O_7$, 10% aq. H_2SO_4, reflux, 2 h, 90-98%.

Scheme 2. Synthesis of 1-substituted and 1,4-disubstituted 6-nitroquinoxaline-2,3-diones **26**–**35**.

concentration (22–25 μM)] than the corresponding derivatives unsubstituted at the mentioned position [11–21% of growth inhibition at 10 μg/mL concentration (23–26 μM) under the same conditions] [30,31]. This remarkable effect of 1-Me substituent is not observed for ω-(dialkylamino)alkyl derivatives **6**–**9**, **12**–**15**, which, except compound **9** (58% of growth inhibition at 18.75 μM concentration), present only moderate trichomonacidal activity (7–33% of growth inhibition at the mentioned concentration) (see Supplementary material, Table S1). On the other hand, in relation to the different ω-substituted alkyl chains introduced at position 4 of quinoxalinone ring, our activity data do not allow the establishment of a clear structure-activity relationship; it is clear, however, that longer chains like pentyl give better results than ethyl or propyl moieties used in the current work.

The second series, composed by 6-nitroquinoxaline-2,3-diones **26**–**35**, showed in general a clear reduction of antiparasitic activity in relation to the previously studied quinoxalin-2-one analogs [28,29]. As before, a direct comparison of the published results with those obtained in the current work is not possible, but *in vitro* activities of 4-(ω-halogenoalkyl)quinoxalin-2-ones **18**, **19**, **21**–**23** at 10 μg/mL concentration (32–35 μM; 73–89% of growth inhibition after 24 h of treatment) [28,29] are rather higher than those found here for the corresponding quinoxaline-2,3-diones **27**, **28**, **30**, **32** and **33** at 37.5 μM concentration (1–46% of growth inhibition under similar conditions) (see Supplementary material, Table S1). Only quinoxaline-2,3-dione **35** [IC_{50} = 25 μM; 53% of growth inhibition at 37.5 μM (Supplementary material, Table S1)] retains a significant activity which is lower, however, than that of the starting quinoxalin-2-one **25** [94% of growth inhibition at 10 μg/mL (ca. 31 μM)] [29]. The introduction of the second CO group affects the pharmacokinetic properties of the resulting quinoxalinediones (section 2.2) as well as the basicity of N-1 of the mentioned ring and, probably, the reduction potential of NO_2 group at position 6, which seems essential for antiprotozoal activity [33].

Moreover, compounds of both series with an interesting *in vitro* activity against the parasite (IC_{50} < 70 μM) were subsequently evaluated for their unspecific toxicity against Vero cells. According to their percentage of cytotoxicity (%C) values, none of these nitroquinoxaline derivatives achieved a cytotoxic profile at the concentrations evaluated (300–9.37 μM). CC_{50} values (648.4 μM and 1235.1 μM, respectively) have only been calculated for compounds **12** and **13**, showing the highest %C at the highest concentration screened (300 μM). The remarkable low unspecific

Table 1
Molecular properties related to bioavailability of new nitroquinoxaline derivatives **6**–**10**, **12**–**17** and **26**–**35**.

Compound	MW	Volume	%Abs	TPSA	Atoms	Log *P*	H_A	H_D	ROTB	Lipinski's violation	Drug-likeness score
6	264.285	237.07	80.92	81.398	19	1.364	7	1	4	0	0.8
7	306.366	287.42	80.92	81.398	22	2.387	7	1	7	0	0.57
8	304.35	277.06	80.92	81.398	22	2.272	7	1	4	0	0.97
9	318.377	293.86	80.92	81.398	23	2.543	7	1	5	0	0.93
10	251.242	215.93	75.06	98.388	18	0.973	7	2	4	0	−0.12
12	278.312	253.96	83.95	72.609	20	1.242	7	0	4	0	0.73
13	320.393	304.36	83.95	72.609	23	2.264	7	0	7	0	0.51
14	318.377	294.01	83.95	72.609	23	2.15	7	0	4	0	1.02
15	332.404	310.81	83.95	72.609	24	2.42	7	0	5	0	0.97
16	265.269	232.87	78.09	89.599	19	0.85	7	1	4	0	−0.08
17	249.226	206.08	78.85	87.394	18	1.057	7	1	1	0	−0.18
26	221.172	176.25	74.26	100.691	16	0.184	7	1	1	0	−0.36
27	297.698	240.43	74.26	100.691	20	1.331	7	1	5	0	−0.57
28	311.725	257.23	74.26	100.691	21	1.836	7	1	6	0	−0.57
29	356.176	261.58	74.26	100.691	21	1.967	7	1	6	0	−0.69
30	325.75	274.04	74.26	100.69	22	2.341	7	1	7	0	−0.57
31	235.2	193.19	78.01	89.834	17	0.699	7	0	1	0	−0.63
32	311.72	257.37	78.01	89.834	21	1.85	7	0	5	0	−0.57
33	325.752	274.18	78.01	89.834	22	2.351	7	0	6	0	−0.62
34	370.203	278.53	78.01	89.834	22	2.482	7	0	6	0	−0.74
35	339.77	290.98	78.01	89.834	23	2.86	7	0	7	0	−0.62

Percentage of absorption is calculated by %Abs = 109 − (0.345 × TPSA) [45]. TPSA: Topological Polar Surface Area. Log *P*: logarithm of partition coefficient of the molecule in a octanol/water system calculated by Molinspiration online tool. H_D: Hydrogen bond donors. H_A: Hydrogen bond acceptors. ROTB: number of rotatable bonds.

Table 2
Trichomonacidal activity and unspecific toxicity of nitroquinoxaline derivatives **6**–**10**, **12**–**17** and **26**–**35**: half maximal inhibitory concentration (IC_{50}) against *T. vaginalis*, percentage of cytotoxicity (%C) at the highest concentration assayed (300 μM), half maximal cytotoxic concentration (CC_{50}) against Vero cells and selectivity indexes (SI).

Compound	IC_{50} (μM)	%C	CC_{50} (μM)	SI
6	36.77	0 ± 0	>>300	>10
7	38.93	0.16 ± 0.22	>>300	>10
8	30.65	1.51 ± 2.13	>>300	>10
9	18.26	3.26 ± 6.48	>>300	>10
10	102.46			
12	50.74	17.29 ± 0.59	648.4	12.77
13	32.44	10.81 ± 6.95	1235.1	38.07
14	29.89	0 ± 0	>>300	>10
15	109.76			
16	60.69	5.07 ± 7.09	>>300	>10
17	41.89	2.08 ± 1.03	>>300	>10
26	111.36			
27	150.90			
28	147.38			
29	145.32			
30	106.08			
31	235.25			
32	178.99			
33	61.39	0 ± 0	>>300	>10
34	255.68			
35	25.41	14.14 ± 1.68	>>300	>10
MTZ	4.16	0 ± 0	>600	>10

cytotoxicity results obtained for both series of nitroquinoxalines were also observed for related 4-[ω-(dialkylamino)pentyl]- [30,31] and 4-(ω-halogenoalkyl)quinoxalin-2-ones [28,29]. The selectivity index (SI) determined as the ratio of unspecific cytotoxicity in cells and activity against the pathogen [SI = CC_{50} (Vero cells)/IC_{50} (parasite)] was also estimated. Due to the absence of cytotoxic effect on Vero cells and the activity against *T. vaginalis* shown by 6-nitroquinoxalin-2-ones **6**–**9**, **12**–**14** and **16**, **17** and 7-nitroquinoxaline-2,3-diones **33** and **35** we can conclude that these compounds exhibit a remarkable specific trichomonacidal activity and good selectivity indexes (SI > 10).

2.3.2. *In vivo assays*

Due to the high *in vitro* activity and the absence of cytotoxicity against Vero cells, compound **9** was screened in a murine model of trichomonosis to determine the *in vivo* activity. Moreover, the computational ADME studies (Section 2.2) indicate that compound **9** displays good bioavailability properties with 80.92% of absorption and a drug-likeness score value of 0.93. Two different doses (50 mg/kg and 100 mg/kg) were administered daily by oral route during 7 days, and the different pathogenicity index scores [49] obtained after observation of peritoneal lesions are shown in Fig. 1. Although a statistical difference was observed with the control group, no divergences were obtained between both experimental groups (46.13% and 50.70% pathogenicity reduction). The similar reduction of pathogenicity in mice observed within both doses could be caused by a saturation on the absorption of the drug; however further pharmacokinetic studies are necessary. No animal died during the experiments at any of the doses examined as was expected due to the absence of cytotoxic behavior achieved on *in vitro* assays towards mammalian cells.

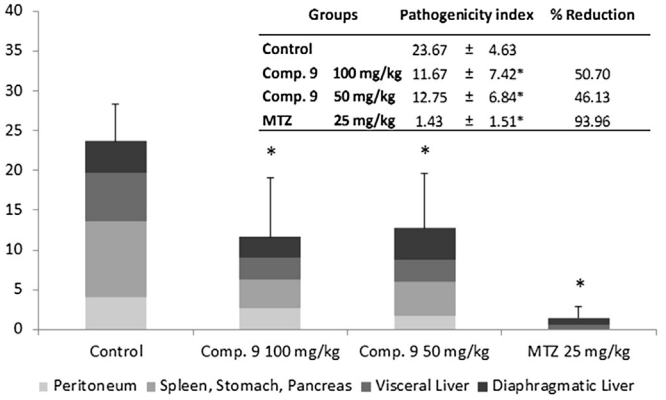


Fig. 1. *In vivo* activity of compound **9** administered orally to mice infected with C-1:NIH isolate of *T. vaginalis*. The colored fractions reflect the different scores based on the injuries observed per experimental group. Pathogenicity indexes of the experimental groups are expressed as mean ± standard deviation. *All the groups showed a statistical difference in comparison with the control of infection ($p < 0.05$).

2.4. Considerations on the mechanism of action

In relation to the mode of action of nitroquinoxaline derivatives, the reference nitroimidazole drugs approved for trichomonosis treatment act at hidrogenosomal level. The nitro group is reduced by the pyruvate:ferredoxin oxidoreductase (PFOR) through a single electron transfer [15], and the produced cytotoxic nitro radicals and intermediates are the responsible for the DNA disruption [50] and for the formation of adducts with parasitic proteins [51]. Trichomonacidal activity of nitroquinoxaline derivatives studied in this article is probably due to the presence in the molecules of NO_2 group. In fact, some related 4-[5-(dialkylamino)pentyl]-7-nitroquinoxalin-2-ones especially active against *Trypanosoma cruzi*, *T. vaginalis* and other protozoa [31] have been characterized by electrochemical and ESR studies corroborating the production of nitro radicals and oxidative stress [33]; furthermore, induction of DNA damage has also been confirmed for a 7-nitroquinoxalinone of this series [52].

3. Conclusions

In the current study new 7-nitroquinoxalin-2-ones (**6**–**10** and **12**–**16**) and 6-nitroquinoxaline-2,3-diones (**26**–**35**) have been synthesized and subsequently evaluated as potential trichomonacidal agents. 7-Nitroquinoxalin-2-ones have shown significant *in vitro* activity against *T. vaginalis* while 6-nitroquinoxaline-2,3-diones display in general low activity. Toxicity assays showed no unspecific cytotoxic behavior of 7-nitroquinoxalin-2-ones against mammalian cells. Compound **9** with the lowest IC_{50} value and a predicted percentage of absorption of 80.92%, was screened *in vivo* in a murine model of intraperitoneal trichomonosis. Two different doses of compound were administered orally during 7 days and the obtained results showed a clear reduction of the pathogenicity index in comparison with the control of infection. All the molecules have been submitted to *in silico* studies to determine diverse physicochemical parameters related to absorption, lipophilicity and drug-likeness properties. Both series of compounds respected Lipinski's rule: low MW (<500 Da), lipophilicity determined by log $P < 5$, less than 5 hydrogen bond donors and less than 10 hydrogen bond acceptors. Furthermore, a reduced number of rotatable bonds (<10) and a high percentage of absorption (>70%) were also displayed by the new nitroquinoxalinone derivatives. Thus, we think that the remarkable activity against *T. vaginalis* displayed by some quinoxalinones and the adequate molecular properties for good oral bioavailability, suggest the interest in continuing the study of this structural core for trichomonosis treatment.

4. Experimental section

4.1. Chemistry

4.1.1. General methods

Mps were determined in a Mettler Toledo Scientific melting point apparatus MP70. Mps of hydrochlorides **6**–**9**, **13** and **15** as well as that of tricyclicic compound **17** are not very well defined; these compounds decompose on heating and the observed mps are frequently heating-rate dependent and previous softening is usual. ^1H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded on a Varian Unity 300 spectrometer. Most NMR spectra were recorded at room temperature (~20 °C); however, owing to solubility problems, those of compound **6** were record, as indicated, at 75 °C. Chemical shifts are reported in ppm from TMS (δ scale) but were measured against the solvent signal. The assignments have been performed by means of different standard 1D and 2D correlation experiments (NOE, COSY, gHSQC and gHMBC). Numbering used in the description of NMR spectra is indicated in Schemes 1 and 2. Mass spectra [MS (ES^+)] were obtained on a Hewlett Packard 1100 MSD32 spectrometer; under these conditions, free amines as well as their hydrochlorides give the signals of the corresponding ammonium ions, which are described in both cases as MH^+. DC-Alufolien silica gel 60 PF_{254} (Merck, layer thickness 0.2 mm) was used for TLC, and silica gel 60 (Merck, particle size 0.040–0.063 mm) for flash column chromatography. Microanalyses were performed on a Heraeus CHN-O-RAPID analyzer and were within ±0.3% of the theoretical values.

4.1.2. Preparation of 4-substituted and 1,4-disubstituted 3,4-dihydroquinoxalin-2(1H)-ones **5**–**16** and fused quinoxalinone **17**

General procedure: a mixture of the corresponding 2-chloroacetanilide (**1** or **2**) [38] (4 mmol) and the required amine (methylamine was used as a 33% w/w solution in ethanol) (8 mmol) in ethanol (10 mL) was stirred at room temperature until consumption of the starting material (TLC, 5–7 days).

4-Methyl (**5**), 1,4-dimethyl (**11**) and 1-methyl-4-(3-hydroxypropyl) (**16**) derivatives precipitated pure from the reaction mixture and were collected by filtration, washed with ethanol (2 × 3 mL) and air-dried; crude compound **10**, however, contained some tricyclic compound **17** which was separated by column chromatography using chloroform/methanol (40:1 to 5:1) mixtures.

In the case of 4-[ω-(dialkylamino)alkyl] derivatives the precipitated solids, collected as described, were shown to be the corresponding hydrochlorides; after recrystallization from an appropriate solvent, compounds **6**–**9**, **13** and **15** were obtained as the mentioned salts, while compounds **12** and **14** were obtained as the free bases.

HPLC-MS study of the reaction of compounds **1** and **2** with methylamine, as well as yields, mps and spectral and analytical data of 7-nitroquinoxalin-2-ones **7**, **9**, **12**, **14** and **16** are given as Supplementary material.

*4.1.2.1. 4-Methyl-7-nitro-3,4-dihydroquinoxalin-2(1H)-one (**5**).* Yield 77%; mp 261–263 °C (pyridine/H_2O); lit. mp 262–264 °C [38]. Spectral data are in agreement with those previously reported [38].

*4.1.2.2. 4-[2-(Dimethylamino)ethyl]-7-nitro-3,4-dihydroquinoxalin-2(1H)-one hydrochloride (**6**).* Yield 53%; mp 230–233 °C (MeOH/H_2O). ^1H NMR [75 °C, $(CD_3)_2SO$]: δ 10.85 (s, 1H, 1-H), 10.81 (br s, 1H, 2′-NH^+), 7.77 (dd, 1H, $J = 9.0$, 2.6 Hz, 6-H), 7.65 (d, 1H, $J = 2.6$ Hz, 8-H), 7.02 (d, 1H, $J = 9.0$ Hz, 5-H), 4.10 (s, 2H, 3-H), 3.79 (t, 2H, $J = 7.0$ Hz, 1′-H), 3.27 (t, 2H, $J = 7.0$ Hz, 2′-H), 2.77 (s, 6H, CH_3); ^{13}C NMR [75 °C, $(CD_3)_2SO$]: δ 163.2 (C-2), 139.3 (C-4a), 137.5 (C-7), 126.0 (C-8a), 119.5 (C-6), 109.5, 109.4 (C-5, -8), 51.7 (C-2′), 51.1 (C-3), 44.1 (C-1′), 42.4 (CH_3); MS (ES^+): *m/z* (%) 265 (100) (MH^+). Anal. calcd. for $C_{12}H_{16}N_4O_3 \times HCl$: C 47.92; H 5.70; N 18.63. Found: C 47.68; H 5.84; N 18.49.

*4.1.2.3. 7-Nitro-4-(2-piperidinoethyl)-3,4-dihydroquinoxalin-2(1H)-one hydrochloride (**8**).* Yield 52%; mp 245–247 °C (MeOH). ^1H NMR [$(CD_3)_2SO$]: δ 11.15 (br s, 1H, 1″-H), 10.59 (s, 1H, 1-H), 7.75 (dd, 1H, $J = 9.0$, 2.6 Hz, 6-H), 7.69 (d, 1H, $J = 2.6$ Hz, 8-H), 7.08 (d, 1H, $J = 9.0$ Hz, 5-H), 4.09 (s, 2H, 3-H), 3.89 (t, 2H, $J = 7.3$ Hz, 1′-H), 3.22 (t, 2H, $J = 7.3$ Hz, 2′-H), 3.05 (m, 4H, 2″-, 6″-H), 1.81 (m, 4H, 3″-, 5″-H), 1.57 (m, 2H, 4″-H); ^{13}C NMR [$(CD_3)_2SO$]: δ 163.1 (C-2), 139.2 (C-4a), 137.6 (C-7), 125.9 (C-8a), 119.4 (C-6), 109.6 (C-5), 109.5 (C-8), 52.1 (C-2″, -6″), 51.1 (C-3), 50.4 (C-2′), 43.5 (C-1′), 21.9 (C-3″, -5″), 21.0 (C-4″); MS (ES^+): *m/z* (%) 305 (100) (MH^+). Anal. calcd. for $C_{15}H_{20}N_4O_3 \times HCl$: C 52.86; H 6.21; N 16.44. Found: C 52.75; H 6.19; N 16.40.

*4.1.2.4. 4-(3-Hydroxypropyl)-7-nitro-3,4-dihydroquinoxalin-2(1H)-one (**10**).* Yield 61%; mp 186–189 °C (2-PrOH). ^{1}H NMR [$(CD_3)_2SO$]: δ 10.76 (s, 1H, 1-H), 7.77 (dd, 1H, J = 9.0, 2.6 Hz, 6-H), 7.59 (d, 1H, J = 2.6 Hz, 8-H), 6.78 (d, 1H, J = 9.0 Hz, 5-H), 4.62 (t, 1H, J = 4.7 Hz, OH), 4.05 (s, 2H, 3-H), 3.43 (m, 4H, 1′-, 3′-H), 1.71 (m, 2H, 2′-H); ^{13}C NMR [$(CD_3)_2SO$]: δ 163.8 (C-2), 140.3 (C-4a), 136.4 (C-7), 125.6 (C-8a), 120.6 (C-6), 109.6, 109.1 (C-5, -8), 58.0 (C-3′), 51.1 (C-3), 46.4 (C-1′), 27.7 (C-2′); MS (ES^+): *m/z* (%) 252 (100) (MH^+). Anal. calcd. for $C_{11}H_{13}N_3O_4$: C 52.59; H 5.22; N 16.73. Found: C 52.38; H 5.19; N 16.62.

*4.1.2.5. 1,4-Dimethyl-7-nitro-3,4-dihydroquinoxalin-2(1H)-one (**11**).* Yield 85%; mp 166–168 °C (EtOH); lit. mp 165–168 °C [38]. Spectral data are in agreement with those previously reported [38].

*4.1.2.6. 4-[3-(Diethylamino)propyl]-1-methyl-7-nitro-3,4-dihydroquinoxalin-2(1H)-one hydrochloride (**13**).* Yield 81%; mp 211–213 °C (MeOH). ^{1}H NMR [$(CD_3)_2SO$]: δ 10.82 (br s, 1H, 3′-NH^+), 7.86 (dd, 1H, J = 9.1, 2.5 Hz, 6-H), 7.70 (d, 1H, J = 2.5 Hz, 8-H), 7.00 (d, 1H, J = 9.1 Hz, 5-H), 4.15 (s, 2H, 3-H), 3.49 (t, 2H, J = 7.5 Hz, 1′-H), 3.32 (s, 3H, 1-CH_3), 3.07 [m, 6H, 3′-H and N(CH_2CH_3)$_2$], 2.00 (m, 2H, 2′-H), 1.21 [t, 6H, J = 7.3 Hz, N(CH_2CH_3)$_2$]; ^{13}C NMR [$(CD_3)_2SO$]: δ 163.2 (C-2), 141.6 (C-4a), 137.1 (C-7), 127.8 (C-8a), 120.6 (C-6), 109.8 (C-5), 109.7 (C-8), 51.1 (C-3), 47.8 (C-3′), 46.5 (C-1′), 46.1 [N(CH_2CH_3)$_2$], 28.3 (1-CH_3), 19.1 (C-2′), 8.4 [N(CH_2CH_3)$_2$]; MS (ES^+): *m/z* (%) 321 (100) (MH^+). Anal. calcd. for $C_{16}H_{24}N_4O_3 \times$ HCl: C 53.85; H 7.06; N 15.70. Found: C 53.77; H 7.18; N 15.72.

*4.1.2.7. 1-Methyl-7-nitro-4-(3-piperidinopropyl)-3,4-dihydroquinoxalin-2(1H)-one hydrochloride (**15**).* Yield 61%; mp 229–232 °C ($MeNO_2$). ^{1}H NMR [$(CD_3)_2SO$]: δ 10.74 (br s, 1H, 1″-H), 7.86 (dd, 1H, J = 9.1, 2.4 Hz, 6-H), 7.70 (d, 1H, J = 2.4 Hz, 8-H), 6.97 (d, 1H, J = 9.1 Hz, 5-H), 4.14 (s, 2H, 3-H), 3.47 (t, 2H, J = 7.3 Hz, 1′-H), 3.35 (m, 2H, 2″-, 6″-H_e), 3.32 (s, 3H, CH_3), 3.04 (m, 2H, 3′-H), 2.81 (m, 2H, 2″-, 6″-H_a), 2.05 (m, 2H, 2′-H), 1.76 (m, 5H, 3″-, 5″-H, and 4″-H_A), 1.37 (m, 1H, 4″-H_B); ^{13}C NMR [$(CD_3)_2SO$]: δ 163.3 (C-2), 141.6 (C-4a), 137.1 (C-7), 127.8 (C-8a), 120.6 (C-6), 109.8, 109.7 (C-5, -8), 53.1 (C-3′), 51.9 (C-2″, -6″), 51.0 (C-3), 46.6 (C-1′), 28.3 (CH_3), 22.2 (C-3″, -5″), 21.4 (C-4″), 19.3 (C-2′); MS (ES^+): *m/z* (%) 333 (100) (MH^+). Anal. calcd. for $C_{17}H_{24}N_4O_3 \times$ HCl: C 55.36; H 6.83; N 15.19. Found: C 55.08; H 7.06; N 14.94.

*4.1.2.8. 8-Nitro-2,3,4a,6-tetrahydro[1,3]oxazino[3,2-a]quinoxalin-5(1H)-one (**17**).* Yield 27%; mp 249–251 °C (1-PrOH). ^{1}H NMR [$(CD_3)_2SO$]: δ 11.20 (s, 1H, 6-H), 7.87 (dd, 1H, J = 9.1, 2.6 Hz, 9-H), 7.73 (d, 1H, J = 2.6 Hz, 7-H), 7.18 (d, 1H, J = 9.1 Hz, 10-H), 5.29 (s, 1H, 4a-H), 4.21 (br d, J = −14.1 Hz, 1H, 1-H_A), 3.98 (m, 2H, 3-H), 3.50 (m, J = −14.1, 14.1, 2.7 Hz, 1H, 1-H_B), 1.92 (m, 1H, 2-H_A), 1.49 (br d, J = −13.5 Hz, 1H, 2-H_B); ^{13}C NMR [$(CD_3)_2SO$]: δ 159.8 (C-5), 139.0 (C-10a), 137.9 (C-8), 126.1 (C-6a), 120.0 (C-9), 112.4 (C-10), 110.3 (C-7), 84.6 (C-4a), 67.8 (C-3), 52.8 (C-1), 44.6 (C-2); MS (ES^+): *m/z* (%) 250 (100) (MH^+). Anal. calcd. for $C_{11}H_{11}N_3O_4$: C 53.01; H 4.45; N 16.86. Found: C 52.98; H 4.35; N 16.69.

*4.1.3. Preparation of 1-substituted and 1,4-disubstituted quinoxaline-2,3-diones **26**–**35***

General procedure: a suspension of the corresponding quinoxalin-2-one (**5**, **11**, **18**–**25**) [38] (1.5 mmol) and potassium dichromate (0.47 g; 1.6 mmol) in 10% aqueous sulfuric acid (30 mL) was refluxed for 2 h. The reaction was then cooled and, after addition of water (30 mL), the solid in suspension was collected by filtration, washed with plenty water and air-dried.

Yields, mps, and spectral and analytical data of 6-nitroquinoxaline-2,3-diones **26**, **28**, **30**, **32** and **34** are given as Supplementary material.

*4.1.3.1. 1-(4-Chlorobutyl)-6-nitroquinoxaline-2,3(1H,4H)-dione (**27**).* Yield 95%; mp 190–193 °C (2-PrOH). ^{1}H NMR [$(CD_3)_2SO$]: δ 12.28 (s, 1H, 4-H), 7.98 (dd, 1H, J = 10.0, 2.6 Hz, 7-H), 7.97 (d, 1H, J = 2.6 Hz, 5-H), 7.59 (d, 1H, J = 10.0 Hz, 8-H), 4.15 (t, 1H, J = 7.3 Hz, 1′-H), 3.68 (t, 2H, J = 6.2 Hz, 4′-H), 1.81 (m, 2H, 3′-H), 1.75 (m, 2H, 2′-H); ^{13}C NMR [$(CD_3)_2SO$]: δ 155.1 (C-2), 153.2 (C-3), 142.2 (C-6), 131.8 (C-8a), 126.4 (C-4a), 118.2 (C-7), 115.5 (C-8), 110.5 (C-5), 45.0 (C-4′), 42.0 (C-1′), 29.2 (C-3′), 23.9 (C-2′); MS (ES^+): *m/z* (%) 298 (100) (MH^+). Anal. calcd. for $C_{12}H_{12}ClN_3O_4$: C 48.41; H 4.06; N 14.12. Found: C 48.19; H 3.91; N 13.98.

*4.1.3.2. 1-(5-Bromopentyl)-6-nitroquinoxaline-2,3(1H,4H)-dione (**29**).* Yield 95%; mp 225–227 °C (EtOH). ^{1}H NMR [$(CD_3)_2SO$]: δ 12.29 (s, 1H, 4-H), 7.98 (dd, 1H, J = 9.9, 2.6 Hz, 7-H), 7.97 (d, 1H, J = 2.6 Hz, 5-H), 7.58 (d, 1H, J = 9.9 Hz, 8-H), 4.11 (t, 1H, J = 7.3 Hz, 1′-H), 3.52 (t, 2H, J = 6.7 Hz, 5′-H), 1.84 (m, 2H, 4′-H), 1.64 (m, 2H, 2′-H), 1.48 (m, 2H, 3′-H). ^{13}C NMR [$(CD_3)_2SO$]: δ 155.0 (C-2), 153.2 (C-3), 142.2 (C-6), 131.8 (C-8a), 126.3 (C-4a), 118.3 (C-7), 115.6 (C-8), 110.5 (C-5), 42.5 (C-1′), 34.9 (C-5′), 31.9 (C-4′), 25.5 (C-2′), 24.8 (C-3′); MS (ES^+): *m/z* (%) 358 (100) ($[MH+2]^+$), 356 (100) (MH^+). Anal. calcd. for $C_{13}H_{14}BrN_3O_4$: C 43.84; H 3.96; N 11.80. Found: C 43.75; H 4.02; N 11.71.

*4.1.3.3. 1,4-Dimethyl-6-nitroquinoxaline-2,3(1H,4H)-dione (**31**).* Yield 93%; mp 255–257 °C (2-PrOH). ^{1}H NMR [$(CD_3)_2SO$]: δ 8.10 (m, 2H, 5-, 7-H), 7.59 (d, 1H, J = 9.8 Hz, 8-H), 3.59 (s, 3H, 4-CH_3), 3.56 (s, 3H, 1-CH_3); ^{13}C NMR [$(CD_3)_2SO$]: δ 153.8 (C-2), 153.4 (C-3), 142.6 (C-6), 132.9 (C-8a), 127.9 (C-4a), 118.8 (C-7), 115.7 (C-8), 110.1 (C-5), 30.4 (4-CH_3), 30.0 (1-CH_3); MS (ES^+): *m/z* (%) 236 (100) (MH^+). Anal. calcd. for $C_{10}H_9N_3O_4$: C 51.07; H 3.86; N 17.87. Found: C 50.98; H 4.02; N 17.91.

*4.1.3.4. 1-(5-Chloropentyl)-4-methyl-6-nitroquinoxaline-2,3(1H,4H)-dione (**33**).* Yield 98%; mp 165–167 °C (2-PrOH). ^{1}H NMR [$(CD_3)_2SO$]: δ 8.11 (d, 1H, J = 2.5 Hz, 5-H), 8.10 (dd, 1H, J = 8.9, 2.5 Hz, 7-H), 7.66 (d, 1H, J = 8.9 Hz, 8-H), 4.16 (t, 1H, J = 7.6 Hz, 1′-H), 3.63 (t, 2H, J = 6.6 Hz, 5′-H), 3.60 (s, 3H, CH_3), 1.76 (m, 2H, 4′-H), 1.65 (m, 2H, 2′-H), 1.49 (m, 2H, 3′-H); ^{13}C NMR [$(CD_3)_2SO$]: δ 153.6 (C-2), 153.4 (C-3), 142.5 (C-6), 131.9 (C-8a), 128.1 (C-4a), 118.9 (C-7), 115.7 (C-8), 110.4 (C-5), 45.2 (C-5′), 42.6 (C-1′), 31.7 (C-4′), 30.1 (CH_3), 25.6 (C-2′), 23.5 (C-3′); MS (ES^+): *m/z* (%) 326 (100) (MH^+). Anal. calcd. for $C_{14}H_{16}ClN_3O_4$: C 51.62; H 4.95; N 12.90. Found: C 51.73; H 5.05; N 12.99.

*4.1.3.5. 1-(6-Chlorohexyl)-4-methyl-6-nitroquinoxaline-2,3(1H,4H)-dione (**35**).* Yield 91%; mp 143–145 °C (2-PrOH). ^{1}H NMR [$(CD_3)_2SO$]: δ 8.10 (d, 1H, J = 2.4 Hz, 5-H), 8.09 (dd, 1H, J = 8.8, 2.4 Hz, 7-H), 7.65 (d, 1H, J = 8.8 Hz, 8-H), 4.14 (t, 2H, J = 7.6 Hz, 1′-H), 3.62 (t, 2H, J = 6.6 Hz, 6′-H), 3.59 (s, 3H, CH_3), 1.71 (m, 2H, 5′-H), 1.62 (m, 2H, 2′-H), 1,41 (m, 4H, 3′-, 4′-H); ^{13}C NMR [$(CD_3)_2SO$]: δ 153.5 (C-2), 153.4 (C-3), 142.4 (C-6), 131.9 (C-8a), 128.1 (C-4a), 118.8 (C-7), 115.6 (C-8), 110.3 (C-5), 45.2 (C-6′), 42.7 (C-1′), 31.9 (C-5′), 30.0 (CH_3), 26.1 (C-2′), 25.9 (C-3′), 25.3 (C-4′); MS (ES^+): *m/z* (%) 340 (100) (MH^+). Anal. calcd. for $C_{15}H_{18}ClN_3O_4$: C 53.02; H 5.34; N 12.37. Found: C 52.97; H 5.38; N 12.41.

4.2. Computational analysis: bioavailability parameters study

A virtual screening analysis was carried out to study diverse physicochemical features associated with oral bioavailability following the Lipinski's "rule of five" [37]. Topological Polar Surface Area (TPSA), a molecular property correlated with molecular transport through membranes [42], log *P* (the partition coefficient of the molecule in a octanol/water system based on group contributions and developed by Molinspiration), molecular weight

(MW), hydrogen bond acceptors (H_A), hydrogen bond donors (H_D), rotatable bonds (ROTB) and drug-likeness prediction were calculated by Molinspiration-Cheminformatics online calculation tool [53] and MolSoft program [54]. Percentage of absorption (%Abs) was calculated following the formula described by Zhao et al. [45].

4.3. Biological assays

4.3.1. T. vaginalis culture

T. vaginalis JH31A#4 [ref. 30,326; American Type Culture Collection (ATCC), MD, USA] and C-1:NIH (ref. 30,001; ATCC, MD, USA) were grown in TYM medium (pH 6.4) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and antibiotic solutions (penicillin 100 U/mL and streptomycin 100 mg/L). Cultures were incubated at 37 °C in a 5% CO_2 atmosphere and sub-cultured every 48–72 h.

4.3.2. Mammalian cells culture

African green monkey kidney epithelial cell line (Vero) (ATCC, MD, USA) was grown in RPMI-1640 medium (Sigma Aldrich Chemical Co., St. Louis, MO) supplemented with 10% FBS and antibiotic solutions. Cultures were maintained at 37 °C in a 5% CO_2 humidified incubator. The cells were grown in plastic flasks and passaged on reaching 80–90% confluence.

4.3.3. Animals

Female NMRI (Naval Medical Research Institute) mice, weighing between 18 and 20 g were used in experimental groups of 10, to determine the *in vivo* activity of the most active nitroquinoxalinone derivative **9**. Animals were kept under temperature, humidity and light controlled environment. The experiments were carried out according to the directive 2010/63/EU of the European Parliament and the Council of the European Union and controlled in Spain by Royal Decree 53/2013 of 1 February, on the protection of animals used for experimental and other scientific purposes.

4.3.4. In vitro determination of trichomonacidal activity

This assay was carried out following the fluorimetric microtitre method recently described by our group [48]. Stock solutions of compounds in dimethyl sulfoxide (DMSO) were prepared and added to cultures containing 10^5 JH31A#4 trophozoites/mL to reach the required final concentrations (300–9.37 μM). Final DMSO concentration in the culture media was always ≤0.2% (v/v). After 24 h of incubation at 37 °C and 5% CO_2, the culture tubes were seeded in sterile 96-well flat-bottom microplates (NUNC, Roskilde, Denmark). After removal of the culture medium, the protozoa were resuspended in 200 μL of phosphate buffer solution supplemented with 0.1% glucose and 20 μL of the redox dye resazurin (3 mM stock solution in the same phosphate buffer-0.1% glucose system). The fluorescence was measured after 1 h of incubation in a plate fluorometer (Infinite 200, TECAN). Half maximal inhibitory concentration (IC_{50}) was calculated by Probit analysis (SPSS v.20, IBM). Experiments were carried out in triplicate and repeated at least twice.

4.3.5. In vitro determination of unspecific cytotoxicity in mammalian cells

Vero cells were seeded into 96-well flat-bottom microplates at a density of 50,000 cells/well in 100 μL of RPMI medium. After overnight culture at 37 °C and 5% CO_2 for cell attaching, the derivatives dissolved in RPMI medium at the same concentrations assayed against *T. vaginalis* were added to the wells. The drugs were incubated with the mammalian cells for 24 h under the same conditions. The cytotoxicity activity was determined fluorimetrically after 3 h of incubation with 20 μL of the redox dye resazurin (stock solution 1 mM). Half maximal cytotoxic concentration (CC_{50}) for compounds with some cytotoxic effect on cells at the highest concentration assayed (300 μM) was determined by Probit analysis (SPSS v.20, IBM). Results were obtained from two independent experiments carried out in triplicate.

4.3.6. In vivo assay against T. vaginalis

Compound **9**, with the highest *in vitro* activity against *T. vaginalis* and low unspecific cytotoxicity against Vero cells, was assayed in a murine model of trichomonosis. According to the modified method described by Nogal-Ruiz et al. [49], 10^7 C-1:NIH trophozoites in logarithmic phase of growth, were intraperitoneally inoculated to NMRI female mice. The active compound was previously dissolved in 2% carboxymethylcellulose (CMC) and administered orally to mice from day 3 to day 10 post-infection (p.i.). At day 15 p.i., the animals were sacrificed and pathogenicity indexes were determined according to the lesions observed in the peritoneum and annexed organs. Two experimental groups, untreated and orally treated during 7 days with 25 mg/kg/day of metronidazole, were included as controls. For the present study two different daily dosages of compound **9** were used: 50 mg/kg and 100 mg/kg. The statistical comparison of experimental groups according to their pathogenicity indexes was calculated by Kruskal Wallis analysis (SPSS v.20, IBM).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.03.002.

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