Anthelmintic activity of benzalphthalides and phthalazinones against *Teladorsagia circumcincta*: synthesis and structure-activity relationship

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Abstract

Infections caused by nematodes are very common in both humans and animals. Among them, those produced by gastrointestinal nematodes (NGI) are one of the most prevalent in grazing ruminants, constituting an important health problem in livestock farms. Several drugs in the therapeutic arsenal for the treatment of GIN are available, however, their indiscriminate use has led to the development of resistance worldwide, which represents an important problem that needs an urgent solution. The aim of this study was to synthesize and tests the ovidical and larvicidal activity against *Teladorsagia circumcincta* (*T.c.*) of some heterocycle compounds, such as benzalphthalides (Bp) and phthalazinones (Pt). The activity of twenty-four Bp and thirteen Pt with modifications on ring B with electron donating or withdrawing groups attached to different positions of the phenyl ring, and bulky fragment such as α- or β-naphthyl were analyzed, they show non-substitution or a methyl in ring A. Pt **26** showed excellent nematocidal activity in the Egg Hatch Assay in both susceptible and resistant strain of *T.c.* at 10 µg/mL, with values of of 75.5 and 99.4%, respectively. **26** was also a good ovicidal, showing 99.0% LMT inhibition at 10 µg/mL in the *T.c* susceptible strain, which was higher than the control levamisole.

Resumo

Infecções causadas por nematóides são muito comuns em humanos e animais. Dentre eles, os produzidos por nematoides gastrointestinais (NGI) são um dos mais prevalentes em ruminantes em pastejo, constituindo um importante problema de saúde nas

fazendas pecuárias. Diversas drogas do arsenal terapêutico para o tratamento da GIN estão disponíveis, entretanto, seu uso indiscriminado tem levado ao desenvolvimento de resistência em todo o mundo, o que representa um problema importante que necessita de solução urgente. O objetivo deste estudo foi sintetizar e testar a atividade ovídica e larvicida contra *Teladorsagia circuncincta* (*T.c.*) de alguns compostos heterocíclicos, como benzalftalidas (Bp) e ftalazinonas (Pt). A atividade de vinte e quatro Bp e treze Pt com modificações no anel B com grupos doadores ou retiradores de elétrons ligados a diferentes posições do anel fenil e fragmentos volumosos como α- ou β-naftil foram analisados, eles mostram não substituição ou a metil no anel A. A Pt 26 apresentou excelente atividade nematocida no Ensaio de Eclosão de Ovos em cepas susceptíveis e resistentes de *T.c*. a 10 μg/mL, com valores de 75,5 e 99,4%, respectivamente. 26 também foi um bom ovicida, mostrando 99,0% de inibição de LMT a 10 μg/mL na cepa suscetível a T.c, que foi maior que o levamisol controle.

Keywords: Phthalazinones, synthesis, *Teladorsagia circumcincta*, in vitro assays, cytotoxicity, resistant strain

Introduction

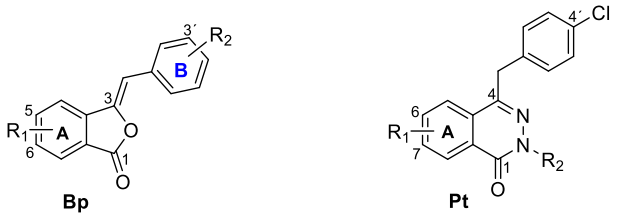
The infections caused by gastrointestinal nematodes (GIN) are one of the most prevalent in grazing ruminants, constituting an important health problem, especially in sheep, due to lower growth, reduction in milk production, and poor quality of wool, decreased fertility and even produce death when the load of worms is very high (Moje *et al*., 2021) (Craig, 2018). All this generates considerable economic losses in livestock farms of grazing animals. Most frequent GIN infections are caused by *Teladorsagia* (Ostertagia) spp., *Haemonchus contortus*, *Trichostrongylus* spp., among others.

Various broad-spectrum anthelmintic agents in the therapeutic arsenal for the treatment of GIN (albendazole, fenbendazole, ivermectin, monepantel, etc.) are available. However, in the last decades, their incorrect and massive use has led to therapeutic failure and the generation of resistances worldwide (Sargison, 2012). In this context, new chemical entities for the GIN treatment are needed, for that, different approaches have been proposed, such as the identification of active natural products, the synthesis of novel chemical compounds, drug repurposing, or the synthesis of new derivatives of established drugs (Zajíčková *et al*., 2020).

The presence of a heterocyclic skeleton is very common in molecules of interest in medicinal chemistry. Among the great variety of heterocyclic compounds we can include those containing hydrazine, which show a wide structural variation and several pharmaceutical applications, such as antihypertensive (Demirayak *et al*., 2004), anticonvulsant (Sivakumar *et al*., 2002) or antimicrobial (Pathak S *et al*., 2013), among others, playing a considerable role in Medicinal Chemistry. Moreover, the phthalazinone skeleton represents a nucleus with a wide variety of applications, such as antihistaminic (azelastine), antidiabetic (ponalrestat) or antitumor (olaparib) activities (Terán *et al*., 2019).

Our research group has a great experience in heterocyclic derivatives obtaining, including arylindazoles (Viña *et al.* 2007), pyrazolophthalazines (Viña, *et al*. 2008) quinoline derivatives (Kouznetsov *et al.* 2012a), or imidazoisoindoles (Arsène *et al*. 2019), among others, some of those compounds were tested as antifungal. Recently, the group has worked on the synthesis of new molecules with nematocidal properties. In this context, they have obtained new derivatives of benzimidazole (BZ) type (Escala *et al*., 2020) (Escala *et al*., 2022), as well as of aliphatic aminoalcohol and diamine type (Valderas *et al*., 2021), some of these compounds showed excellent in vitro activity in sensitive and resistant strains of *Teladorsagia circumcincta* and were selected for in vivo tests in gerbils and sheep (Valderas *et al*., 2022). Following with this approach, we have decided to explore the nematocide profile of other heterocyclic nucleus, such benzalphthalides and phthalazinones, the former contains oxygen as a heteroatom and the latter an hydrazine group. In previous works, compounds from these families were tested as antiparasitics (*Leishmania* spp (del Olmo *et al.,* 2001), *Trypanosoma cruzi* (del Olmo *et al.,* 2001), *Plasmodium falciparum* (del Olmo *et al.,* 2003), or anti-HIV (Bedoya *et al*., 2006). Some benzalphthalides showed excellent anti-anxiety activity (Zamilpa *et al.,* 2005), and phthalazinones good vasorelaxant (del Olmo *et al.,* 2006), and antifungal results (Derita *et al.,* 2013).

Therefore, we describe here the synthesis and in vitro activity against *Teladorsagia circumcincta* of some benzalphthalides and phthalazinones, the latter are obtained from the former, and the influence of the substituents in rings A and B in relation to the activity were analyzed (Fig. 1). The compounds obtained were tested against eggs and larvae (L1) of a sensitive strain of *T. circumcincta*. The most potent compounds were selected to be tested on a resistant strain of *T. circumcincta.*



R1 = H, CH_3
R2 = H, 4´-OMe, 3´,4´-diOMe, etc.
B = α-naphthyl, β-naphthyl

R1 = H, CH_3
R2 = Me, Et, Allyl, But, etc.

Figure 1. General structure of the benzalphthalides and phthlazinones obtained.

Material and Methods

Chemistry. All commercial chemicals (Aldrich, Alpha, Fischer, SDS) were used as purchased and solvents (Fischer, SDS, Scharlau) purified by the standard procedures prior to use. Reactions were monitored by Thin Layer Chromatography (TLC) (Kieselgel 60 F254 precoated plates, E. Merck, Germany), the spots were detected by exposure to UV light at λ 254 nm, and colorization with 10% phosphomolybdic acid spray, with further heating of the plate. Melting points (Mp) were determined with Mel-Temp apparatus in open capillaries and were uncorrected. Separations by flash column chromatography were performed on Merck 60 silica gel (0.063-0.2 mesh). Infrared spectra were recorded on a FT-IR spectrometer Perkin Elmer System BX, using NaCl or KBr disks. NMR spectra were recorded on a Bruker Avance or Varian Mercury 200 and 400 MHz (200 MHz for ^{1}H, 50 MHz for ^{13}C; 400 MHz for ^{1}H, 100 MHz for ^{13}C). The spectra were measured either in $CDCl_3$, methanol-d4 or DMSO-d6, using tetramethylsilane (TMS) as internal standard, chemical shifts (δ) are given in ppm and coupling constants (*J*) in Hertz (Hz). High resolution mass spectra (HRMS) were obtained by electron spray ionisation-mass spectrometry (ESI-MS) technique (5 kV) on a QSTAR XL mass spectrometer. Microwave synthesis procedures were performed under selected conditions in a Monowave 300 microwave reactor (AP-MW; Anton Parr).

General procedures for preparation of compounds

Procedure for the synthesis of benzalphthalides 13, 14, 20-23 by Dean-Stark system. We have followed a procedure similar to that indicated by Zamilpa *et al*. (2005). 324.0 mg (2.0 mmol) of 5-methylphthalic anhydride, 459.0 mg (2.7 mmol) of 4-chlorophenylacetic acid and 25.6 mg (0.26 mmol) of potassium acetate were mixed in a two-necked flask. Then, dry toluene (5 mL) was added, and the mixture was maintained at 220 ºC in a Dean-Stark apparatus with magnetic stirring for 24 h. After completion of the reaction (monitored by

TLC), it was taken to dryness in a rotary evaporator, and the obtained crude was extracted with ethyl acetate (2 x 10 mL), the combined organic layers were washed with saturated sodium bicarbonate, and water to neutral pH, then, were dried with sodium sulphate, filtered and the solvent was removed under vacuum to provide a crude mixture that was purified by column chromatography with the properly movile phase. The reaction yield ranged from 46 to 65%.

Procedure for the synthesis of benzalphthalides 15-19 and 24 by microwave. We have applied a modification of the procedure indicated by Viña *et al*. (2009). 1 mmol of the phthalic anhydride, 1.4 mmol of the corresponding phenylacetic acid derivative and 0.13 mmol (13 mg) of potassium acetate were homogeneous mixed and introduced in a G4 MW flask, and the mixture was heated in a microwave at 230 ºC for 20 minutes. After the completion of the reaction (monitored by TLC), the crude was extracted in a separatory funnel with dichlorometane (3 x 10 mL) and washed with saturated sodium bicarbonate and water until neutral pH. Then, it was dried with sodium sulphate, filtered and the solvent was removed under vacuum to provide a crude mixture. Reaction crudes were purified by crystallization from CH_2Cl_2/ether. The reaction yield ranged from 51 to 80%.

*(Z)-3-(4-Chlorobenzylidene)-5(6)-methylisobenzofuran-1(3*H*)-one* **19**, Yield 70%; yellow crystal; mp 125-127 ºC; IR υ_{max} 3058, 2920, 1775, 1662, 1491, 1276, 1082, 976, 879, 284, 777 cm^{-1}. HRMS (ESI) *m/z*, calcd. for $C_{16}H_{11}ClO_2$ $[M+H]^+$: 271.0526, found: 271.0560.

*(Z)-3-(4-Chlorobenzylidene)-5-methylisobenzofuran-1(3*H*)-one* **19a**, ^{1}H-NMR (400 Hz, $CDCl_3$) δ 7.79 (d, *J* = 8.7 Hz, H-7), 7.54 (brs, H-4), 7.53 (d, *J* = 8.7 Hz, H-6), 7.76 (d, *J* = 8.8 Hz, H-2´+6´), 7.36 (d, *J* = 8.8 Hz, H-3´+5´), 6.31 (s, CH), 2.53 (s, CH_3); ^{13}C-NMR (100 Hz, $CDCl_3$) δ 167.0 (C-1), 146.0 (C-3), 145.1 (C-5), 140.9 (C-3a), 134.1 (C-1´), 131.7 (C-6), 131.5 (C-4´), 131.2 (C-2´+6´), 129.0 (C-3´+5´), 120.0 (C-4), 125.5 (C-7), 121.1 (C-7a), 105.3 (C-H), 22.3 (CH_3).

*(Z)-3-(4-Chlorobenzylidene)-6-methylisobenzofuran-1(3*H*)-one* **19b**, ^{1}H-NMR (400 Hz, $CDCl_3$) δ 7.82 (brs, H-7), 7.63 (d, *J* = 7.7 Hz, H-7), 7.74 (d, *J* = 8.8 Hz, H-2´+6´), 7.53 (d, *J* = 7.7 Hz, H-5), 7.35 (d, *J* = 8.8 Hz, H-3´+5´), 6.28 (s, CH), 2.49 (s, CH_3); ^{13}C-NMR (100 Hz, $CDCl_3$) δ 167.0 (C-1), 146.0 (C-3), 140.9 (C-3a), 138.2 (C-6), 135.9 (C-5), 134.1 (C-1´), 131.5 (C-4´), 131.2 (C-2´+6´), 129.0 (C-3´+5´), 119.7 (C-4), 125.5 (C-7), 123.6 (C-7a), 104.9 (C-H), 21.6 (CH_3).

Procedure for the synthesis of phthalazinones 25, 26, 30 and 32. In a round button flask fitted with a condenser, a methanol solution of 174 mg (0.68 mmol) of **7**, 2.03 mmol of the corresponding hydrazine and excess of triethylamine was heated under reflux at 80 °C

for 24 hours. Once the reaction was finished (monitored by TLC), it was extracted with ethyl acetate (3 x 10 mL) and washed with hydrochloric acid and water to neutral pH. Then, the combined organic phases were dried with sodium sulphate, filtered and the solvent removed under vacuum to provide a crude mixture that was purified by column chromatography with the properly solvent. Reaction yields ranged from 23 to 93%.

The spectroscopic data of compound **26** are in agreement with those indicated by Derita *et al*. (2013).

*4-(4-Chlorobenzyl)-2-methylphthalazin-1(2*H*)-one*, **26**. Yield 73%; light yellow crystal; mp 272-274 ºC; IR υ_{max} 3068, 2920, 1650, 1587, 1489, 1262, 1093, 815, 797, 749, 700 cm^{-1}. HRMS (ESI) *m/z*, calcd. for $C_{16}H_{13}ClN_2O$ $[M+H]^+$: 285.0795, found: 285.0776. ^{1}H-NMR (200 Hz, $CDCl_3$) δ 8.42 (m, 1H), 7.70 (m, 3H), 7.26 (d, *J* = 8.7, 2H), 7.20 (d, *J* = 8.7, 2H), 4.25 (s, 2H), 3.87 (s, 3H); ^{13}C-NMR (50 Hz, $CDCl_3$) δ 159.6, 144.5, 136.4, 132.8 (2C), 131.3, 129.8 (2C), 129.2, 128.9 (2C), 128.3, 127.2, 125.0, 39.4, 38.3.

Synthesis of compounds 27-29, 31 and 33-37 by reaction of compound 25 with the corresponding halide. In a one-necked frosted flask, 33.4 mg (0.24 mmol) of K_2CO_3 were added to a solution of 54 mg (0.20 mmol) of **25** in acetonitrile (7 mL) and the mixture was maintained at room temperature for 15 minutes. Then, 0.22 mmol of the corresponding halogen derivative was added dropwise and the mixture maintained at room temperature for 16 h under magnetic stirring. The reaction was controlled by TLC. Once the reaction was finished, the solvent was removed, and the resulting solid was purified by column chromatography. Yields ranged from 72 to 93%.

Biology

Animals. Two animals were used to obtain the necessary material to carry out the in vitro experiments. Two months-old Merino lambs were infected with 20,000 L3 of *T. circumcincta*. All protocols carried out were performed according to current national and European regulations of animal wellbeing (R.D 53/2013 and EU Directive 2010/63/EU) at the facilities of the Instituto de Ganadería de Montaña (IGM, CSIC, León, Spain).

Egg Hatch Assay (EHA). The eggs were extracted from fresh faecal material of infected animals by sieving, centrifugation, and flotation in a solution of saturated sodium chloride. Each compound was first tested in a susceptible strain of the GIN at concentration of 50 µg/mL to select those with activities higher than 96%. Then, their half maximal effective

concentration (EC_{50}) was calculated using eight serial dilutions (1:2) ranging from 50 to 10 µM. Thiabendazole at 0.49 µM (equivalent to 0.1 µg/mL) the known cut-off value used to classify field susceptible and resistant strains according to Coles *et al.,* (2006) was used as positive control and 0.5% DMSO as negative. The EHA was performed using a similar protocol described by Coles *et al*. Briefly, each compound was incubated with fresh eggs in 24-well culture plates for 48 hours at 23 ºC. The concentration of eggs per well was 150 in a final volume of 2 mL. All compounds were tested in duplicate during three different days to ensure the accuracy of results. After 48 hours of incubation, all eggs and larvae present in each well were counted and the ovicidal activity was estimated by following the formulas:

% Egg Hatching per well = (number of L1 / number of L1 larvae and eggs) x 100.

% Ovicidal activity = [100 – (% Egg hatching per well / % Egg hatching in control well)] x 100.

Dose-response curves were fitted by nonlinear regression using the computer program Sigma Plot V 10.0 (Systat Software, Inc., San José, California; USA). The EC_{50} values were calculated. The results were expressed as the mean of the EC_{50} and the standard error of the mean (SEM).

Larval mortality test (LMT). This test was carried out only on those compounds in which dead larvae appeared during the EHT reading (**19** and **26**), to discard a possible activity against this parasite stage. With the aim to obtain L1, fresh eggs (previously extracted from the faeces) were incubated during 24 h at 23 ºC. Then, larvae were collected in a known volume of water to obtain a density of 100-150 larvae per mL. The LMT was performed in the same way as the EHT but using the L1 instead. In this case a stock solution of levamisole at 10 mg/mL was added as positive control on every plate to reach a final concentration of 1 mg/mL per well. The number of dead and alive L1 present per well was counted using an inverted microscope to determine the larvicidal activity of the compound ([number of dead L1/number of dead and alive L1] x 100). Apparently motionless and rod-shaped larvae were considered dead, while those that presented some kind of movement or curvature in their body were considered alive. The efficacy of the compound was expressed by the percentage of viability inhibition using the following formula:

% Viability inhibition = (% larvicidal activity per well/% larvicidal activity in control well) x 100.

Cytotoxicity assay. Cytotoxicity assays were carried out on two human cell lines: the human colorectal adenocarcinoma Caco-2 (ATCC® HTB-37™) and the human hepatocarcinoma HepG2 (ATCC® HB-8065™), to assess the toxicity on a cell line of intestinal origin and to assess the systemic toxicity of the compounds, respectively. Briefly, 10,000 cells were seeded on 96 well-plates containing RPMI 1640 Medium supplemented with 2.0 g/L sodium bicarbonate (Fisher Scientific®), 1% (w/v) L-glutamine (Sigma-Aldrich®) and 25 mM HEPES buffer, pH 7.6, 10% (v/v) inactivated foetal bovine serum and antibiotic cocktail containing 100 U/mL penicillin, and 100 mg/mL streptomycin. Cultures were incubated at 37 ºC in a humidified atmosphere containing 5% CO_2. After 24 h, different concentrations of testing compounds (ranging 1 to 100 µM) were added for a period of 72 h. After this time, the viability of the cells was assessed using the Alamar Blue (Thermo Fisher) staining method according to manufacturer's recommendations.

Cell viability expressed as the fluorescence emitted by resorufin at 590 nm was plotted against the corresponding concentration added to cell culture and fitted using the software package for scientific data analysis SigmaPlot 10.0 with the aim to estimate the half cytotoxic concentration (CC_{50}) of each compound. As an estimation of the safety of each compound, selectivity index (SI) with respect to HepG2 cells was calculated by dividing CC_{50} by EC_{50}.

Results and Discussion

Chemistry. We have first obtained the corresponding benzalphthalide (Bp), and then, the phthalazinones (Figure 2). Initially, Bp without substitution on ring A were obtained (**1**-**12**), that included twelve modifications in ring B. Then, the same derivatives with a methyl in ring A were obtained (**13**-**24**). Next, Pt derivatives (**25**-**37**) based on the activity results of Bps were obtained.

Two different procedures for the synthesis of Bp were used. Method *A*) which was similar to that indicated by Zamilpa *et al*. (2005) that involves the use of toluene to improve the elimination of water generated during the reaction process. Thereby, the phthalic anhydride, the corresponding phenylacetic acid and potassium acetate were reacted in a Dean-Stark apparatus at 220 ºC during 2 h, to provide Bp derivatives **13**,**14** and **20**-**23** in 46-65%. On the other hand, Method *B*) was used to obtain compounds **15**-**19** and **24**, in which a homogeneous mixture of the reagents and potassium acetate were irradiated in a MW apparatus at 230 ºC for 20 minutes to provide the derivatives in 51 to 80%. In general, better yields were obtained using Method *B* than Method *A*.

Then, Bp compounds were reacted with different hydrazines (hydrazine, methyl-, *t*-butyl- and phenyl-hydrazine) and triethylamine at 80 ºC during 24 h to obtain phthalazinones (Pt) **25**, **16**, **30** and **32**, with yields ranging from 23 to 77%. Compounds **27** to **29**, **31** and **33** to **36** were obtained by an S_N2 reaction between amide **25** and the corresponding halide and in basic conditions. Compound **37** was obtained from **19** with methylhydrazine with the same procedure.

All the compounds obtained were properly characterized according to their physicochemical properties. MS, NMR and IR spectral data for Bp and Pt are reported in Supplementary Material.

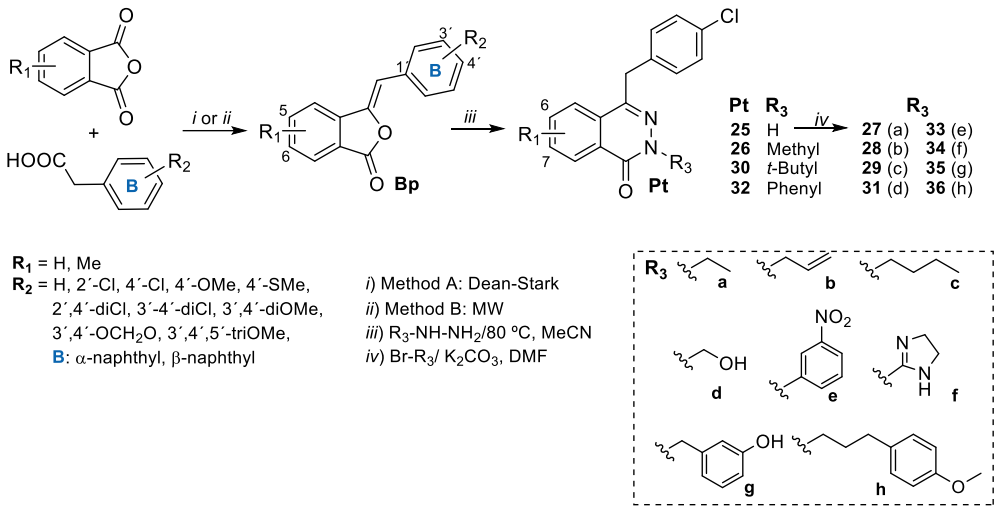


Figure 2. Synthesis procedure for benzalphthalide and phthalazinone derivatives.

Bioactivity. The nematocidal activity of all the compounds was evaluated at 50 µg/mL against *T. circumcincta* and EC_{50} values were determined for those compounds with egg hatching (EHT) higher than 96% inhibition. To determine their selectivity indexes (SI) cytotoxicity on HepG2 and Caco-2 cells was calculated.

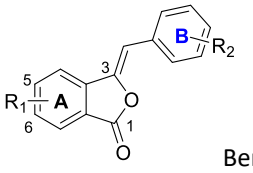
Table 1 shows the inhibition percentage of egg hatching and larval migration of *T. circumcincta*. Twenty-four benzalphthalides were obtained and evaluated, twelve of them without substitution on ring A (**1** to **12**), and another twelve with a methyl group on ring A, which are mixtures of 5-Me/6-Me regioisomers (**13** to **24**). Regarding ring B, Bp compounds show non-substitution, or electron donating (Me, OMe, SMe) or withdrawing groups (Cl) attached to different positions of the phenyl ring, or moreover a bulky fragment such as α- or β-naphthyl.

Bearing in mind the results of Bp, we decide to obtain Pt without substitution on ring A combined with a 4´-chloro on ring B, and to explore the convenience of different size chains on the amide nitrogen (alkyl, aromatic, heteroaromatic), compounds **25** to **35**, Table 1. Regarding the methyl derivatives in ring A, only the result of compound **37** is indicated.

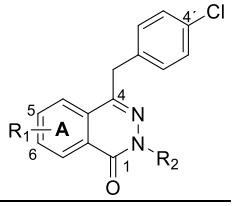
Among the compounds without substitution in ring A (**1** to **12**), **1** (R= H) and **7** (R = 4´-Cl) stand out due to their Egg Hatching Inhibition (EHI) of 97.7 and 96.7%, respectively, with EC_{50} values of 27.9 and 32.1 µM. The methoxy di-substituted **3**, and tri-substituted **5**, showed mild EHI with values of 66.8 and 36.7%, respectively. Similar results were obtained in the methyl analogues **13** to **24**, thus, **13** and the 4´-chloro **19** displayed 99.4 and 100% EHI, with EC_{50} values of 17.9 and 30.6 µM, respectively, and also the dimethoxy **15** showed moderated EHI of 57.0 %.

For compounds **1**, **7**, **13** and **19** the cytotoxicity on HepG2 and Caco-2 cells were calculated, in order to determine the safety of each compound, expressed as its selectivity index (SI). Cytotoxicity values were similar on HepG2 and Caco-2 cells, and those respecting to Caco-2 cells are shown in table 1. SI indexes rang from 3.70 for compound **1** to 9.14 and 10.23 for compounds **7** and **19**. Therefore, we have decided to obtained and tested Pt derivatives with ring A= H, and ring B = 4´-Cl and modification on the amide nitrogen with different aliphatic chains (Me, Et, allyl, *n*-But, CH_2CH_2OH), bulky (*t*-But), and aromatic groups (phenyl, 3´-nitrophenyl, 3´-hydroxibencyl, 3´-(4´-methoxyphenyl)propyl) or heteroaromatic fragments (imidazolidine-2-yl). Unluckily, only the methyl amide **26** (R_1 = H; R_2 = Me) showed 100% EHI activity with EC_{50} equal to 26.7 and SI of 13.64, subsequently, we obtained the methyl analogue **37**, which displayed slightly lower activity with 96.0% EHI, EC_{50} of 34.87and SI of 13.64.

Table 1. Results of egg hatching test (EHT) for obtained compounds against a susceptible strain of *T. circumcincta*. Cytotoxicity and selectivity index.



Benzalphthalides (Bp)



Phthalazinones (Pt)

Compound			*T. circumcincta* susceptible strain			
	R_1	R_2	EHT inhib. % at 50 µg/mL	Ovicidal EC_{50} µM	CC_{50}	SI
		Benzalphtalides				
1	H	H	**97.7**	**27.9**	103.2	3.70

2	H	4´-OMe	12.7	nc		
3	H	3´,4´-diOMe	**66.8**	nc		
4	H	3´,4´-OCH_2O	1.85	nc		
5	H	3´,4´,5´-triOMe	36.7	nc		
6	H	2´-Cl	4.54	nc		
7	H	4´-Cl	**96.7**	**32.1**	293.4	**9.14**
8	H	2´,4´-diCl	1.88	nc		
9	H	3´,4´-diCl	3.58	nc		
10	H	4´-SMe	4.72	nc		
11	H	α-naphthyl	0.74	nc		
12	H	β-naphthyl	0.33	nc		
13	Me	H	**99.4**	**17.9**	146.1	8.19
14	Me	4´-OMe	3.78	nc		
15	Me	3´,4´-diOMe	57.0	nc		
16	Me	3´,4´-OCH_2O	1.21	nc		
17	Me	3´,4´,5´-triOMe	0.05	nc		
18	Me	2´-Cl	0.00	nc		
19	Me	4´-Cl	**100**	**30.6**	313.0	**10.23**
20	Me	2´,4´-diCl	0.03	nc		
21	Me	3´,4´-diCl	0.03	nc		
22	Me	4´-SMe	0.86	nc		
23	Me	α-naphthyl	1.04	nc		
24	Me	β-naphthyl	2.56	nc		
		Phthalazinones				
25	H	H	5.02	nc		
26	H	Me	**100**	**26.7**	363.2	**13.64**
27	H	Et	3.94	nc		
28	H	Allyl	10.5	nc		
29	H	*n*-But	5.09	nc		
30	H	*t*-But	4.74	nc		
31	H	Et-OH	1.02	nc		
32	H	Ph	2.24	nc		
33	H	3´-nitroPh	3.31	nc		
34	H	Imidazolidin-2-yl	1.52	nc		
35	H	3´-hydroxiBn	1.02	nc		
36	H	3´-(4´-OCH_3Ph)Prop	2.54	nc		
37	Me	Me	**96.2**	**34.87**	241.7	6.93
Thiabendazole			**100**			

nc: not calculated. Hatching inhibition values > 96%, the EC_{50} values, and the SI values > 10 have been bolded to facilitate comparisons. Cytotoxicity (CC_{50}) in evaluated against HepG2 and Caco-2 for all compounds was >100 μM.

Bp **19** and Pt **26** were selected to be evaluated at lower doses of 10 μg/mL in eggs and larvae (L1) of a susceptible strain of *T. circumcincta* in the EHI and larval migration inhibition (LMT) tests, and in egg of a resistant strain of *T. circumcincta*, Table 2. Compounds **19** and **26** almost maintained the previous inhibition values at 10 μg/mL in the susceptible strain, with 76.3 and 76.1% EHI, respectively, without reaching the value of thiabendazol

used at 1mg/mL, however, in the resistant strain, this relationship is reversed, and we have found that Pt **26** shows almost complete inhibition of eggs hatching, 99.4% EHI, which is almost double the activity of the reference compound. In addition to that, Pt **26** in the LMT at 10 µg/mL was more potent than control levamisole, with also a nearly complete inhibition of larvae migration, 99.0% LMT.

Table 2. Results of EHT and LMT inhibition at 10 µg/mL against a susceptible and resistant strain of *T. circumcincta*.

Compound			*T. circumcincta* susceptible strain		*T. circumcincta* resistant strain
	R^1	R^2	EHT inhib. % at 10 µg/mL	LMT inhib % at 10 µg/mL	EHT inhib. % at 10 µg/mL
19	Me	4´-Cl	**76.3**	23.7	**75.5**
26	H	Me	**76.1**	**99.0**	**99.4**
Thiabendazole			**100**[a)]	-	55.0
Levamisole			-	nc	-

[a)] Thiabendazole tested at 1 µg/mL; levamisole 1 used at 1 mg/mL. nc: not calculated.

Druglikeness and toxicity risk predictions.

To analyse the druggability of the most active compounds, a prediction study by the online free webs ADMETsar (lmmd.ecust.edu.cn/admetsar2/) and Molinspiration (www.molinspiration.com/) was performed (Table 3). Briefly, compounds **19** and **26** fulfil the Lipinski´s Rule of Five with MW values between of 270.7 and 284.8 amu, clogP values in the range of 3.18 and 4.32, and the H-bond acceptors between 2 and 4, although they did not show H-bond donors. To ensure the distribution of the drug values as solubility (Log S = -3.06 and -4.63), topological polar surface area (TPSA = 30.21 and 34.90), intestinal absorption (HIA >97%) and plasma protein binding (100% and 96.4%) were predicted. The most active compound Pt **26** was neither mutagenic nor tumorigenic.

Table 3. *In silico* predicted physicochemical and ADMET properties of compounds **19** and **26**.

				Physicochemical properties				
Compounds	**MW**	**H-A**[a]	**H-D**[a]	**Log *P***	**Rot. bonds**	**TPSA**	**Druglikeness**	**Leadlikeness**
19	270.7	2	0	4.32	1	30.21	Yes	No
26	284.8	3	0	3.18	2	34.90	Yes	Yes
				ADMET properties				
Compounds	**Log S**	**HIA**	**BBB**	**PPB**		**P-g substrate**	**Mutagenic**	**Tumorigenic**

19	-3.06	High	Yes	100%	No	Yes	No
26	-4.63	High	Yes	96.4%	No	No	No

MW, molecular weight; [a] H-A; Acceptor, H-D; Donor; Log *P*, lipophilicity; PSA, Topological Polar Surface Area Å^2; Log S, aqueous solubility; HIA, Human intestinal absorption %; BBB, Blood-Brain Barrier; PPB, Plasma Protein Binding %.

Conclusion

In summary, in this study we described the synthesis and anthelmintic activity of series of benzaphthalide and phthalazinone derivatives. Among them, the benzaphthalide **19** and the phthalazinone **26** exhibited remarkable nematocidal activity on eggs of *T. circumcincta* in both susceptible and resistant strain at 10 µg/mL, especially in the latest results of 75.5 and 99.4% EHI were higher than the one found for the reference thiabendazole of 55.0%. Compound **26** showed excellent results in the LMT with 99.0% inhibition at 10 µg/mL in the susceptible strain with respect to the control levamisole. Compound **26** fulfil the Lipinski´s Rule of Five, and its ADME/toxicity-risks predictions, were in agreement with a leadlikeness, either **19** or **26** could be selected as an interesting starting point for the development of new anthelmintic compounds.

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Conflict of interest

The authors declare they have no conflict of interest.

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Supplementary material

Supplementary material accompanies this paper.

1. Physicochemical properties some obtained compounds

   1a. Benzalphthalides (**13** to **24**)

   1b. Phthalazinones (**25** to **37**).

2. Spectroscopic data of benzalphthalide **19** and phthalazinone **26**.