Unexpected Rearrangement and Aromatization on Dehydration Reaction of the Bioactive Diterpenequinone 7α-Hydroxyroyleanone

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7α-Hydroxyroyleanone is a bioactive diterpene isolated from leaves of Tetradenia riparia, active against tuberculosis and T. cruzi. In order to use this compound as starting material for the synthesis of compounds with enhanced bioactivity we aimed the dehydration of the 7-alpha-hydroxyl group of royleanone. The reaction performed in acetic acid and hydrochloric acid under reflux gave an unexpected chlorinated naphtoquinone, with aromatization and opening of one of the rings of the starting material, as major product, besides 6,7-dehydroroyleanone and a complex mixture of other products. The reaction products were purified by high-speed countercurrent chromatography with hexane-acetonitrile 1:1. Compounds were tested for their anti-leishmanial activity (L. amazonensis) pointing out a potential leishmanicidal agent which may favor the development of more effective therapies.

Keywords: 5-(4-chloro-4-methylpentyl)-3-hydroxy-2-isopropyl-8-methylnaphthalene-1,4-dione, horminone, diterpenequinone, Tetradenia riparia, leishmanicidal activity, 6,7-dehydroroyleanone

Introduction

The benzoquinone 7α-hydroxyroyleanone (I) is a bioactive diterpene from leaves of Tetradenia riparia (Hochstetter) Codd, a member of the family Lamiaceae. It is a common shrub throughout Africa, introduced in Brazil as an ornamental plant, growing in gardens and parks. In South Africa, it is one of the most popular herbs and medicinal plants in folk medicine. In Brazil, it is used in religious rituals and is commercialized in open markets. The plant is used as a remedy against a wide range of diseases including malaria, angina, gastroenteritis, diarrhea, influenza, respiratory problems and symptoms such as headaches, fever, stomachaches, etc. Studies have shown that extracts of leaves of T. riparia as well as its essential oil exhibited antimicrobial activities against bacteria and fungi such as Mycobacterium tuberculosis, M. smegmatis, M. simiae, Staphylococcus aureus and Candida albicans. The diterpenequinone 7α-hydroxyroyleanone, also known as horminone, was shown to be one of the active compounds against tuberculosis (TB). It also displays activity against T. cruzi, the Chagas’ disease parasite. Due to previously assayed activities our group has developed a strategy for the selective isolation of the target compound by high-speed countercurrent chromatography (HSCCC). Our goal was to use this compound as starting material for the synthesis of compounds with enhanced bioactivity. As quinones are described as very reactive compounds, our first experiment aimed the dehydration reaction of the 7-alpha-hydroxyl group of royleanone.

Leishmaniasis is a neglected disease, which affects millions of people in 98 countries in tropical and subtropical regions worldwide. This disease is caused by parasites of the genus Leishmania and displays three major clinical forms:
cutaneous, mucocutaneous and visceral leishmaniasis.\textsuperscript{9} Pentavalent antimonials, amphotericin B and miltefosine are the main drugs currently available for leishmaniasis treatment. However, all of them present problems that limit their use such as adverse side-effects, induction of parasite resistance and high costs.\textsuperscript{10,11} Thus, the discovery of new drugs, which are effective against parasites with reduced or absent side effects for the treatment of this pathology is important.

**Experimental**

**General**

All organic solvents used for the preparation of crude extracts and for the chemical reaction were of analytical grade. Solvents for the HSCCC separation were of high performance liquid chromatography (HPLC) grade. All solvents were purchased from Tedia Brazil (Brazil). All aqueous solutions were prepared with ultra-pure water (18.2 MΩ cm) produced by a PURELAB® classic system (ELGA, USA) water system. Sodium carbonate was purchased from Vetec Química Fina (Brazil).

Thin-layer chromatography (TLC) analyses were performed on silica gel 60 F254 aluminum plates from Merck, Germany.

Collection of plant material (*Tetradenia riparia* Schodd.) from a private garden in the city of Teresópolis, a mountainous region in Rio de Janeiro state, Brazil, in April 2012, preparation of the dichloromethane extracts as well as extraction of 7α-hydroxyroyleanone were performed according to the methods previously described by our group.\textsuperscript{8} A voucher specimen of *T. riparia* is deposited at the Herbarium of Universidade Federal do Rio de Janeiro under the No. RFA 36979.

HSCCC fractionations were performed on the 80 mL multi-layer column of a P.C. Inc. countercurrent chromatograph (Potomac, MD, USA). The rotation speed was adjusted to 865 rpm. The HSCCC system was connected to a constant flow pump Jasco® PU-2089S Plus (Japan Spectroscopic Corporation, Japan). Fractions were collected with a Merck® fraction collector L-7650 (Merck, Germany).

Gas chromatography (GC) with flame ionization detector (FID) was used to analyze the purity of HSCCC fractions. Analyses were performed in a GC-2010 plus gas chromatograph coupled to an FID detector (Shimadzu, Japan) and auto injector AOC-20i (Shimadzu, Japan). Injections were made in splitless (1 μL) mode, detector temperature was 290 °C. A DB-5 (5% phenylmethylpolysiloxane) (Agilent Technologies, USA) fused silica capillary column (30 m, 0.25 mm internal diameter, 0.25 μm film thickness) was used and hydrogen was the carrier gas with a flow rate of 1.0 mL min\(^{-1}\). The oven temperature was programmed at 3 °C min\(^{-1}\) from 150 °C to 250 °C. The FID detector temperature was set at 300 °C. Data were acquired and processed by the GC Solution Postrun® version 2.41 (Shimadzu, Japan) software.

High-resolution electrospray ionization time-of-flight mass spectrometry (HR-ESI-TOF/MS) of (2) was carried out in a MicroTOF II mass spectrometer (Bruker Daltonics Inc., MA, USA) in negative ion mode, at 3800 V (capillary voltage), 180 °C.

Unidimensional (\(^1\)H and \(^13\)C) and bidimensional (correlated spectroscopy (COSY) \(^1\)H-\(^1\)H, heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC)) nuclear magnetic resonance (NMR) spectra were acquired through a Varian VNMRS spectrometer at 31 °C, operating at 299.74 MHz for \(^1\)H and 100.53 MHz to \(^13\)C. Chemical shifts are given on the \(\delta\) scale, and \(J\) values are given in Hz. Tetramethylsilane (TMS) was used as internal reference.

Dehydration reaction of 7α-hydroxyroyleanone (1)

\[ 7\alpha\text{-Hydroxyroyleanone (487 mg, 1.45 mmols) and 0.5 mL of concentrated hydrochloridric acid (37% m/v, 5 mmols) were dissolved in 30 mL of acetic acid and heated by two hours at 100 °C under reflux. The TLC analysis showed that the reaction provided a mixture of products. The reaction mixture was poured over cold water and a precipitate was formed which was filtered under vacuum. The solid residue (321.7 mg) was purified by countercurrent chromatography. }\]

Countercurrent chromatography purification of (2) and (3)

The crude reaction mixture (321.7 mg) was purified by HSCCC on the 80 mL column of the P.C. Inc. equipment with the solvent system hexane-acetonitrile 1:1. The mobile phase was the upper phase, 2 mL min\(^{-1}\), 850 rpm. The column was first filled with the stationary lower phase without rotation. Then rotation started, and the column was equilibrated with the upper mobile phase until hydrodynamic equilibrium occurred (which is observed when the excess of stationary phase is displaced, \(V_m = 15\) mL, and only mobile phase elutes from the column). The retention of the stationary phase was 81.2%. The sample was dissolved in a 1:1 mixture of the biphasic solvent system (5 mL) and injected with a 6-port medium pressure injection valve model V-450 (IDEX Health & Science, US) after hydrodynamic equilibrium occurred.
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Fractions of 4 mL were collected, in a total of 117 fractions which were monitored by thin layer chromatography yielding 10 sub-fractions. Rotation stopped at fraction 91. Compound 2 (111.4 mg) was recovered from fractions 65-91 (sub-fraction 7) and compound 3 (23.2 mg) was recovered from fractions 24-36 (sub-fraction 3).

Spectral data for compounds (2) and (3)

5-(4-Chloro-4-methylpentyl)-3-hydroxy-2-isopropyl-8-methyl-naphthalene-1,4-dione (2)

Orange solid, melting point 122-124 °C; UV (ethyl acetate) λ/ nm 353.8, 285.8, 256.4, 249.6 (shoulder); 1H NMR (400.1 MHz, CDCl3) δ 1.27 (d, J 7.2 Hz, 6H, CH3, H-2b/H-2c), 1.58 (s, 6H, CH3, H-5/e/H-5f), 1.67 (m, 2H, H-5b), 1.96 (m, 2H, H-5c), 2.42 (s, 3H, CH3, ArH-8b), 3.11 (t, J 8 Hz, 1H, H-5a), 3.35 (sept, J 7.2, 1H, H-2a), 7.48 (d, J 8 Hz, 1H, H-7), 7.93 (d, J 8 Hz, 1H, H-6); 13C NMR (100.6 MHz, CDCl3) δ 184.42 (C-1), 183.19 (C-4), 153.06 (C-3), 144.40 (C-8a), 143.01 (C-4a), 136.32 (CH, C-7), 133.24 (C-8), 126.48 (C-2), 126.06 (C-5), 125.50 (CH, C-6), 70.90 (C-5d), 46.33 (CH2, C-5c), 32.41 (CH3, C-5e/C-5f), 30.15 (CH2, C-5a), 24.70 (CH2, C-5b), 24.33 (CH, C-2a), 20.26 (CH3, C-8b), 19.80 (CH3, C-2b/C-2c); HR-ESI-MS m/z, calcd. for C20H25ClO3 [M – H]−: 347.141946, found: 347.141946 [M – H – Cl]−.

6,7-Dehydro-royleanone (3)

1H NMR (400 MHz, CDCl3) δ 0.95 (s, CH3, H-18), 1.02 (s, CH3, H-19), 1.03 (s, CH3, H-20), 1.21 (d, J 7Hz, CH3, H-16), 1.22 (d, J 7Hz, CH3, H-17), 2.12 (t, J 5 Hz, H-5) 3.17 (m, H-15), 6.46 (dd, J 10 and 5 Hz, H-6), 6.81 (dd, J 10 and 5 Hz, H-7); 13C NMR (100 MHz, CDCl3) δ 186.20 (C-14), 183.31 (C-11), 151.19 (C-12), 140.7 (C-9), 139.46 (C-6), 138.35 (C-8), 122.60 (C-13), 121.09 (C-7), 52.11 (C-5), 40.52 (C-3), 39.25 (C-10), 35.16 (C-15), 33.26 (C-4), 32.60 (C-C-18), 24.08 (C-15), 22.80 (C-19), 20.01 (C-17), 19.81 (C-16), 18.70 (C-2), 15.17 (C-20). Chemical shifts are in accordance with literature.12

Biological evaluation of (1) and (2)

Parasite culture

Leishmania amazonensis (WHOM/BR/75/Josefa) promastigotes were cultured at 26 °C in Schneider insect medium, supplemented with 10% fetal calf serum (Gibco-BRL, USA) and 100 U mL−1 penicillin and 100 μg mL−1 streptomycin (all from Sigma, USA).

Ethics statement

The animal experiments were performed in strict accordance with the Brazilian animal protection law (Lei Arouca, No. 11.794/08) of the National Council for the Control of Animal Experimentation (CONCEA, Brazil). The protocol was approved by the Committee for Animal Use of the Universidade Federal do Rio de Janeiro (Permit No. IMPPG 128/15).

Anti-promastigote activity

Leishmania amazonensis promastigotes were incubated in Schneider’s insect medium containing 10% fetal bovine serum at 26 °C in the absence or presence of the tested substances for 48 h. The parasite growth was evaluated using 1 mg mL−1 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxinilide inner salt (XTT) and 20 μM phenazinmethosulfate (PMS, Sigma, USA). The reaction product was read at 450 nm after 3 h, and results were expressed in percentage of viable promastigotes compared to untreated control. Dimethyl sulfoxide (DMSO) at 1% was used as vehicle control in all experiments.

Cytotoxicity for host macrophages

Peritoneal macrophages from BALB/c mice stimulated with 3% thioglycollate for 3 days were harvested in RPMI 1640 medium (LGCBiotec, Brazil) and cultured in 96-well plates for 2 h adherence at 35 °C, 5% CO2. Non-adherent cells were removed, and macrophages were incubated with the tested substances for 24 h. Cell viability was determined by the XTT assay as described above. Results are expressed in percentage of viable cells compared to untreated control.13

Anti-amastigote activity

Peritoneal macrophages from BALB/c mice obtained as above cultured in 24-well plates were infected with L. amazonensis promastigotes (stationary growth phase) at a 10:1 parasite/macrophage ratio during 1 h at 35 °C, 5% CO2. Free parasites were washed out with 0.01 M phosphate buffered saline (PBS), and the cultures maintained for 24 h at 35 °C, 5% CO2. Infected macrophage cultures were treated with different concentrations of the compounds for an additional 24 h at 35 °C, 5% CO2. Cultures were then PBS washed and incubated with 0.01 M phosphate dodecyl sulfate for 10 min followed by addition of 1 mL of Schneider’s medium supplemented with 10% fetal calf serum (FCS) and maintained at 26 °C for 2 days. The relative intracellular load of viable L. amazonensis amastigotes was measured, after promastigote transformation, using Alamar blue (Invitrogen, USA), according to the manufacturer’s instructions. After 4 h of incubation, the fluorescence was read at 540/610 nm excitation/emission in a SpectraMax Paradigm (Molecular Devices, USA). Positive controls
were done using amphotericin B (1 μM, Cristália, Brazil) and Miltefosine (10 μM, Cayman, USA), which killed 80 and 74% of the amastigotes.

**Results and Discussion**

**Chemical transformation of (1)**

The reaction was performed in acetic acid and hydrochloric acid under reflux for 2 h, giving an unexpected chlorinated product (2), with aromatization and opening of one of the rings of the starting material, as major product, besides 6,7-dehydroroyleanone (3), and a complex mixture of other products (Figure 1). The reaction products were purified by high-speed countercurrent chromatography (HSCCC) (Figure S1, Supplementary Information (SI) section).

Although no experiment or theoretical calculation were done to elucidate the reaction’s mechanism, due to the strong acid media, it is reasonable to suppose that the diterpene structure may generate a carbocation which undergoes rearrangement leading to methyl group migration and aromatization. Another possibility to aromatization is a reaction with atmospheric oxygen.

**Structural identification of (2)**

The identification of the major product was done based on spectrometric and spectroscopic data (Table 1 and Figures S2-S11, SI section). The molecular formula was determined as C_{20}H_{25}ClO_{3} based on the deprotonated ion m/z 347.1421 (error –0.5 ppm). The 1:3 ratio of the ions m/z 347.1421: 349.1403 is indicative of the presence of one chlorine atom. The proton NMR spectrum of (2) shows the lack of the carbinolic hydrogen at position 7 of -hydroxyroyleanone, and the appearance of signals at δ_{H} 7.48 ppm. In the 1'H-1'H COSY spectrum we can see the correlation between the aromatic protons H-6 and H-7 (7.48 and 7.93 ppm, respectively). The other four remaining methyl groups appear at δ_{H} 1.27 ppm (d, J 7.2 Hz, H-2b and 2c, which show correlation with H-2a at δ_{H} 3.35 ppm in the 1'H-1'H COSY spectrum) and δ_{H} 1.58 (s, H-5e and 5f). Proton 5a, adjacent to the aromatic ring, appears at δ_{H} 3.11 ppm (t, J 8Hz) and correlates with H-5b, at δ_{H} 1.67 ppm (m). H-5b also shows correlation in the 1'H-1'H COSY spectrum with H-5c at δ_{H} 1.96 ppm. The characteristic isopropyl group of the original abietane skeleton is maintained unaltered in compound (2) and the signal for H-2a can be seen at δ_{H} 3.35 ppm (sept), correlating with H-2b and 2c.

The quinone system is also maintained, which could be observed at the 13C NMR spectra (Table 1, C-1 to C4 and C-4a and 8a). The attached proton test (APT) experiment allowed to distinguish carbon type, where it was possible to confirm the six aromatic carbons, four quaternary and two methinic. The HSQC showed that the aromatic protons at δ_{H} 7.48 and 7.93 ppm correspond to the carbons at δ_{C} 136.32 and 125.5 ppm, respectively, and the rearranged methyl group at δ_{H} 2.42 ppm in the 1'H NMR spectrum corresponds to the carbon signal at δ_{C} 20.4 ppm. The HMBC spectrum allowed us to place the chlorinated side chain and the rearranged methyl group at the correct positions by selected J and J_C,H correlations. Figure 2 shows the selected J and J_C,H correlations for the methyl protons at δ_{H} 2.42 ppm and the methylene protons at δ_{H} 3.11 ppm. The protons of the methyl group at δ_{H} 2.42 ppm present correlations with the carbons of the naphthoquinone moiety, C-8a and C-7, the latter being the one bearing the hydrogen at δ_{H} 7.48 ppm. The selected correlations between the methylene protons at δ_{H} 3.11 ppm and the other two naphthoquinone quaternary carbons (C-5 and C-4a) place the side chain at carbon C-5.

**Leishmanicidal activity of (2) and (3)**

Recently, activity against *Mycobacterium tuberculosis*, *Leishmania amazonensis*, *Staphylococcus aureus* and *Caenorhabditis elegans* was demonstrated for the essential oil from *T. riparia* and some of its isolated constituents. Here, we evaluated the activity of 7α-hydroxyroyleanone (1), extracted from *T. riparia* leaves and the product of its dehydration reaction (2), against *Leishmania amazonensis*, a parasite that can cause different clinical disease manifestations.

![Figure 1. Chemical conversion of (1) to a new naphthoquinone (2) and 6,7-dehydroroyleanone (3).](image-url)
such as cutaneous leishmaniasis, which may evolve to the severe anergic diffuse cutaneous disease, and the visceral leishmaniasis as well.\textsuperscript{18-21}

Our results demonstrated that the diterpenequinone (1) exhibits a dose dependent anti-\textit{Leishmania} promastigote activity with half maximal inhibitory concentration (IC\textsubscript{50}) of 2.96 μg mL\textsuperscript{-1} (Table 2). Compound (2) also showed dose-dependent leishmanicidal activity with an IC\textsubscript{50} of 8 μg mL\textsuperscript{-1} (Table 2). Our data show that (1) presents similar anti-promastigote activity to that of 6,7-dehydroroyleanone (3), previously isolated from the essential oil of the same plant by Demarchi et al.\textsuperscript{14} (IC\textsubscript{50} of 2.45 μg mL\textsuperscript{-1}), which is one of the products of the dehydration reaction (compound 3), while (2) was 3.2 folds less powerful. The cytotoxicity evaluation through the dehydrogenase enzymes activity test has shown that (1) and (2) have similar toxicity, with half maximal cytotoxicity concentration (CC\textsubscript{50}) of 4 and 3.1 μg mL\textsuperscript{-1} (Table 2), which were higher than those observed for the essential oil and 6,7-dehydroroyleanone from \textit{T. riparia},\textsuperscript{14} 0.17 and 0.53 μg mL\textsuperscript{-1}, respectively, obtained by Demarchi et al.\textsuperscript{14} Evaluating anti-\textit{Leishmania} activity against intracellular amastigote forms, we demonstrated a dose-dependent effect of 7α-hydroxyroyleanone with an IC\textsubscript{50} of 1.36 μg mL\textsuperscript{-1}, an activity well above that demonstrated by 2, which significantly inhibited the amastigotes only at 9.8 μg mL\textsuperscript{-1}. These results point to 7α-hydroxyroyleanone as a more potent compound than the reaction product (2). Moreover, the latter presents similar activity to 6,7-dehydroroyleanone (3),\textsuperscript{14} while 7α-hydroxyroyleanone was 7.3 folds more powerful. The selectivity index (SI) calculated for 7α-hydroxyroyleanone (1), and the reaction product (2), were 1.35 and 0.38 respectively, (Table 2). These selectivity indexes (SI) were lower than 5.67 calculated for the essential oil from \textit{Tetradenia riparia}.\textsuperscript{14} On the other hand, the SI of 7α-hydroxyroyleanone (1), was better than that observed for 6,7-dehydroroyleanone (3) (1.35 versus 0.22, respectively).

\section*{Conclusions}

The reaction performed with the diterpenequinone 7α-hydroxyroyleanone (1), showed it to be very reactive, suggesting that (1) may constitute a starting material for a series of derivatives for novel bioactive compounds. The

\begin{table}[h]
\centering
\begin{tabular}{lcccc}
\hline
Position & $^1$H δ / ppm (J / Hz) & $^{13}$C δ / ppm & \\
\hline
1 & – & 184.42 & \\
2 & – & 126.48 & \\
2a & 3.35, sept, (7.2) & 24.33 & \\
2b & 1.27, d, (7.2) & 19.80 & \\
2c & 1.27, d, (7.2) & 19.80 & \\
3 & – & 153.06 & \\
4 & – & 183.19 & \\
4a & – & 143.01 & \\
5 & – & 126.06 & \\
5a & 3.11, t, (8) & 30.15 & \\
5b & 1.67, m & 24.70 & \\
5c & 1.96, m & 46.33 & \\
5d & – & 70.90 & \\
5e & 1.58, s & 32.41 & \\
5f & 1.58, s & 32.41 & \\
6 & 7.93, d, (8) & 125.50 & \\
7 & 7.48, d, (8) & 136.32 & \\
8 & – & 133.24 & \\
8a & – & 144.40 & \\
8b & 2.42, s & 20.26 & \\
\hline
\end{tabular}
\caption{$^1$H and $^{13}$C NMR spectroscopic data of (2) in CDCl\textsubscript{3}.}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{lcccc}
\hline
Compound & Promastigotes IC\textsubscript{50} (μg mL\textsuperscript{-1}) & Peritoneal macrophages CC\textsubscript{50} (μg mL\textsuperscript{-1}) & Amastigotes IC\textsubscript{50} (μg mL\textsuperscript{-1}) & Selectivity index \\
\hline
1 & 2.96 ± 0.29 & 4.0 ± 0.15 & 1.36 ± 0.4 & 1.35 \\
2 & 8.0 ± 0.15 & 3.1 ± 0.14 & 9.8 ± 0.32 & 0.38 \\
\hline
\end{tabular}
\caption{Anti-\textit{Leishmania amazonensis} and macrophage cytotoxicity effects of (1) and (2)}
\end{table}

\textsuperscript{18}Measured at 400 MHz; $^a$measured at 100 MHz.
counter-current chromatography (CCC) technique was a valuable tool for purifying the reaction mixture, providing the main product in some quick purification steps. Our results contribute to pointing out an interesting candidate for future assessments of its potential as a leishmanicidal agent and may favor the development of more effective therapies.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as a PDF file.

Acknowledgments

This work was supported by Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Finance Code 001. One of the authors (AGL) is thankful to CAPES for a scholarship.

References

1.  Coopoosamy, R. M.; Naidoo, K. K.; Afr. J. Microbiol. Res. 2011, 5, 2942.
2.  Leitão, F.; Fonseca-Kruel, V. S.; Silva, I. M.; Reinert, F.; Braz. J. Pharm. 2009, 19, 333.
3.  Baldin, V. P.; Scodro, R. B. L.; Lopes-Ortiz, M. A.; Almeida, A. L.; Gazim, Z. C.; Ferarrese, L.; Faioes, V. D. S.; Torres-Santos, E. C.; Pires, C. T. A.; Caleffi-Ferracioli, K. R.; Siqueira, V. L. D.; Cortez, D. A.G.; Cardoso, R. F.; Pires, T. A.; Caleffi-Ferracioli, K. R.; Gazim, Z. C.; Curr. Microbiol. 2018, 74, 1453.
4.  Leitão, G. G.; Figueiredo, F. S.; Dantas, S. W. R. M.; Groll, A.; Silva, R. S. F.; Silva, P. E. A.; Planta Med. 2012, 78, 1267.
5.  Liu, D.; Nanjing Zelang Medical Technology Co., Ltd., CN102826988 (A), 2012.
6.  Milato, J. V.; Silva, R. S. F.; Figueiredo, F. S.; Azevedo, D. A.; Ribeiro, C. A. B.; Leitão, G. G.; J. Chromatogr. A 2018, 1537, 135.
7.  Alvar, J.; Velez, I. D.; Bern, G.; Herrero, M.; Desjeux, P.; Cano, J.; Jannin, J.; Boer, M.; PLoS One 2012, 7, e35671.
8.  Bekhit, A. A.; El-Agroudy, E.; Helmy, A.; Ibrahim, T. M.; Shavandi, A.; Bekhit, A. E. A.; Eur. J. Med. Chem. 2018, 160, 229.
9.  Ghorbani, M.; Farhoudi, R.; Drug Des., Dev. Ther. 2017, 12, 25.
10.  Tezuka, Y.; Kasimu, R.; Li, J. X.; Basnet, P.; Tanaka, K.; Namba, T.; Kadota, S.; Chem. Pharm. Bull. 1998, 46, 107.
11.  Roehm, N. W.; Rodgers, G. H.; Hatfield, S. M.; Glasebrook, A. L.; J. Immunol. Methods 1991, 142, 257.
12.  Demarchi, I. G.; Thomazella, M. V.; Terron, M. S.; Lopes, L.; Gazim, Z. C.; Cortez, D. A.; Donatti, L.; Aristides, S. M.; Silveira, T. G.; Lonardoni, M. V.; Exp. Parasitol. 2015, 157, 128.
13.  Demarchi, I. G.; Terron, M. S.; Thomazella, M. V.; Mota, C. A.; Gazim, Z. C.; Cortez, D. A.; Aristides, S. M.; Silveira, T. G.; Lonardoni, M. V.; Parasite Immunol. 2016, 38, 64.
14.  Fernandez, A. C. A. M.; Rosa, M. F.; Fernandez, C. M. M.; Bortolucci, W.; Melo, U. Z.; Siqueira, V. L. D.; Cortez, D. A. G.; Gonçalves, J. E.; Linde, G. A.; Gazim, Z. C.; Curr. Microbiol. 2017, 74, 1453.
15.  Puyvelde, L.; Liu, M.; Veryser, C.; Borggraewe, W. M.; Muganulire, J.; Mukazayire, M. J.; Luyten, W.; J. Ethnopharmacol. 2018, 216, 229.
16.  Barral, A.; Pedral-Sampaio, D.; Grimaldi Jr., G.; Momen, H.; Mcmahon-Pratt, D.; Jesus, A. R.; Almeida, R.; Badaro, R.; Barral-Netto, M.; Carvalho, E. M.; Johnson Jr., W. D.; Am. J. Trop. Med. Hyg. 1991, 44, 536.
17.  Silveira, F. T.; Lainson, R.; Corbett, C. E.; Mem. Inst. Oswaldo Cruz 2004, 99, 239.
18.  Alexio, J. A.; Nascimento, E. T.; Monteiro, G. R.; Fernandes, M. Z.; Ramos, A. M. O.; Wilson, M. E.; Pearson, R. D.; Jeronimo, S. M. B.; Trans. R. Soc. Trop. Med. Hyg. 2006, 100, 79.
19.  Souza, C. S. F.; Calabrese, K. S.; Abreu-Silva, A. L.; Carvalho, L. O. P.; Cardoso, F. O.; Dorval, M. E. M. C.; Oshiro, E. T.; Quaresma, P. F.; Gontijo, C. M. F.; Pacheco, R. S.; Rossi, M. I. D.; Costa, S. C. G.; Valle, T. Z.; Histol. Histopathol. 2018, 33, 705.

Submitted: April 2, 2019
Published online: September 17, 2019