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Article

Synthesis of leading chalcones with high antiparasitic, against *Hymenolepis nana*, and antioxidant activities

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The hymenolepiosis by *Hymenolepis nana* is a major public health problem in developing countries, and the commercial drugs against this parasitosis are not enough effective. The combination of antiparasitic and antioxidant agents has improved the treatment of some parasitoses. Thus, the development of new cestocidal and antioxidant agents to treat the hymenolepiosis cases is important. In the present study, four hydroxy- and four dihydroxy-chalcones were synthesized using the catalyst boron trifluoride diethyl etherate (BF_3•OEt_2). The antioxidant activity and antiparasitic against *H. nana* of chalcones were tested, as well as the toxicity by the brine shrimp lethality bioassay and the method of Lorke. The antioxidant activity was measured by three radical scavenging assays: 2,2'-azino-*bis*-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP). The hydroxyl substitution pattern (number and position), mainly in ring B, was responsible for the chalcone antiparasitic activity. At least one *meta* or *para* hydroxyl group in ring B was essential for activity of the synthetic chalcones against *H. nana*; The time taken for the parasite to die by the **3b** and **3e** chalcones (20 mg/mL) treatment was up to six times lower than the control drug Praziquantel. On the other hand, chalcones with catechol structure in ring B (**3g** and **3h**) showed the highest antioxidant values. The toxicity evaluations suggests that synthetic hydroxychalcones with cestocidal (**3b** and **3e**) and antioxidant (**3g** and **3h**) activities are safe compounds and potential *in vivo* agents to treat this parasitosis.

Keywords: *Hymenolepis nana.* Chalcone/synthesis/antiparasitic/antioxidant activity.

INTRODUCTION

Intestinal parasitoses are one of the most important public health concerns, around one fifth of the world population is infected with at least one parasite (Puente *et al.*, 2011). Hymenolepiosis by *H. nana* is mainly common in children, but *H. diminuta* infections have been also reported (Kim *et al.*, 2014). Hymenolepiosis are usually asymptomatic, however, heavy infections with more than 2000 worms can induce a wide range of gastrointestinal symptoms, such as abdominal pain and diarrhea (Rim *et al.*, 1978). High *H. nana* prevalences have been registered in Africa (17.5%) (Nwele *et al.*, 2013), Asia (0.81%) (Shrestha, Maharjan, 2013), Europe (2.1%) (Calik, Karaman, Colak, 2011), and in the Americas (11.3-23%) (Jacobsen *et al.*, 2007; Quihui *et al.*, 2006). Praziquantel is the drug of choice against the hymenolepiosis but is not available at public health institutions of many underdeveloped countries (King-Charles, Mahmoud-Adel, 1989; Yadav, 2012) and the development of drug resistant parasites has complicated this situation (Chai, 2013), therefore the introduction of new cestocidal treatments is important.

Some parasites induce oxidative stress in the infected host (Gabrashanska *et al.*, 2010; Niwa, Miyazato, 1996). For example, in murine hymenolepiosis, the host produces oxygen radicals and shows an impaired antioxidant state (Kosik-Bogacka *et al.*, 2011; Niwa, Miyazato, 1996). Consequently, combining antiparasitary agents with

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feeding antioxidants can result in better efficiency in the treatment of parasitosis, as demonstrated in murine infections with *Trichinella spiralis* (Gabrashanska *et al.*, 2010) and *Leishmania amazonensis* (Gasparotto *et al.*, 2015).

Chalcones have shown a range of biological activities such as anti-inflammatory (Yadav *et al.*, 2011), anticancer (Syam *et al.*, 2012), antifungal (Konduru *et al.*, 2013), antibacterial (Tran *et al.*, 2012), antioxidant (Kim *et al.*, 2008), and antiparasitary (Sissouma *et al.*, 2011). Hence, organic chemists are interested in the synthesis of chalcones with improved biological activities. Anthelminthic activity has been associated with the substituent group patterns of the aromatic rings in the chalcone's structure (Go *et al.*, 2004), and chalcones with one hydroxyl group in ring A have demonstrated an increased activity (Laliberté *et al.*, 1968).

Chalcones also show good antioxidant activities that are associated with their structures, they have an α,β-unsaturated functional group as well as aromatic rings with variations in the number and position of hydroxyl substituent groups (Lakshmi, Rama-Rao, Basaveswararao, 2014; Todorova *et al.*, 2011). In particular, chalcone antioxidant activity has been correlated with its ability to transfer hydrogen atoms (Sivakumar, Prabhakar, Doble *et al.*, 2011).

The aim of this research was the synthesis of leading chalcones with differences in the hydroxylation pattern in both rings, and in their antiparasitary activity against *H. nana,* as well as their antioxidant properties.

MATERIAL AND METHODS

Experimental

The highest quality available reagents were purchased and used without further purification. The solvents used in column chromatography were obtained from commercial suppliers and used without distillation. Melting points were determined on a Stuart apparatus model SMP30; the reported value is the average of three separate experiments. Infrared spectra were recorded on a Cary 660 series FTIR-ATR spectrophotometer. Nuclear magnetic resonance spectra of ^{1}H (200 MHz) and ^{13}C (50 MHz) were recorded on a Varian Mercury 200 MHz Spectrometer in DMSO-d_6 with TMS as the internal standard. The chemical shifts were expressed as δ values in parts per million (ppm) and the coupling constants (*J*) were given in hertz (Hz). Electrospray ionization mass spectra were obtained with an ion trap equipment (ESI-MS, Thermo Scientific, LTQ XL, USA), and the peak intensities were presented next to the corresponding *m/z* value, as a percentage relative to the height of the base peak. Chemical ionization mass spectra were obtained with a Varian Titan 4000 ion trap GC-MS.

Synthesis of hydroxychalcones using BF_3•OEt_2

A solution of acetophenone 1 (1.2 g, 10 mmol) and benzaldehyde 2 (1.1 g, 10 mmol) was prepared, kept stirred for 10 min, and BF_3•OEt_2 (0.6 mL, 2.5 mmol) was gradually added at room temperature. Dry dioxane (3-4 mL) was used as solvent. The solution was stirred for 120 min at room temperature, subsequently washed with acidified water (2 x 50 mL), and the organic phase was extracted with ethyl acetate. The organic phase obtained was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The chosen chalcone was purified from the crude mixture by silica gel column chromatography.

The chalcones **3c**, **3i**, **3k**, **3l**, and **3m** were prepared according to a published general procedure (Liu, Wilairat, Go, 2001; Montes-Avila *et al.*, 2009).

(E)-3-(4-hydroxyphenyl)-1-phenylprop-2-en-1-one (**3a**) (Karki *et al.*, 2010). Obtained as a yellow solid compound (1.07 g, 4.77 mmol); yield = 58%; mp 106-108 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 10.2 (br, 1H), 8.14 (d, *J* = 8.2 Hz, 2H), 7.78-7.57 (m, 7H,), 6.87 (d, *J* = 8.8 Hz, 2H) ppm; ^{13}C NMR (50 MHz, DMSO-d_6) δ 189.0, 160.1, 144.5, 137.9, 132.8, 131.0, 128.7, 128.3, 125.7, 118.4, 115.8 ppm; IR (FTIR-ATR**)** ν 3161, 3017, 1648, 1579, 1283, 1157 cm^{-1}; GC-MS *m/z* 225 $[M+H]^+$.

(E)-3-(3-hydroxyphenyl)-1-phenylprop-2-en-1-one (**3b**) (Karki *et al.*, 2010). Obtained as a yellow solid compound (0.493 g, 2.22 mmol); yield = 32%; mp 83-85 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 10.4 (br, 1H), 8.08 (d, *J* = 8.4 Hz, 2H), 7.97-7.64 (m, 5H), 7.46-7.43 (m, 2H), 6.90 (d, *J* = 8.0 Hz, 2H) ppm; ^{13}C NMR (50 MHz, DMSO-d_6) δ 187.1, 162.3, 142.8, 134.9, 131.3, 130.8, 129.1, 128.9, 128.7, 128.5, 122.1, 115.4, 115.2 ppm; IR (FTIR-ATR) ν 3298, 3070, 1654, 1568, 1214, 1164 cm^{-1}; GC-MS *m/z* 225 $[M+H]^+$.

(E)-3-(2-hydroxyphenyl)-1-phenylprop-2-en-1-one (**3c**) (Karki *et al.*, 2010). Obtained as a greenish yellow solid compound (1.19 g, 5.3 mmol); yield = 80%; mp 149-151 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 10.3 (br, 1H), 8.12-8.03 (m, 3H), 7.90-7.82 (m, 2H), 7.67-7.53 (m, 3H), 7.32-7.24 (m, 1H), 6.94 (d, *J* = 8.4 Hz, 1H), 6.86 (d, *J* = 7.4 Hz, 1H) ppm; ^{13}C NMR (50 MHz, DMSO-d_6) δ 189.4, 157.2, 139.5, 137.8, 132.9, 132.0, 128.7, 128.3, 121.2, 120.8, 119.3, 116.1 ppm; IR (FTIR-ATR) ν 3204, 3082, 1638, 1560, 1228, 1150 cm^{-1}; GC-MS *m/z* 225 $[M+H]^+$.

(E)-1-(4-hydroxyphenyl)-3-phenylprop-2-en-1-one (**3d**) (Karki *et al.*, 2010). Obtained as a pale yellow solid

compound (0.52 g, 2.3 mmol); yield 33%; mp 166-168 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 10.4 (br, 1H), 8.09 (d, J = 8.6 Hz, 2H), 7.97-7.81 (m, 3H), 7.68 (d, J = 15.6 Hz, 1H), 7.47-7.43 (m, 3H), 6.91 (d, J = 8.6 Hz, 2H) ppm; ^{13}C NMR (50 MHz, DMSO-d_6) δ 187.1, 162.2, 142.8, 134.9, 131.2, 130.4, 129.1, 128.9, 128.7, 122.1, 115.4 ppm; IR (FTIR-ATR) ν 3117, 3066, 1642, 1562, 1215, 1165 cm^{-1}; GC-MS *m/z* 225 $[M+H]^+$.

(E)-1,3-bis(4-hydroxyphenyl)prop-2-en-1-one (**3e**) (Karki *et al.*, 2012). Obtained as a yellow solid compound (0.89 g, 3.7 mmol); yield = 47%; mp 164-166 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.04 (d, J = 8.8 Hz, 2H), 7.84-7.59 (m, 4H), 6.89 (d, J = 8.6 Hz, 2H), 6.83 (d, J = 8.8, 2H) ppm; ^{13}C NMR (50 MHz, DMSO-d_6) δ 187.5, 162.4, 160.3, 143.6, 131.4, 131.2, 129.9, 126.4, 119.0, 116.2, 115.8 ppm; IR (FTIR-ATR) ν 3286, 3160, 1642, 1575, 1212, 1157 cm^{-1}; GC-MS *m/z* 241 $[M+H]^+$.

(E)-1-(3-hydroxyphenyl)-3-(4-hydroxyphenyl)prop-2-en-1-one (**3f**) (Karki *et al.*, 2012). Obtained as a yellow solid compound (1.46 g, 6.1 mmol); yield = 71%; mp 195-197 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 10.1 (br, 1H), 9.98 (br, 1H), 7.75-7.57 (m, 5H), 7.44-7.31 (m, 2H), 7.06-7.01 (dd, J_1 = 7.6, J_2 = 2.4 Hz, 1H), 6.83 (d, J = 8.6 Hz, 2H) ppm; ^{13}C NMR (50 MHz, DMSO-d_6) δ 188.9, 160.1, 157.7, 144.4, 139.4, 131.0, 129.8, 125.8, 120.0, 119.4, 118.6, 115.8, 114.5; IR (FTIR-ATR) ν 3335, 3285, 3076, 1648, 1556, 1233, 1167 cm^{-1}; ESI-MS *m/z* 239 $[M-H]^+$.

(E)-3-(3,4-dihydroxyphenyl)-1-phenylprop-2-en-1-one (**3g**) (Kim *et al.*, 2008). Obtained as a yellow solid compound (0.26 g, 1.1 mmol); yield = 21%; mp 192-193 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 9.50 (br, 2H), 8.10 (d, J = 8.0 Hz, 2H), 7.65-7.51 (m, 5H), 7.27-7.16 (m, 2H), 6.81 (d, J = 8.0 Hz, 1H) ppm; ^{13}C NMR (50 MHz, DMSO-d_6) δ 189.6, 149.4, 146.2, 145.6, 138.7, 133.4, 129.4, 128.9, 126.9, 122.9, 119.0, 116.4, 116.2 ppm; IR (FTIR-ATR) ν 3478, 3296, 3054, 1644, 1559, 1276, 1167 cm^{-1}; GC-MS *m/z* 297 $[M+C_4H_9]^+$.

(E)-3-(3,4-dihydroxyphenyl)-1-(4-hydroxyphenyl) prop-2-en-1-one (**3h**) (Kim *et al.*, 2008). Obtained as a yellow solid compound (0.192 g, 0.8 mmol); yield = 18%; mp 190-192 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 10.3 (br, 1H), 9.69 (br, 1H), 9.11 (br, 1H), 8.02 (d, J = 8.8 Hz, 2H), 7.56 (dd, J_1 = 15.4, J_2 = 15.6 Hz, 2H), 7.24-7.13 (m, 2H), 6.88 (d, J = 8.8 Hz, 2H), 6.80 (d, J = 8.0 Hz, 1H) ppm; ^{13}C NMR (50 MHz, DMSO-d_6) δ 187.0, 161.9, 148.4, 145.5, 143.6, 130.9, 129.5, 126.4, 121.8, 118.3, 115.6, 115.7, 115.2 ppm; IR (FTIR-ATR) ν 3387, 3131, 3037, 1641, 1598, 1271, 1160 cm^{-1}; ESI-MS *m/z* 255 $[M-H]^+$.

2-phenylchroman-4-one (**3i**). Obtained as a pale yellow solid compound (1.54 g, 6.9 mmol); yield = 63%; mp 66-68 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 7.80 (dd, J_1 = 7.8, J_2 = 1.6 Hz, 1H), 7.65-7.41 (m, 6H), 7.13-7.07 (m, 2H), 5.68 (dd, J_1 = 13, J_2 = 2.8 Hz, 1H), 3.27 (m, 1H), 2.83 (dd, J_1 = 16.8, J_2 = 3.0 Hz, 1H); ^{13}C NMR (50 MHz, DMSO-d_6) δ 191.6, 161.0, 138.8, 136.2, 128.5, 126.6, 126.3, 121.4, 120.6, 118.0, 78.7, 43.4; IR (FTIR-ATR) ν 3036, 2896, 1685, 1602, 1223, 1149 cm^{-1}; GC-MS *m/z* 225 $[M+H]^+$.

(E)-1-phenyl-3-(4-(tetrahydro-2H-pyran-2-yloxy) phenyl)prop-2-en-1-one (**3j**) (Liu *et al.*, 2001). Obtained as a pale yellow solid compound (2.59 g, 8.3 mmol); yield = 84%; mp 76 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.14 (d, J = 7.0, 2H), 7.86-7.53 (m, 7H), 7.08 (d, J = 8.6 Hz, 2H), 5.58 (s, 1H), 3.79-3.53 (m, 1H), 1.87-1.56 (m, 7H); ^{13}C NMR (50 MHz, DMSO-d_6) δ 189.9, 158.5, 143.9, 137.7, 132.9, 130.6, 128.7, 128.3, 119.8, 116.5, 95.4, 61.5, 29.6, 24.5, 18.4; IR (FTIR-ATR) ν 3063, 2942, 2873, 2846, 1652, 1592, 1566, 1212, 1172 cm^{-1}; GC-MS *m/z* 225 $[M-C_5H_8O]^+$.

(E)-3-(4-nitrophenyl)-1-phenylprop-2-en-1-one (**3k**) (Montes-Avila *et al.*, 2009). Pale brown solid (1.11 g, 4.40 mmol); yield = 88%; mp 155-157 **°C**; ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.28 (d, J = 8.8 Hz, 2H), 8.04 (m, 2H), 7.83 (d, J = 15.8 Hz, 1H), 7.79 (d, J = 8.8 Hz, 2H), 7.71-7.48 (m, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 189.6, 141.5, 141.0, 137.5, 133.4, 128.9, 128.8, 128.6, 125.7, 124.2; IR (FTIR-ATR) ν 3067, 2928, 1658, 1599,1515 cm^{-1}; ESI-MS *m/z* 254 $[M+H]^+$.

(E)-3-(4-methoxyphenyl)-1-phenylprop-2-en-1-one (**3l**) (Montes-Avila *et al.*, 2009). Pale yellow solid (1.07 g, 4.49 mmol); yield = 90%; mp 70-73 **°C**; ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.00 (dd, J_1 = 8.3 Hz, J_2 = 1.8, 2H), 7.80 (d, J = 15.6 Hz, 1H), 7.60 (d, J = 8.8 Hz, 2H), 7.55-7.48 (m, 3H), 7.41 (d, J = 15.6 Hz, 1H), 6.93 (d, J = 8.8 Hz, 2H), 3.84 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 190.6, 161.7, 144.7, 138.5, 132.6, 130.2, 130.2, 128.6, 128.4, 127.6, 119.8, 55.4; IR (FTIR-ATR) ν 3055, 2932, 1656, 1598, 1262 cm^{-1}; ESI-MS *m/z* 239 $[M+H]^+$.

(E)-3-(4-methylphenyl)-1-phenylprop-2-en-1-one (**3m**) (Montes-Avila *et al.*, 2009). Pale yellow solid (0.755 g, 6.79 mmol); yield = 68%; mp 95-97 **°C;** ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.02 (d, J = 8.1 Hz, 2H), 7.80 (d, J = 15.7 Hz, 1H), 7.60-7.44 (m, 6H), 7.22 (d, J = 8.1 Hz, 2H), 2.40 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 190.6, 145.0, 141.1, 138.3, 132. 7, 132.1, 129.7, 128.6, 128.5, 128.4, 121.1, 21.5; IR (FTIR-ATR) ν 3051, 2916, 1655, 1597 cm^{-1}; ESI-MS *m/z* 223 $[M+H]^+$.

Biological assays

H. nana adult worms

Fecal samples of humans infected with *H. nana*

were obtained, and the eggs of *H. nana* were concentrated by a modification of the coproparasitoscopic method of Sheather (Zajac *et al.*, 2012). Sheather solution was prepared by dissolving 300 g of sugar in 340 mL of distilled water, and its density was adjusted to 1.20 g/mL. Fecal material was rinsed twice in tap water by centrifugation for 5 min at 2500 rpm. The sediment was mixed with Sheather solution (1:9 w/v), homogenized, and centrifuged for 5 min at 1500 rpm. The eggs were collected from the surface film and their viability checked by Evan's blue dye exclusion method. Three groups of 10 BALB/c mice 5-8 weeks old were used. Each mouse was fed 500, 1000 or 2000 viable eggs suspended in 100 µL of phosphate-buffered saline. At 15-21 days post-infection, one fecal sample from each mouse was examined on three consecutive days, using the methods of Faust and Ritchie (Faust *et al.*, 1938; Ritchie, 1948). The appearance of eggs in fecal material indicated the presence of adult worms in the small intestine of infected mice. Infection efficiency varied from 60 to 80%. A dose of 600-1000 eggs was used to maintain the human isolate of *H. nana* in mice, as well as to obtain adult parasites for the antiparasitary *in vitro* assay. The adult parasites were recovered from the small intestine, washed thrice in phosphate buffered saline (PBS, 0.03 M, pH 7.4), and incubated in Hank's medium mixed with 0.5% peptone and 0.2% antibiotic-antimycotic solution (penicillin G, streptomycin sulfate and amphotericin B). To determine viability and morphological changes of *H. nana*, the worms were immersed for 5 min in a 0.4% Evan's blue solution, washed three times in PBS, and observed with a stereoscopic microscope. Dead parasites were stained with blue violet color, whereas live parasites remained unstained. The parasites in the negative control remained viable and with normal structures for at least 72 h.

In vitro determination of antiparasitic activity of hydroxychalcones

Antiparasitary evaluations were made at one concentration that corresponds to the praziquantel solubility (20 mg/mL) in Hank's medium. *Hymenolepis nana* worms were exposed to hydroxyl chalcones or Praziquantel (positive control) in 24-well sterile plates, 5 worms/well in 1 mL of medium. Parasites in Hank's medium were used as negative control. The plates were monitored by light microscope for every hour up to 12 h and then every 24 h up to 72 h; morphology, mobility and vitality were registered (Montes-Avila *et al.*, 2017). Concentration-response assays of selected chalcones (1, 5, 10, 15 and 20 mg/mL) on the antiparasitic activity were performed. The chosen chalcones were those that killed the *Hymenolepis* in the shortest time and caused less toxicity to *Artemia salina*.

Toxicity assays, brine shrimp lethality and the Lorke´s method

Brine shrimp eggs (*Artemia salina*) were hatched in artificial seawater prepared with 38 g/L of sea salt (Instant Ocean®, Blacksburg, VA, USA) and oxygenated with an aquarium pump. After incubation for 48 h at room temperature, nauplii were attracted to one side of the vessel with a light source (white neon, 70 Watt) and collected with a micropipette. Crustaceans were transferred to a 96-well microplate (10-15 larvae or nauplii/100 µL), and compounds at different concentrations (500, 375, 250, 125, 50 µg/mL) were added in 100 µL of seawater. The plates were incubated for 24 h under artificial light, examined with a stereoscopic microscope, and the number of dead nauplii per well was counted. The nauplii were considered dead if they were immobile for at least 10 s (Fernández *et al.*, 2009). Then, 100 µL of formalin (10%) per well was added and the total number of shrimp was counted. The median lethal dose (LD_{50}) was calculated with Minitab®(Minitab 2000).

The acute toxicity of the chalcones with the highest antiparasitic activities was measured by the method reported by Lorke in 1983. The experiment was carried out with Balb/C mice in two phases. The assayed doses were 10, 100 and 1000 mg/kg of body weight in the first phase (three mice/dose); if toxicity was not achieved then 1600, 2900 and 5000 mg/kg were assayed in the second phase (1 mice/dose). Treatments were given by intragastric administration. The mortality and general behavior of mice were registered for 24 h, and the LD_{50} was calculated as the geometric media $\bar{X}_g = \sqrt{X_1 \cdot X_2}$, where X_1 and X_2 were the doses at which the mice were alive and death, respectively.

In vitro **evaluation of the antioxidant activity**

DPPH radical scavenging assay

The radical scavenging activity was estimated according to the method described in literature with minor modifications (Sivakumar, Prabhakar, Doble, 2011). The DPPH radical was dissolved in ethanol (0.1 mM). An aliquot of the solution (1.95 mL) was mixed with 50 *µL* of each ethanol sample solution (50 *µg/mL*). Caffeic acid was used as a positive control. The reaction was stored at room temperature for 20 min and readings were performed at 517 nm in a spectrophotometer. The results were expressed as % of the radical disappearance (% Inhibition) considering control absorbance (abs control) and sample absorbance (abs sample):

$$\% \text{ Inhibition} = \left(\frac{(abs\ control - abs\ sample)}{abs\ control} \right) \times 100$$

ABTS/persulfate assay

ABTS assay was carried out according to the method described previously in literature with minor modifications (Dueñas *et al.*, 2010). The $ABTS^{\bullet+}$ radical was generated by combining 14 mM ABTS with 4.9 mM potassium persulfate (1:1 v/v), the mixture was allowed to stand for 16-24 h at room temperature and darkness. The radical $ABTS^{\bullet+}$ was diluted with phosphate buffered saline (PBS, 0.03 M, pH 7.4) to obtain absorbance values of 1.0 to 1.2 at 734 nm.

For the determination of the antioxidant activity, 50 µL of the tested compound was mixed with 1.95 mL of the $ABTS^{\bullet+}$ (50 µg/mL final concentration), allowed to stand for 10 min at room temperature in darkness, and readings were carried out at 734 nm. Caffeic acid was used as a positive control. The absorbance of the control was obtained by mixing 50 µL of ethanol and 1.95 mL of diluted $ABTS^{\bullet+}$. The results were expressed as µmol of Trolox equivalents/µmol of compound.

The Ferric Reducing Antioxidant Power (FRAP) assay

The ferric-reducing antioxidant power of the hydroxychalcones was determined by methodologies described in the literature with minor modifications (Zhang *et al.*, 2010). The FRAP reagent was prepared by mixing 10 mM TPTZ solution (2.5 mL) with 40 mM HCl, 20 mM $FeCl_3$ (2.5 mL) and 0.3 M acetate buffer (pH 3.6, 25 mL). Freshly prepared FRAP reagent (1.95 mL) was mixed with the test compound (50 µL) or ethanol (as blank). The mixture was incubated for 5 min at 25 °C and the absorbance was measured at 593 nm. The results were expressed as µmol Trolox equivalents/µmol of compound.

RESULTS AND DISCUSSION

Chemistry

The appropriate acetophenone and benzaldehyde were condensed following published procedures (Karki *et al.*, 2010; Montes-Avila *et al.*, 2009; Narender, Reddy, 2007) to obtain the hydroxychalcones **3a-m** (Figure 1 and Table I).

In general, chalcone synthesis catalyzed with $BF_3 \bullet OEt_2$ was successful because they were obtained in moderate yields (Table I), and purification was easy. According to the literature, the aldol condensation under basic conditions is difficult when the aromatic aldehyde reagent shows an hydroxyl substituent, because the basic catalysts decrease the reactivity of the aldehyde by the relocation of the anion (Patil, Mahajan, Katti, 2009).

The synthetic chalcones were yellow solids with characteristic mass spectra peaks, which included the peak for the molecular ion. The infrared spectra signals (cm^{-1}) of chalcones were assigned as follows: 3000-3200 phenolic hydroxyl (-OH); 1640-1690 double bonds of the α,β-unsaturated carbonyl bond (C=O); 1590-1600 aromatic rings; and finally 1150-1190 for the C-O single bond.

The proton NMR data of chalcones showed the signals of the following groups: hydroxyl, δ_H 9.0–10; aromatic rings, δ_H 7.0–8.0; and finally the α and β unsaturated system, δ_H 7.0–7.5. Chalcones were geometrically pure with *trans* configuration (J H_α-H_β = 15.5–16.0 Hz), the latter confirmed that the condensation reaction took place. All ^1H and ^{13}C NMR spectra signals agreed with the chalcone structure, which was confirmed by comparison to literature data (Karki *et al.*, 2010; Karki *et al.*, 2012; Kim *et al.*, 2008).

Biology

Antiparasitic activity of hydroxychalcones

The antiparasitic activity of compounds **3a-m** was evaluated *in vitro* against adult *H. nana* worms (Table II). At least one *meta* or *para* hydroxyl group in ring B was essential for the cestocidal activity. Evaluated at 20 mg/mL, synthetic compounds **3a-b** and **3e** exhibited better antiparasitary activities than praziquantel (positive control).

Compared with the anti-*H. nana* activity of praziquantel, the activities of chalcone **3b**, showing one *meta* hydroxyl group in ring B, and **3a** were 6-fold and

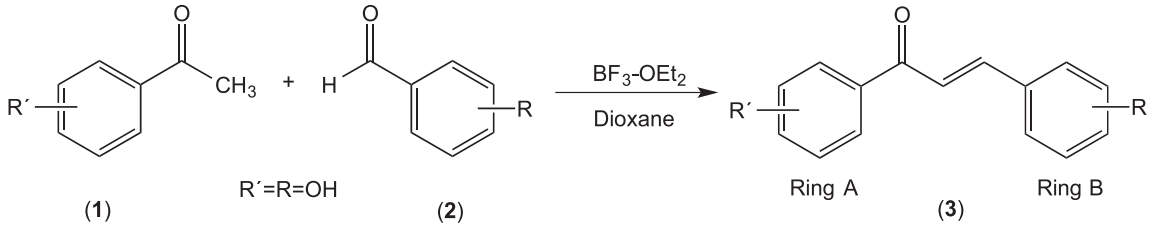


FIGURE 1 - Synthetic chalcones prepared with $BF_3 \bullet OEt_2$.

TABLE I - Reaction time, yield, and melting point (mp) of the prepared chalcones

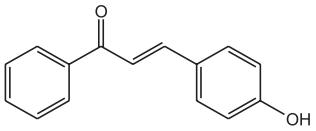
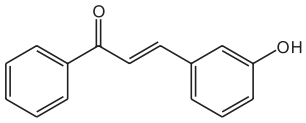
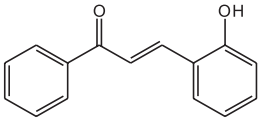
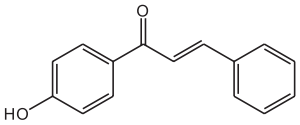
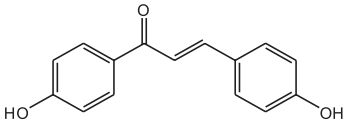
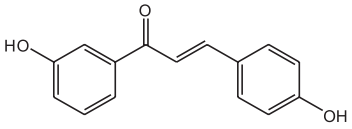
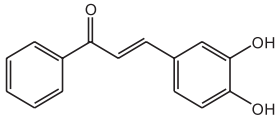
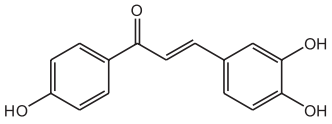
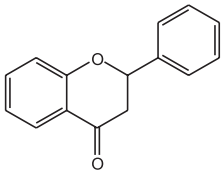
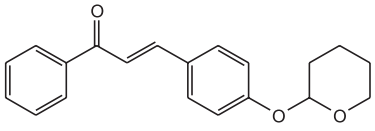
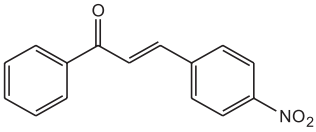
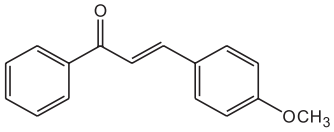
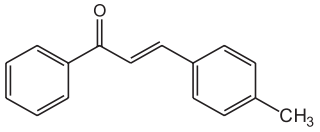
Chalcone	Structure	Time (min)	Yield (%)[a]	mp (°C)
3a		120	58	106-107
3b		120	32	83-84
3c		30	80	149-150
3d		120	33	166-167
3e		120	47	164-165
3f		1200	71	195-196
3g		2700	21	192-193
3h		1080	18	190-191
3i		1320	63	66-68
3j		1140	84	76
3k		720	88	155-157

TABLE I - Reaction time, yield, and melting point (mp) of the prepared chalcones (cont.)

Chalcone	Structure	Time (min)	Yield (%)[a]	mp (°C)
3l		720	90	70-73
3m		720	68	95-97

[a]Yields refer to isolated products

TABLE II - Effect of the treatment of adult *H. nana* parasites with 20 mg/mL of chalcones[1]

Chalcone	Substitution pattern		Time	
	R'	R	Paralysis (seconds)	Death (minutes)
3a	H	4-OH	60	10
3b	H	3-OH	10	5
3c	H	2-OH	N/D	N/D
3d	4-OH	H	N/D	N/D
3e	4-OH	4-OH	10	5
3f	3-OH	4-OH	1800	60
3g	H	3,4-OH	60	40
3h	4-OH	3,4-OH	300	45
3i			900	960
3j			1800	N/D
3k	H	4-NO_2	N/D	N/D
3l	H	4-OCH_3	N/D	N/D
3m	H	4-CH_3	N/D	N/D
Praziquantel	-	-	1200	30
Control (-)	-	-	N/D	N/D

[1] R' and R are the substituent groups as indicated in Figure. N/D indicates that parasites were still moving or alive up to 16 h of treatment.

2-fold higher, respectively, whereas chalcone **3c** was inactive. Moreover, **3i** structure did not have hydroxyl groups, and the time taken for *H. nana* to die with **3i** was about 200 times higher than the time taken with **3b** and **3e**. The inactive **3d** compound gained anthelminthic activity after the introduction of an -OH group in ring B (**3e**), **3e** showed higher activity than **3a**. Considering the dihydroxy compounds **3g** and **3h**, they paralyzed *H. nana* in short times but induced higher times taken for the parasite to die (40 and 45 min, respectively) than praziquantel (30 min) (Table II). In addition, **3j-m** chalcones lacked of hydroxyl groups and of antiparasitic activity (Table II). Thus, hydroxyl groups in chalcones were important for their activity against *H. nana*, and that in the ring B of our chalcones was the pharmacophore. In this respect, weak anthelmintic chalcones increase their activity by the addition of a hydroxyl group at the *ortho* position of ring A. Differences in the number, type, and position of substituent groups of chalcones modify their antiparasitic activities. Imidazopyridinyl chalcones with a *meta* alkyl group in ring B show two times higher activity against the nematode *Haemonchus contortus* than those with the group in *ortho* or *para* (Sissouma *et al.*, 2011). The replacement of a strongly by a weakly electron donating

group (*e.g.*, hydroxyl and methoxyl group, respectively) in the chalcone molecule increases its nematicidal activity from LC_{100}= 424 μg/mL (LC_{100} means absolute lethal concentration) to LC_{100}= 2.87 μg/mL (Sissouma *et al.*, 2011). On the other hand, ten synthetic chalcones were evaluated *in vitro* at concentrations of 3 to 1000 μM against the intracellular protozoa *Leishmania braziliensis* and *Trypanosoma cruzi*. Chalcone trypanocidal activity decreased by incorporating a chlorine atom in position *para* to both rings unlike the unsubstituted chalcone (Lunardi *et al.*, 2003); based on these observations, authors report that the electronic effect of the *para* substituents in ring A of chalcones is not crucial for displaying antiprotozoal activity. Moreover, the *in vitro* antiplasmodial activity of chalcones depends on the type of ring substituents in ring B (alkoxy, methoxy, hydroxyl, and fluorine), but also the ring A substituents modify the activity (Go *et al.*, 2004).

The mode of action of chalcones against *H. nana* is so far unknown; however, mitochondria have been proposed as antiparasitic drug targets, and specifically by the inhibitory effect of chalcones on enzymes of the respiratory chain (Brophy *et al.*, 1989; Monzote, Gille, 2010). In this respect, several reports have demonstrated the antifilarial activity of chalcones against *Setaria cervi*, targeting the glutathione-*S*-transferase (GST); this enzyme is significantly inhibited by chalcones with chlorine and methoxy substituents (Awasthi *et al.*, 2009). Additionally, scientific evidence supports the importance of ring B substituents in the chalcones for their antiparasitary activities; chalcones inhibit mitochondrial *Leishmania* enzymes such as fumarate reductase, succinate dehydrogenase, NADH dehydrogenase, and NADH-cytochrome *c* reductase (Chen *et al.*, 2001). It has been proposed that chalcones react with essential thiol groups of the target enzymes via Michael addition to the ketovinyl double bond, and enzymes are inactivated. Considering this mechanism, the electronic effect of the substituent in ring B is of great importance. Electron donating substituent groups (*e.g.,* hydroxyl, methoxyl) will favor the antiparasitic activity because the electrophilicity of C-β will be increased, and the nucleophilic attack to the thiol groups in the enzyme will be facilitated (Aponte *et al.*, 2008).

Hymenolepis nana worms treated with **3e** registered paralysis and rigidity at 5 min. The death of the parasite was evidenced with a vital staining, where the tegument of *H. nana* parasites treated with **3e** and praziquantel was blue but did not show visible structural alterations under light microscopy; in contrast untreated parasites remained unstained. It has been suggested that chalcone (*E*)-1-(2,6-dihydroxy-4-methoxyphenyl)-3-phenylprop-2-en-1-one alters the membrane structure of *Leishmania amazonensis* (Torres-Santos *et al.*, 2009). Due to the chalcones induced short times of paralysis on *H. nana*; the mechanism of action could share similarities to that of Praziquantel, which increases the permeability of the cell membrane and the loss of intracellular calcium, and consequently, parasites show contractions and muscle paralysis (Chai, 2013; King, Mahmoud, 1989). In fact, the active component (2′,6′-dihidroxy-4′-methoxychalcone) of the methanol extract of *Psidium sartorianum* induces severe damage to the tegument and basement membrane of *H. nana* (Montes-Avila *et al.*, 2017).

The effects of praziquantel in various trematodes (*i.e., Clonorchis sinensis*, *Opisthorchis viverrini*, *Schistosoma japonicum*, *Metagonimus yokogawai*, and *Paragonimus westermani*) has been assessed. Treatment with praziquantel (1 μg/mL) of *C. sinensis*, *S. japonicum*, and *O. viverrini* generates vacuolization at 5 min and more severe damage at longer times. *Paragonimus westermani* is the least sensitive to praziquantel, which shows less vacuolization at 100 μg/mL (Mehlhorn *et al.*, 1983). The effect of treatment with 0.1-100 μg/mL of praziquantel on cestodes (*i.e., Hymenolepis diminuta*, *Hymenolepis microstoma*, *Hymenolepis nana*, and *Echinococcus multilocularis*) is similar to that described for trematodes (Becker *et al.*, 1981).

Considering the results of the brine shrimp lethality bioassay, compound **3b** was toxic (LD_{50} of 255 μg/mL**)** but not **3e** (LD_{50} > 500 μg/mL**).** Toxicity of phenolic compounds has been related with the formation of electrophilic metabolites that affect DNA and enzymes (Kumar *et al.*, 2014). In particular, several chalcones inhibit the tubulin synthesis that affect the microtubule formation and parasite mobility, mode of action suggested for albendazole (Ducki, 2009; King, Mahmoud, 1989). On the other hand, the acute toxicity assay of Lorke showed that our synthetic chalcones **3b** and **3e** were nontoxic (LD_{50} > 5000 mg/kg). Moreover, thirty days after dose administration, mice did not show damage in internal organs (*i.e.* heart, thymus, lungs, spleen, liver, stomach, and small and large intestine).

Chalcones **3b** and **3e** were chosen for their killing effect on *H. nana* and for their low toxicity. Both compounds showed concentration-response effects related to the times required to paralyze and to kill *H. nana* (Table III). Compared with the activity of **3e** against *H. nana*, the activity of **3b** was higher, registering lower times to reach the paralysis and the same time to kill the parasite, both evaluated at 15 mg/mL. Imidazopyridinyl chalcones are anthelmintic against *Peritima posthuma* with times taken for such parasite to die of less than 100 min at 1 mg/mL

(Lakshmi, Rama-Rao, Basaveswararao, 2014). Several 3-(3-arylpropenoyl)imidazopyridine derivatives are active against *Haemonchus contortus,* the nematicidal effect evaluated at 0.0005 and 0.002 μg/mL is similar to those registered for fenbendazole and ivermectin, and activity of these compounds is lost when an alkyl group is attached to the phenyl ring (Sissouma *et al.*, 2011). In addition, chalcones **3b** and **3e** comply with the Lipinski Rules, suggesting their oral bioavailability (Lipinski *et al.*, 2001).

Antioxidant activity

In the DPPH assay, chalcones with two hydroxyl groups showed greater antioxidant activity (**3e**-**3h**), from which **3g** and **3h** with *meta* hydroxyl group in ring B were the best compounds (91 and 90% of inhibition, respectively). The antioxidant activities as μmol TE/μmol compound of **3g** and **3h** also showed to be the highest by the methods ABTS (33 and 35, respectively) and FRAP (2.4 and 2.7, respectively). Moreover, the activities of **3g**, **3h**, and caffeic acid (positive control) were similar (Table IV). The facility to transfer the hydrogen atoms of chalcones has shown a positive association with their DPPH scavenging activity (Sivakumar, Prabhakar, Doble, 2011). As known, the chalcone hydroxyl groups are important for trapping radicals; and their antiradical activity is influenced by the number, substitution pattern, hydrophobicity, and polarity of the molecules (Kim *et al.*, 2008; Todorova *et al.*, 2011; Wu *et al.*, 2007). Hydroxylated chalcones react with radicals and are easily converted to stable phenoxyl radicals. Catechol systems, *ortho-meta-* or *ortho-para-* di-hydroxy chalcones, are very efficient to delocalize electrons by transformation to quinines (Kim *et al.*, 2008; Todorova *et al.*, 2011). The highest antioxidant values for compounds **3g** and **3h** were attributed to the presence of the 3,4-hydroxycinnamoyl group (catechol) in their structures, as it was for caffeic acid. On the other hand, *para* hydroxyls in both rings of chalcones have little effect on the DPPH antioxidant activity (Kim *et al.*, 2008). Moreover, for chalcones with one or two hydroxyl groups in their ring B, the antioxidant activity decreases in the following order 3,4-di-OH >> 4-OH >> 3-OH > 2-OH (Todorova *et al.*, 2011), as it happened with the chalcones included in this research (Table IV).

Treatment of hymenolepiosis with antiparasitary drugs could be improved by the administration of exogenous antioxidants. In mice infected with *H. nana*, parasite induces host intestinal eosinophilia that produces oxygen radicals as mechanism of defense. In fact, previously challenged mice show greater production of radicals as well as of malondialdehyde, product of the lipid peroxidation (Niwa, Miyazato, 1996). In rats with hymenolepiosis by *Hymenolepis diminuta*, the intestine of host increases the production of oxygen radicals, shows an impairment of the superoxide dismutase activity, and an increased glutathione reductase activity; in addition, it is increased the reduced glutathione concentration. These parameters are interpreted as intestines of host are not enough protected against the excess of hydrogen peroxide (Czeczot *et al.*, 2012; Kosik-Bogacka *et al.*, 2011). On the other hand, the rat-*H. diminuta* model shows that *H. diminuta* increases the activities of superoxide dismutase and glutathione peroxidase, enzymes which protect the parasite of the host immunologic response (Czeczot

TABLE III - Concentration-response effect of selected chalcones **3b** and **3e** against *H. nana*

Chalcone	Substitution pattern[1]		Concentration (mg/mL)	Time	
	R'	R		Paralysis (s)	Death (min)
3b	H	3-OH	1	3,000	50
			5	1,500	25
			10	600	10
			15	30	5
			20	10	5
3e	4-OH	4-OH	1	>7,860	180
			5	6,600	120
			10	2,400	40
			15	1,200	20
			20	10	5

[1] R' and R are the substituent groups as indicated in Figure 1.

TABLE IV - Antioxidant activity of the synthesized hydroxychalcones evaluated at 50 µg/mL[a]

Compound	Substitution pattern		DPPH	ABTS	FRAP
	R'	R	(% Inhibition)	(µmol TE/µmol compound)[1]	
3a	H	4-OH	2 ± 0.001	0.32 ± 0.001	0.07 ± 0.005
3b	H	3-OH	8 ± 0.006	0.30 ± 0.005	0.01 ± 0.004
3c	H	2-OH	7 ± 0.001	0.32 ± 0.001	0.08 ± 0.025
3d	4-OH	H	7 ± 0.004	0.31 ± 0.003	0.01 ± 0.003
3e	4-OH	4-OH	21 ± 0.001	0.34 ± 0.000	0.15 ± 0.015
3f	3-OH	4-OH	20 ± 0.011	0.34 ± 0.000	0.16 ± 0.015
3g	H	3,4-OH	91 ± 0.002	33 ± 0.001	2.4 ± 0.008[c]
3h	4-OH	3,4-OH	90 ± 0.002	35 ± 0.003	2.7 ± 0.053[c]
3i			18 ± 0.005	0.04 ± 0.002	0.01 ± 0.000
3j			4 ±0.005	0.43± 0.002	0.05 ±0.020
3k	H	4-NO_2	4±0.005	0.01 ± 0.010	0.01 ±0.015
3l	H	4-OCH_3	3±0.005	0.03 ± 0.013	0±0.003
3m	H	4-CH_3	3±0.005	0.03 ± 0.025	0.001±0.013
Caffeic acid[b]			96 ± 0.006	5.5 ± 0.001	2.2 ± 0.012[c]

[1] TE: Trolox equivalents; [a] Values are the mean ± SD (n=3); [b] Used as a positive control; [c] Evaluated at 0.5 µg/mL

et al., 2013; Skrzycki *et al.*, 2011). Thus, feeding the parasitized host with antioxidants could improve the antiparasitary treatments; this has not been demonstrated with hymenolepiosis but with other parasitoses. In rats infected with *Trichinella spiralis*, Se supplementation decreases the worm burden by 63% and increases both the glutathione peroxidase activity and the vitamin E level (Gabrashanska *et al.*, 2010). In *Leishmania* infected mice, host presented up-regulation of the receptor of advanced glycation end products (involved in the production of reactive species), increased protein carbonylation, decreased IFN-γ, and impaired antioxidant defenses. Mice treatment with the antioxidant *N*-acetyl cysteine improves the redox state and recovers the IFN-γ levels. IFN-γ is required to have an adequate Th1 response to control the parasite (Gasparotto *et al.*, 2015).

Several *in vivo* studies have shown that hydroxylated chalcones disrupt the mitochondrial oxidative phosphorylation, increasing the production of reactive oxygen species (ROS). High levels of ROS and an impaired antioxidant defensive system could lead to cell death via apoptosis (Guzy *et al.*, 2010). However, hydroxylated chalcone analogs are free radical scavengers *in vitro*, and they show antioxidant protective effect in a free radical-injury Alzheimer's model in mice (Pan *et al.*, 2013). **3g** and **3h** showed high antioxidant activity *in vitro* and future studies must demonstrate their *in vivo* antioxidant activity.

CONCLUSION

We have synthetized chalcones with high activity against *H. nana* (**3a**, **3b** and **3e**). These were better than the control drug Praziquantel evaluated at the same dose. At least one hydroxyl *meta* or *para* in ring B of chalcones was essential for the anti-*H. nana* activity, and *meta* substitution was associated with the highest activities. On the other hand, the *meta*- and *para*-dihydroxy substitution patterns in ring B of chalcones, as in **3g** and **3h**, were the best combinations for the highest antioxidant activity. Further studies are in progress to demonstrate the *in vivo* antiparasitary and antioxidant effect of the selected chalcones. Moreover, these chalcones will be used to design more active molecules.

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