UNIVERSIDADE ESTADUAL PAULISTA

FACULDADE DE ENGENHARIA

CAMPUS DE ILHA SOLTEIRA – FEIS

Texto Sistemático Crítico Evidenciando a Evolução das Pesquisas Realizadas e o Seu Contexto Cronológico na Área de Produtos Naturais e de Síntese Orgânica.

Livre Docência

Rosangela da Silva de Laurentiz

Grupo de Síntese Orgânica e Produtos Naturais

Ilha Solteira 2020

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Rosangela da Silva de Laurentiz

Texto Sistemático Crítico Apresentado à Faculdade de Engenharia de Ilha Solteira da Universidade Estadual Paulista – UNESP, para o Concurso de Livre Docência junto ao Departamento de Física e Química na disciplina de Química Orgânica

Grupo de Síntese Orgânica e Produtos Naturais

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Dedicatória

Dedico a Deus pelo dom da vida e pelas bençãos recebidas

Dedico ao meu marido Antonio e aos meus filhos Lucas e Gabriel por fazerem parte da minha vida, pelo apoio e amor incondicional.

Dedico aos meus pais Benedito da Silva e Aparecida Paes da Silva por me ensinarem os reais valores da vida, pela educação, apoio e amor em todos os momentos.

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Sumário

1. Resumo

As plantas são uma fonte inesgotável de compostos orgânicos com as mais variadas estruturas químicas e propriedades biológicas. Esses compostos são chamados de metabólitos secundários e estão envolvidos no metabolismo secundário das plantas sendo produzidos em resposta a agentes externos como predadores, parasitas, outras plantas, fatores ambientais e de solo (nutrientes). A diversidade de propriedades biológicas que eles apresentam nos vegetais chama a atenção para uma possível aplicação no controle de algumas enfermidades que afetam também humanos e animais.

Dentre as principais classes de metabólitos secundários produzidos pelas plantas as lignanas passaram a ser alvo de estudo de vários grupos de pesquisa depois da descoberta das propriedades anticâncer da podofilotoxina. Desde então, outras lignanas foram isoladas, identificadas e avaliadas quanto às suas propriedades biológicas. Tais propriedades estão estritamente relacionadas aos esqueletos químicos de cada subclasse, estereoquímica, natureza e posição dos substituintes. Devido às propriedades biológicas e ao desafio sintético relacionado aos centros assimétricos, inúmeras metodologias para a síntese de lignanas e de derivados têm sido propostas com o objetivo de obter moléculas que possam ser utilizadas como alvos para o desenvolvimento de novos fármacos. Com o avanço científico, ferramentas de modelagem molecular aliadas a ensaios *in vitro* vem facilitando o planejamento dos alvos sintéticos, guiando a síntese para a obtenção de compostos com maior probabilidade de serem ativos *in vivo* contra o alvo biológico avaliado *in sílico*.

No decorrer da minha pesquisa tenho trabalhado com extratos de plantas, lignanas naturais, síntese total de lignanas, síntese parcial de derivados de lignanas naturais, síntese de análogos heterocíclicos de lignanas e com a avaliação biológicas desses compostos. Para alguns alvos biológicos a modelagem molecular tem sido utilizada como guia para definir o tipo de composto a ser sintetizado e avaliado *in vivo*. Tenho usado os frutos da *Piper cubeba* para obtenção da lignana cubebina a qual foi avaliada em vários ensaios biológicos e usada como material de partida para a síntese de derivados e de outras lignanas. A partir dessa lignana publiquei muitos dos meus artigos científicos e ainda hoje esse composto continua sendo fonte de inspiração para outros dois projetos que desenvolvo. As lignanas também tem sido inspiração para a síntese de análogos heterocíclicos que possuem interessantes propriedades biológicas e luminescentes.

Neste texto, comento os artigos científicos vinculados tanto à área de produtos naturais quanto à área de síntese que publiquei nos últimos anos com o intuito de evidenciar a evolução das pesquisas realizadas e o seu contexto cronológico.

Abstract

Plants are an inexhaustible source of organic compounds with the most varied chemical structures and biological properties. These compounds are called secondary metabolites and are involved in the secondary metabolism of plants being produced in response to external agents such as predators, parasites, other plants, environmental factors and soil (nutrients). The diversity of biological properties that they present in plants calls attention to a possible application in the control of some diseases that also affect humans and animals. Among the main classes of secondary metabolites produced by plants, lignans became the target of study by several research groups after the discovery of the anticancer properties of podophyllotoxin. Since then, other lignans have been isolated, identified and evaluated for their biological properties. Such properties are strictly related to the chemical skeletons of each subclass, stereochemistry, nature and position of the substituents. Due to the biological properties and the synthetic challenge related to asymmetric centers, numerous methodologies for the synthesis of lignans and derivatives have been proposed in order to obtain molecules that can be used as targets for the development of new drugs. With scientific advances, molecular modeling tools combined with in vitro assays have facilitated the planning of synthetic targets, guiding the synthesis to obtain compounds with greater probability of being active in vivo against the biological target evaluated in silico. In the course of my research, I have worked with plant extracts, natural lignans, total synthesis of lignans, partial synthesis of derivatives of natural lignans, synthesis of heterocyclic analogues of lignans and the biological evaluation of these compounds. For some biological targets, molecular modeling has been used as a guide to define the type of compound to be synthesized and evaluated in vivo. I have used the fruits of *Piper cubeba* to obtain the cubebin lignan which has been evaluated in several biological assays and used as a starting material for the synthesis of derivatives and other lignans. From this lignan I published many of my scientific articles and even today this compound continues to be a source of inspiration for two other projects that I develop. Lignans have also been the inspiration for the synthesis of heterocyclic analogues that have interesting biological and luminescent properties. In this text, I comment on the scientific articles linked to both the area of natural products and the area of synthesis that I have published in recent years with the aim of highlighting the evolution of the research carried out and its chronological context

2. Estudos com extratos de plantas e suas propriedades biológicas

Extratos de plantas apresentam em sua composição metabolitos secundários que podem atuar sobre diversos alvos biológicos de forma individual, em sinergismo com outros metabolitos ou ainda por efeito somatório de vários metabolitos ativos. A composição fitoquímica desses extratos pode guiar os ensaios biológicos mais adequados para a avaliação biológica. Por exemplo, extratos ricos em flavonoides são candidatos a avaliação da atividade antioxidante, atividade anti-inflamatória e analgésica, enquanto extratos com altas concentrações de alcaloides podem ser avaliados em ensaios sobre o sistema nervoso central (analgesia e sedação) e taninos em ensaios de atividade anti-helmíntica.

Meu primeiro contato com extratos de planta se deu com a *Piper cubeba* no meu Pós Doutorado e depois no Jovem Pesquisador. Na UNESP passei a trabalhar também com outras plantas e dentre elas o *Psidium Cattleianum* Sabine (araçá) e a *Opuntia fícus* indica (figo da Índia) com as quais publiquei os trabalhos que apresento nesse tópico.

2.1. In vivo analgesic activity, toxicity and phytochemical screening of the hydroalcoholic extract from the leaves of Psidium cattleianum Sabine

Essa planta é utilizada na medicina tradicional por várias comunidades no Brasil e em outros países para várias doenças e também como analgésico, entretanto seu uso como analgésico ainda não havia sido provado cientificamente. Essa planta foi alvo do estudo de um aluno de Mestrado em Biotecnologia da UNIMONTES do qual fui coorientadora. Um estudo fitoquímico da planta indicou a presença de substâncias com potencial ação antioxidante, analgésico e anti-helmíntico. Essa composição fitoquímica aliada ao uso tradicional da planta também guiou nossos estudos.

Neste trabalho investigamos a atividade analgésica *in vivo* do extrato hidroalcóolico das folhas do araçá e determinamos sua toxicidade com a finalidade de validar seu uso tradicional. Neste trabalho também foi avaliada a atividade antioxidante do extrato. Os ensaios *in vivo* confirmaram ação analgésica periférica do extrato que pode estar relacionada à presença de substâncias antioxidantes atuando na inibição de mediadores periféricos. No modelo de DPPH o extrato apresentou atividade antioxidante semelhante à atividade observada para o ácido ascórbico. Vários estudos na literatura demonstram a relação entre radicais livres e a dor e que substâncias antioxidantes têm a capacidade de eliminar esses radicais livres e, assim, evitar danos oxidativos intracelulares que resultam em dor. O estudo fitoquímico mostrou a presença de várias classes de metabólitos secundários com propriedades antioxidantes. O extrato não apresentou toxicidade sobre fibroblastos de acordo com o modelo de ensaio utilizado. Em conclusão, o extrato apresentou atividade analgésica,

de acordo com os modelos de atividade analgésica *in vivo* utilizados, e esses resultados dão suporte ao uso tradicional desta planta.

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In vivo analgesic activity, toxicity and phytochemical screening of the hydroalcoholic extract from the leaves of *Psidium cattleianum* Sabine

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ABSTRACT

Ethnopharmacological relevance: *Psidium cattleianum* Sabine is extensively used in Brazilian traditional medicine to treat several diseases including painful disorders. *Aim of the study* to investigate the toxicity and the possible analgesic activities of the hydroalcoholic extract from the leaves of *Psidium cattleianum* Sabine (ELPCS), to support its use in folk medicine. To screen the major phytochemical constituents of this extract and evaluate their antioxidant activity.
Materials and methods: ELPCS was assessed for its antioxidant activity using the DPPH model. Its analgesic activity was examined using mouse models of acetic acid-induced writhing and hot plate paw licking models. The major phytochemical constituents of the extract were screened; their toxicity on LLC-MK2 mammalian cells was evaluated.
Results: ELPCS exhibited significant peripheral analgesic activity at doses of 60, 80, 100, 200 and 400 mg/kg in mice, but it did not display central analgesic activity and not was toxic to LLC-MK2 cell ($LD_{50} > 400$ μg/mL). The extract exhibited free radical scavenging activity as evidenced by IC_{50} values (15.9 μg/mL) obtained by the DPPH method. Phytochemical screening detected flavonoids, saponins, cardiac glycosides, anthraquinones, and tannins.
Conclusions: The results of the experimental studies proved the analgesic activity of ELPCS and supported the traditional use of this plant.

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1. Introduction

Psidium cattleianum Sabine (PCS) belonging to the *Myrtaceae* family is native to Brazil and occurs in the Atlantic Forest, especially in dense rain forest, restinga, and the Southern Plateau, but it can also be found in several other countries. Popularly known in Brazil as araça-rosa, araça-vermelho, or araça do campo, it is a medium-sized shrub measuring no more than nine feet tall. PCS produces ovoid or oblong fruit weighing less than 20 g; the fruit can be yellow or red. The fruits has white pulp and tart taste, is rich in vitamin C (Luximon-Ramma et al., 2003; Lapcik et al., 2005; Pino et al., 2001), and contains a large amount of phenolic compounds such as epicatechin and gallic acid as the main component (Medina et al., 2011). Recent studies have showed that the PCS extracts potentially reduce metastasis of lung cancer cells (Im et al., 2012; Jun et al., 2011), display antimicrobial activity (Brighenti et al., 2008; Desoti et al., 2011; Gaetti-Jardim et al., 2011; Souza et al., 2004), and exert anti-caries effects in rats (Menezes et al., 2010). PCS is used in folk medicine in French Polynesia and Brazil for several use as adstringent, hepatoprotective, antidiarrheal e analgesic (Hanazaki et al., 2000; Pétard, 1986; Ritter et al., 2002; Vendruscolo et al., 2005; Vendruscolo and Mentz, 2006). The leaves of PCS are used as tea, directly by chewing, kept in cold water or ethanol for topical use of the plant or as drink for relief of pains as toothache, bellyache, throat pain, and abdominal pain (Pétard, 1986; Vendruscolo et al., 2005; Vendruscolo and Mentz, 2006).

Despite the publications on other biological activities of PCS there is no scientific evidence on activities related to traditional use as analgesic. Therefore, it is considered important to study the analgesic activity of hydroalcoholic extract from the leaves of PCS as well as determining its main constituents to provide scientific support to the safe use of the plant for this purpose.

2. Methodology

2.1. Plant material

Psidium cattleianum Sabine specimens were collected in Glaucilândia (Minas Gerais, Brazil) and identified by Prof. Dr. Rubens

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Manoel dos Santos in UEMC (Universidade Estadual de Montes Claros), Minas Gerais. A voucher specimen was deposited in the UEMC herbarium under number 3533. The material was dried naturally in the shade for 96 h, pulverized, stored in paper bags, and refrigerated at −10 °C.

2.2. Plant extracts

The hydroalcoholic extract was prepared by maceration exhaustive method in which 100 g of powdered leaf were placed in 1000 mL of ethanol/water (7:3 v/v). The extract was stored in the dark at room temperature for 7 days, with occasional shaking. The extract was filtered, evaporated and refrigerated at −10 °C. This extraction process was repeated with the residue for another three times (yield 12.15%. w/w).

2.3. Preliminary phytochemical analysis

Plant materials were screened for the presence of alkaloids, saponins, tannins, total phenols, anthraquinones, flavonoids, and sterols using the methods previously described by Tona et al. (1998).

2.4. Toxicity to mammalian cells

The LLC-MK2 fibroblast cells were grown in RPMI 1640 medium supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 5% of inactivated fetal calf serum, and maintained at 37 °C in 5% CO_2. A cell suspension was seeded at a concentration of 1 × 106 cells/mL in a 96-well microplate containing RPMI 1640 medium. Thereafter, the cells were treated with ELPCS at different concentrations (6.25, 12.5, 25, 50, 100, 200, and 400 μg/mL). The plates were incubated at 37 °C for 24 h, and the biological activity was evaluated by using the MTT colorimetric method [MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] in a microplate reader at 540 nm. RPMI 1640 medium plus DMSO and RPMI 1640 medium were used as positive and negative controls, respectively (Twentyman and Luscombe, 1987). All the experiments were performed in triplicate. The percentage of cell viability was determined by the formula: % cell viability $= 1 - [(Y-N)/(N-P)] \times 100$, where Y = absorbance of wells containing cells and ELPCS at different concentrations; N = negative control; P = positive control.

2.5. Antioxidant assay

To measure the antioxidant activity of the extract and standards (gallic acid and ethanol), the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay was carried out according to the procedure described previously (Blois, 1958), with slight modifications. Briefly, the DPPH radical scavenging activity was measured in a reaction mixture containing 0.1 mL of 1 mM DPPH radical solution, 0.8 mL of ethanol 99%, and 0.1 mL of ELPCS (in methanol). The same mixture was used for the standards. The solution was rapidly mixed, and the scavenging capacity was measured spectrophotometrically by monitoring the decrease in the absorbance at 517 nm. The antioxidant activity was expressed as IC_{50}, which was defined as the concentration of the ELPCS required to inhibit the formation of DPPH radicals by 50%.

2.6. Animals

Male albino mice (20–25 g) were used for the experimental study. The animals were maintained under standard husbandry conditions in polypropylene cages with water *ad libitum* and food until 8 h before the experiment. All the procedures were approved by the Ethics Committee for Animal Use of Universidade Estadual Paulista FEIS, Brazil (no. 001/2012) and were carried out in accordance with international guidelines for the care and use of laboratory animals, published by the US National Institute of Health.

2.7. Analgesic activity

2.7.1. Acetic acid-induced writhing in mice

The assay was performed as described by Koster et al. (1959). The nociceptive effect caused by injection of acetic acid was detected by observing abdominal writhing associated with stretching of the whole body. Animals were treated with different doses of ELPCS (60, 80, 100, 200, and 400 mg/kg, p.o.), saline, or indomethacin (5 mg/kg), which was used as a reference compound. Thirty minutes after treatment, all the animals received 0.6% acetic acid i.p.; 10 min later, the number of abdominal constrictions was recorded for 20 min, by visual observation of the animals.

2.7.2. Hot plate method

This method was used to evaluate the central analgesic activity (delay in the latency of pain response) according to a procedure described earlier (Eddy and Leimback, 1953). The mice were treated with different doses of ELPCS (100, 200, and 400 mg/kg, p.o.). After 30 min, the animals were placed on a hot plate maintained at 55 ± 0.5 °C. The reaction time was recorded as the time taken by the animals to blow or lick the fore or hind paw or jump off the plate. A 5% Tween saline solution was used as normal control; morphine (5 mg/kg) was employed as reference drug.

3. Data analysis

The results were statistically analyzed by analysis of variance (ANOVA) followed by the Tukey test, with significant differences being considered if $p < 0.05$. All values are presented as mean ± - standard error of the mean (SEM). The level of significance used in analysis of the data was less than 0.05 ($p < 0.05$).

4. Results

4.1. Acetic acid-induced writhing method

ELPCS presented significant peripheral analgesic activity at all the evaluated doses (Fig. 1). The inhibition percentage of the number of writhing of acetic acid-induced writhing in mice was 86, 91, 81, 99, and 73% at doses of 60, 80, 100, 200, and 400 mg/kg, respectively. The smallest dose of ELPCS showed significant analgesic activity with 86% ($p < 0.0001$) inhibition of acetic acid writhing compared to control, but the effect was lower than produced by indomethacin (99%). An amount of 200 mg/kg ELPCS showed the highest analgesic activity with 99% ($p < 0.0001$), same value presented by standard drug indomethacin. The inhibitory effect of the extract is not dose-dependent: increasing the extract dose from 200 to 400 mg/kg reduces the inhibition percentage from 99 to 73%.

4.2. Hot plate test

The hot plate tests were realized with the ELPCS at the doses 100, 200, and 400 mg/kg. The results shown in Fig. 2, demonstrated that there is no significant differences between the ELPCS-treated mice and the control group. Hence, we assume that ELPCS has no analgesic effect on the central nervous system that would contribute to its peripheral analgesic effect.

4.3. Phytochemical study

The phytochemical study did not detect any alkaloids but identified high concentrations of saponins, flavonoids, cardiac glycosides, anthraquinones, and tannins.

4.4. Toxicity to mammalian cells

The cultures of LLC-MK2 mammalian fibroblast cells were treated with ELPCS at concentrations of 6.25, 12.5, 25.0, 50.0, 100, 200 and 400 μg/mL for 24 h. The viability of the cultures was determined by establishing a relation between the absorbance values obtained in the treated and untreated (control) groups, as shown in Fig. 3. No significant cytotoxicity was observed in the concentrations evaluated after 24 h of treatment, only control 25% DMSO display significant toxicity with $p < 0.05$. These results showed that the ELPCS no present toxicity at concentration evaluated with $IC_{50} > 400$ μg/mL.

4.5. Antioxidant activity

DPPH is a free radical compound that is widely used to test the free radical scavenging ability of various samples. Antioxidants interacting with DPPH, they can either transfer an electron or hydrogen atom to DPPH, thus neutralizing its free radical character. The color of the reaction changes from purple to yellow, which can be quantified by the change in the absorbance at 517 nm. The ELPCS was capable of scavenging DPPH radicals in a concentration-dependent manner. The scavenging effect on DPPH radicals increased with increasing concentrations from 3.3 to 13 μg/mL, respectively, with values of 14 and 41%. The ELPCS displayed $IC_{50} = 15.9$ μg/mL, whereas the value of IC_{50} found for gallic acid was 4.8 μg/mL, these results shown that DPPH radical scavenging activity of the ELPCS, though lower than gallic acid, is very satisfactory, indicating that their compounds may contribute to neutralize the oxidant agents produced during pain and inflammatory states.

5. Discussion

The analgesic activities of ELPCS were evaluated by two mouse models, which could provide response to two different grades of noxious stimuli including thermal stimulus and chemically induced tissue damage (Zhou et al., 2008). The classification of antinociceptive drugs is usually based on their mechanism of action; these drugs can act on either the peripheral nervous system or the central nervous system (Planas et al., 2000).

The acetic acid writhing test is a standard sensitive test for both opioid and non-opioid analgesics (Steranka et al., 1987), and the

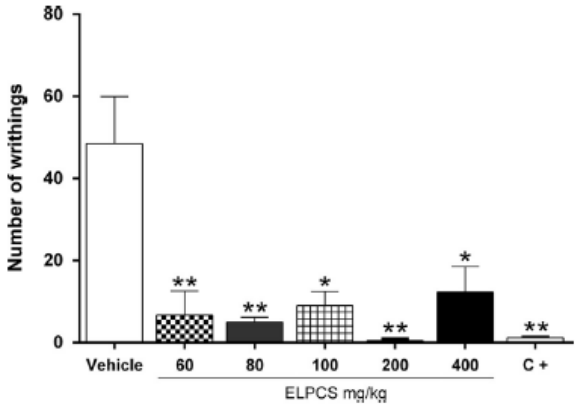

Fig. 1. Effects of ELPCS on the acetic acid-induced writhing assay. Vehicle (control), ELPCS (60, 80, 100, 200, and 400 mg/kg), indomethacin (C+, 5 mg/kg) were administered i.p. 0.5 h before acetic acid injection. Each column represents mean ± SEM ($n=6$). *$p < 0.001$ **$p < 0.0001$ vs. Control (ANOVA followed by Tukey's test).

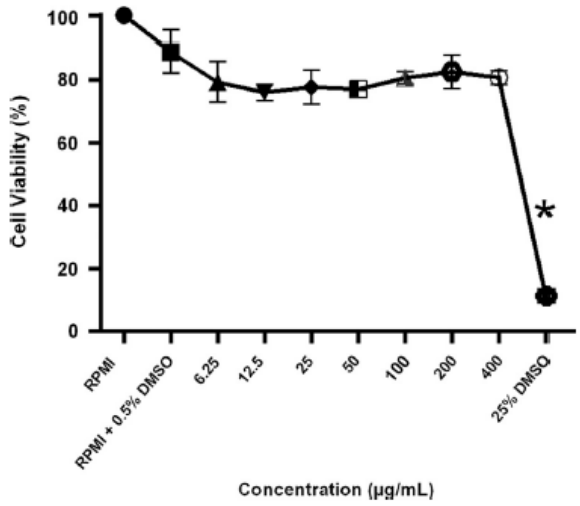

Fig. 3. Effects of ELPCS on the viability of LLC-MK2 mammalian fibroblast cells. The percentage cell viability was determined using MTT assay after 24 h treatment with the indicated concentrations. Values are expressed by mean ± S.D. *$p < 0.05$ vs. control indicates statistically significant differences (ANOVA followed by Tukey's test).

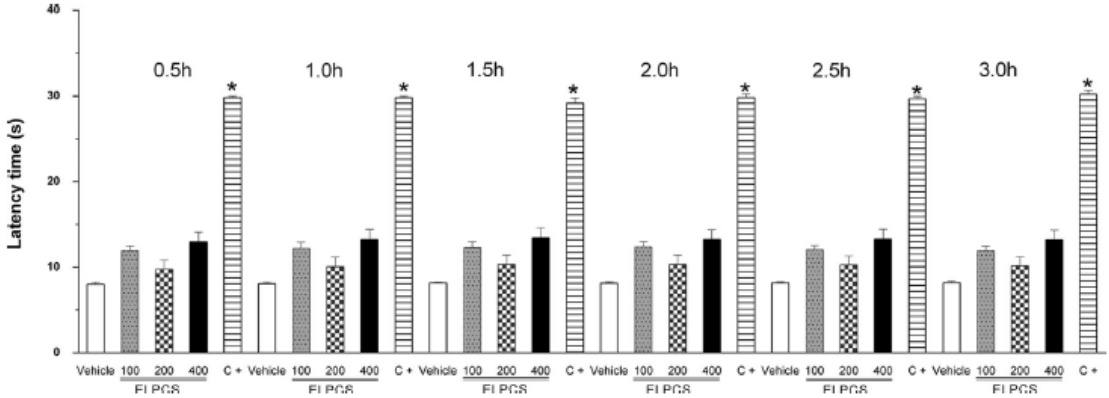

Fig. 2. Antinociceptive effect of ELPCS or morphine (C+) on the hot plate test in mice. Each column represents mean ± SEM ($n=6$). *$p < 0.001$ vs. Control (ANOVA followed by Tukey's test).

most commonly used for screening peripheral analgesics (Bentley et al., 1981; Derardt et al., 1980; Habib and Waheed, 2013; Wang et al., 2013). When animals are intraperitoneally injected with acetic acid, a painful reaction and acute inflammation emerge in the peritoneal area. The stimulation of peritoneal nociceptors is indirect and occurs with the release of endogenous substances, which stimulate nervous endings (Berkenkopf and Weichman, 1988; Gyires and Torna, 1984). In this study, ELPCS significantly inhibited the number of writhing responses in mice intraperitoneally injected with acetic acid in all concentration evaluated, showing a significant anti-nociceptive activity. However, this writhing test alone does not substantiate the non-central analgesic effect of ELPCS, as central analgesic drugs such as morphine also display a potent analgesic effect in the writhing test. The hot-plate test, when associated with the writhing test, can usually distinguish the central from peripheral effects (Srinivasan et al., 2003). Hot-plate test is one of the most common tests of nociception that are based on a phasic stimulus of high intensity (Mandegary et al., 2004). Pain induced by thermal stimulus of the hot-plate is specific for centrally mediated nociception (Magaji et al., 2008). Opioid analgesics, as well as drugs of predominantly central action, such as tricyclic antidepressants and antiepileptic drugs, increase the latency time of the animals on the hot plate, a result that is interpreted as antinociception (Freitas et al., 2009). Our results exhibited that ELPCS insignificantly prolonged latency period of mice in the hot-plate test, suggesting its non-central analgesic activity. The effective peripheral analgesic activity displayed by ELPCS is probably mediated via the inhibition of peripheral mediators which could be related to its antioxidant effect observed *in vitro*. The DPHH model demonstrated that ELPCS exerted significant antioxidant activity with $IC_{50} = 15.9$ μg/mL. Recent studies have shown that free radicals are responsible for mechanisms that cause inflammation and pain, and that high levels of ROS can induce pain. ROS are also involved in persistent pain, including neuropathic and inflammatory pain (Fidanboylu et al., 2011; Kumar, 2011; Yowtak et al., 2011) are generated from many redox processes and are known to be major free radicals in the human body. Antioxidants substances have the ability to scavenge these free radicals and thus prevent intracellular oxidative damage that result in pain (Valko et al., 2007). The pharmacological inhibition of ROS was reported to have anti-nociceptive effects in neuropathic and inflammatory pain models (Gao et al., 2007).

The phytochemical study did not detect any alkaloids but identified high concentrations of saponins, flavonoids, cardiac glycosides, anthraquinones, and tannins. The presence of saponins and flavonoids can be directly related to peripheral analgesia elicited by ELPCS (Cody et al., 1985; Mutalik et al., 2003; Owoyele et al., 2008; Zayachkivska et al., 2005). However, the central analgesia promoted by many plant extracts is generally attributed to alkaloids (Almeida et al., 2001), which were not detected in the EPLPCS which could explain the absence of central analgesia of the extract.

In addition, we also evaluated the toxicity of ELPCS using MTT assays on LLC-MK2 mammalian fibroblast cells. This test provides important information on the toxic effect of chemical compounds in direct contact with the cell culture. Viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance maximum near 570 nm. When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells. The exact cellular mechanism of MTT reduction into formazan is not well understood, but likely involves reaction with NADH or similar reducing molecules that transfer electrons to MTT (Marshall et al., 1995). It is one of the most commonly used tests for determining cytotoxicity of drugs (Riss et al., 2013). In this assay the ELPCS no showed toxicity at the concentrations evaluated with $IC_{50} > 400$ μg/mL.

In conclusion, ELPCS exhibits strong analgesic effect and no toxicity, which justifies the traditional use of this plant to treat some pain disorders.

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2.2. Ovicidal and larvicidal activity of extracts of Opuntia ficus-indica against gastrointestinal nematodes of naturally infected sheep

As plantas apresentam uma grande quantidade de substâncias com potencial anti-helmíntico, por isso é crescente o número de trabalhos utilizando plantas como alternativas ao uso de anti-helmínticos sintéticos para o controle da parasitose em ovinos. A escolha da planta para esse estudo teve como base à grande quantidade de taninos e flavonoides (substâncias com propriedades anti-helmínticas) presentes nos extratos dos cladódios e da polpa dos frutos da *Opuntia ficus* indica, além do fato da planta também ser fornecida aos animais como alimento em períodos de seca em algumas regiões do Brasil.

Neste trabalho, o extrato bruto dos cladódios, da polpa dos frutos e frações de ambos foram submetidos a ensaios de eclodibilidade de ovos, desenvolvimento larval e ensaios de migração larval utilizando ovos e larvas de nematoides gastrointestinais obtidos de animais doadores com contaminação natural sendo 95% *Haemonchus contortus*. Dentre todos os extratos e frações, as frações residuais aquosas obtidas ao final dos fracionamentos dos extratos brutos dos cladódios e dos frutos foram as mais ativas em todos os ensaios. Os resultados do trabalho demonstraram que os extratos dos cladódios e da polpa dos frutos apresentaram atividade anti-helmíntica, reforçando o interesse nesta planta com o objetivo de aliar seu potencial nutricional a um possível efeito anti-helmíntico *in vivo*. A ação anti-helmíntica apresentada pela planta pode ter relação com a presença de taninos, pois as frações mais ativas foram aquelas em que os taninos são mais solúveis. A dificuldade em se trabalhar com extratos é justamente tentar relacionar a atividade biológica apresentada pelos extratos e frações a seus componentes, ou seja, determinar se o feito biológico observado é devido a um único composto, ao sinergismo entre vários compostos ou efeito somatório de vários componentes ativos.

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Short communication

Ovicidal and larvicidal activity of extracts of *Opuntia ficus-indica* against gastrointestinal nematodes of naturally infected sheep

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ABSTRACT

This study describes the *in vitro* anthelmintic activity of extracts from *Opuntia ficus indica* against gastrointestinal nematodes of sheep. The anthelmintic activity was evaluated by inhibition of egg hatching, larval development and larval migration assays. The residual aqueous fractions from cladodes and fruits showed higher ovicidal activity with EC_{50} values of 7.2 mg/mL and 1.5 mg/mL, respectively. The aqueous, hexane, and ethyl acetate fractions from fruits and the aqueous fraction from cladodes inhibited 100% of larval development at the lowest concentration tested (1.56 mg/mL). The crude cladode and fruit ethanolic extracts inhibited larval migration and showed EC_{50} values of 0.74 mg/mL and 0.27 mg/mL, respectively. Phytochemical screening detected high concentrations of alkaloids, tannins, flavonoids, and saponins in the fruits and cladodes. The results demonstrated that *O. ficus* exhibits anthelmintic activity *in vitro*, suggesting that, beyond its nutritional potential, this plant can also be an ally for parasite control in sheep.

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1. Introduction

The indiscriminate use of synthetic anthelmintics has selected resistant parasites, further aggravating the problem of parasitosis in small ruminants (Botura et al., 2013).

Medicinal plants have emerged as an alternative to the use of synthetic anthelmintics worldwide, but there is a lack of scientific evidence regarding their effectiveness and their excess ingestion may pose a risk to animals (Rajeswari, 2014). Therefore, it is mandatory to evaluate whether medicinal plants are safe to use for the control of parasitosis in small ruminants.

Opuntia ficus-indica, belonging to the family Cactaceae, is a common plant in the Cerrado and Caatinga biomes (Griffith, 2004). This plant displays anti-inflammatory, analgesic, antioxidant and antiviral activities (Stintzing and Carle, 2005). Furthermore, a review on folk veterinary medicine demonstrated the use of *O. ficus* as a plant for ethnoveterinary medicine in Italy (Viegi et al., 2003), and in Brazil this species is widely used in feeding sheep (Veras et al., 2002). Considering that sheep feed on *O. ficus* in several places, and that finding alternative ways to control parasitosis is necessary, this study evaluated the ovicidal and larvicidal activity of *O. ficus* by means of *in vitro* assays.

2. Materials and methods

2.1. Plant materials

Plant materials were collected in the municipality of Ilha Solteira in the state of São Paulo, Brazil in September 2014. Cladodes were dried in a circulating air oven (50 °C). The pulp was removed from ripe fruits and frozen. The crude cladode ethanolic extract (CCE) was prepared from 28 g of powdered cladodes in 200 mL of ethanol (70%) by the exhaustive maceration method for seven days. The crude fruit ethanolic extract (CFE) was obtained by centrifuging the pulp (2054g) with 10 mL of ethanol (70%) for 15 min. The supernatant was filtered and evaporated. The partition of the extracts was performed by diluting 1.5 g of the crude extract with 20 mL of ethanol (70%) and successively extracting with hexane, dichloromethane, and ethyl acetate. The extraction process was repeated three times for each solvent. All extracts were frozen.

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Table 1
Percentages of inhibition of egg hatching (mean ± SD) of sheep gastrointestinal nematodes (95% *Haemonchus contortus*) by the *O. ficus* extracts and EC_{50} of the *O. ficus* extracts.

Concentration(mg/mL)	Fruit extract					Cladodes extract				
	Ethanol	Hexane	Dichloro-methane	Ethyl acetate	Residualaqueous	Ethanol	Hexane	Dichloro-methane	Ethyl acetate	Residualaqueous
100	100.0 ± 0.0^{Aa}	–	–	–	–	93.0 ± 2.2^{Bb}	–	–	–	–
50	98.2 ± 0.5^{Aa}	–	–	–	–	90.5 ± 1.5^{Bb}	–	–	–	–
25	58.5 ± 2.6^{Bc}	47.0 ± 2.2^{Bd}	32.2 ± 2.3^{Be}	98.7 ± 1.5^{Aa}	100.0 ± 0.0^{Aa}	77.5 ± 2.1^{Cb}	14.2 ± 1.7^{Bf}	32.7 ± 1.7^{Be}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}
12.5	24.7 ± 1.2^{Ce}	26.5 ± 1.0^{Ce}	22.5 ± 2.1^{Ce}	58.5 ± 1.9^{Bc}	90.0 ± 0.8^{Bb}	58.7 ± 2.3^{Dc}	6.7 ± 0.9^{Cf}	24.5 ± 2.6^{Ce}	51.5 ± 1.3^{Bd}	100.0 ± 0.0^{Aa}
6.25	14.0 ± 0.8^{Ce}	28.2 ± 2.3^{Cd}	15.0 ± 0.8^{De}	32.7 ± 1.9^{Cc}	84.2 ± 0.9^{Ca}	37.0 ± 2.2^{Eb}	7.5 ± 0.9^{Cf}	11.0 ± 0.9^{De}	15.0 ± 0.8^{Ce}	30.7 ± 0.9^{Bc}
3.12	13.0 ± 0.8^{Cc}	7.5 ± 0.3^{Dd}	6.7 ± 0.9^{Ed}	19.2 ± 0.9^{Db}	69.0 ± 0.8^{Da}	13.7 ± 0.9^{Fc}	7.5 ± 0.5^{Cd}	6.2 ± 1.3^{Ed}	7.0 ± 0.8^{Dd}	$16.5 \pm 0.6^{Cb,c}$
Albendazole 0.025	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}
5% DMSO	6.0 ± 1.4^{D}	6.0 ± 1.4^{D}	6.0 ± 1.4^{E}	6.0 ± 1.4^{E}	6.0 ± 1.4^{E}	6.0 ± 1.4^{G}	6.0 ± 1.4^{C}	6.0 ± 1.4^{E}	6.0 ± 1.4^{D}	6.0 ± 1.4^{D}
EC_{50}	9.90	27.3	8.90	55.7	1.50	9.90	71.5	11.9	50.7	7.12

Small letters compare mean between lines and capital letters between columns ($p < 0.05$). – Not tested.

Table 2
Percentages of inhibition of larval development (mean ± SD) of sheep gastrointestinal nematodes (95% *Haemonchus contortus*) by the *O. ficus* extracts and EC_{50} of the *O. ficus* extracts.

Concentration (mg/mL)	Fruits extracts					Cladodes extracts				
	Ethanol	Hexane	Dichloro-methane	Ethyl acetate	Residualaqueous	Ethanol	Hexane	Dichloro-methane	Ethyl acetate	Residualaqueous
100	100.0 ± 0.0^{Aa}	–	–	–	–	100.0 ± 0.0^{Aa}	–	–	–	–
50	100.0 ± 0.0^{Aa}	–	–	–	–	100.0 ± 0.0^{Aa}	–	–	–	–
25	96.2 ± 0.9^{Aa}	100.0 ± 0.0^{Aa}	85.0 ± 0.8^{Bb}	85.7 ± 1.7^{Bb}	100.0 ± 0.0^{Aa}	99.7 ± 0.5^{Aa}	6.1 ± 0.5^{Bc}	5.6 ± 1.5^{Bc}	100.0 ± 0.0^{Aa}	100.0 ± 0.00^{Aa}
12.5	86.7 ± 2.2^{Bb}	100.0 ± 0.0^{Aa}	74.5 ± 1.7^{Cc}	83.2 ± 0.7^{Bb}	100.0 ± 0.0^{Aa}	98.7 ± 1.0^{Aa}	5.1 ± 0.8^{Bd}	5.9 ± 0.5^{Bd}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}
6.25	68.5 ± 2.1^{Cc}	100.0 ± 0.0^{Aa}	68.3 ± 1.3^{Dc}	77.1 ± 1.1^{Cc}	100.0 ± 0.0^{Aa}	73.7 ± 1.7^{Bb}	5.8 ± 0.3^{Bd}	4.9 ± 1.3^{Bd}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}
3.12	49.2 ± 0.9^{Dc}	100.0 ± 0.0^{Aa}	63.2 ± 0.9^{Db}	67.0 ± 2.1^{Db}	100.0 ± 0.0^{Aa}	51.2 ± 0.96^{Cc}	6.2 ± 1.0^{Bd}	5.2 ± 0.8^{Bd}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}
1.56	12.5 ± 0.7^{De}	86.0 ± 1.4^{Bb}	53.2 ± 1.7^{Ec}	44.2 ± 1.7^{Ed}	100.0 ± 0.0^{Aa}	50.3 ± 1.9^{Cc}	5.9 ± 0.7^{Bf}	5.0 ± 0.7^{Bf}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}
Ivermectin 0.010	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}
5% DMSO	5.1 ± 1.4^{E}	5.1 ± 1.4^{C}	5.1 ± 1.4^{F}	5.1 ± 1.4^{F}	5.1 ± 1.4^{B}	5.1 ± 1.4^{D}	5.1 ± 1.4^{B}	5.1 ± 1.4^{B}	5.1 ± 1.4^{B}	5.1 ± 1.4^{B}
EC_{50}	3.70	<1.56	1.70	1.20	<1.56	3.40	>25	>25	<1.56	<1.56

Small letters compare mean between lines and capital letters between columns ($p < 0.05$). – Not tested.

2.2. Phytochemical analysis

Phytochemical assays were performed to detect flavonoids, alkaloids, anthraquinones, saponins, coumarins and tannins in the fruit and cladode extracts according to Harborne (1998).

2.3. Bromatological composition

The moisture content, mineral matter, crude fat, dry matter, crude protein and crude fibre were determined according to Silva and Queiroz (2006).

2.4. Egg hatch assay (EHA)

Eggs were collected from the donor sheep (95% *Haemonchus contortus* and 5% *Trichostrongylus* sp.) and washed repeatedly with distilled water according to Coles et al. (1992). The egg suspension (100 μL/100 eggs), and 100 μL of extract at different concentrations (3.12, 6.25, 12.5, 25.0, 50.0 and 100 mg/mL) were incubated for 48 h at room temperature. The eggs and L1 were counted under a microscope. The controls were performed in parallel. Five replicates for each concentration of the extracts and for each control were performed.

2.5. Larval development assays (LDA)

The egg suspension (100 μL/100 eggs), 90 μL of nutritive media (1 g of yeast in 90 mL of normal saline and 10 mL of Earle's balanced salt) and 10 μL of amphotericin (25 μg/mL) were added to each well of a 24-well plate. The plates were incubated at 25 °C for 48 h. Then, 200 μL of extract was added at the same concentrations used for the EHA. Ivermectin (10 μg/mL) and distilled water were used as positive and negative controls, respectively. Five replicates were carried out for each concentration and the controls. The plates were incubated for five days. The number of L1 and L3 in each well was counted under a microscope (Costa et al., 2008).

2.6. Larval migration inhibition assays (LMIA)

One thousand live L3 were added to Falcon® tubes containing the negative control (PBS; pH 7.2), an anthelmintic control (levamisole at 1.25 mg/mL) or the extract at different concentrations (3.12, 6.25, 12.5, 25.0, 50.0 and 100 mg/mL) to be tested. After incubation for 3 h at 28 °C, the L3 in each tube were washed with PBS and centrifuged (2054*g*) three times. The tubes were capped with a 25 μm steel mesh and placed on a Petri dish. After 3 h of incubation, the number of larvae that migrated through the mesh was counted at 40× magnification by means of the 15% aliquot technique (Rabel et al., 1994).

2.7. Statistical analysis

The data obtained during the *in vitro* assays were analyzed by ANOVA and compared by the Dunnet test (5%). The EC_{50} values (at a concentration that yielded a response of 50%) were calculated by means of nonlinear regression analysis conducted with the aid of the GraphPad Prism version 5.0.

3. Results

3.1. Yield of extracts

After the extraction with ethanol (70%), 28 g of the cladodes furnished 3.0 g of CCE. The extraction of 30 g of fruit with ethanol (70%) produced 3.8 g of CFE. The partition of 1.5 g of CCE in solvents of increasing polarity furnished 0.27 g, 0.28 g, 0.30 g and 0.31 g,

Table 3
Percentages of inhibition of larval migration (mean ± SD) of sheep gastrointestinal nematodes (95% *Haemonchus contortus*) by the crude extracts from cladodes (CCE) and fruits (CFE) of *O. ficus*.

Extracts	Concentrations (mg/mL)								Controls		EC_{50}
	100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	Levamisole	PBS	
CCE	95.6 ± 0.42^{Aa}	89.9 ± 0.48^{Ba}	86.2 ± 0.18^{Ca}	83.7 ± 0.47^{Da}	82.4 ± 0.6^{Db}	79.9 ± 0.49^{Eb}	66.3 ± 0.34^{Fb}	44.9 ± 1.8^{Gb}	38.1 ± 0.19^{Hi}	96.2 ± 0.62^{A}	2.34 ± 0.67^{I}	0.74
CFE	95.5 ± 0.9^{Aa}	90.2 ± 0.55^{Ba}	85.6 ± 0.42^{Ca}	84.6 ± 0.54^{Da}	83.8 ± 0.26^{Da}	83.2 ± 0.24^{Da}	74.5 ± 0.45^{Ea}	64.9 ± 0.53^{Fa}	48.9 ± 1.27^{Ga}	96.2 ± 0.91^{A}	3.2 ± 0.94^{H}	0.27

Small letters compare mean between columns and capital letters lines columns ($P < 0.05$). Levamisole (0.025 mg/mL) is a positive control; PBS is a negative control.

respectively, of the fraction in hexane (HFC), dichloromethane (DFC), ethyl acetate (EAFC) and residual aqueous solution (AFC). The same procedure was repeated with 1.5 g of CFE to obtain 0.60 g, 0.63 g, 0.24 g and 0.27 g, respectively, of the fraction in hexane (HFF), dichloromethane (DFF), ethyl acetate (EAFF) and residual aqueous (AFF) solution.

3.2. Phytochemical analysis

The phytochemical analysis of CCE and CFE revealed the presence of alkaloids, flavonoids, coumarins, tannins, phenolic compounds and saponins in high quantity.

3.3. Bromatological composition

For the fruits, the values for crude fibre, crude protein, ether extract, dry matter and moisture were 17.06%, 5.85%, 3.92%, 7.75% and 92.24%, respectively. For cladodes, these values were 9.91%, 3.79%, 1.51%, 5.28% and 94.72, respectively, with the SD lower than 1% for all parameters. These values are within an acceptable range of variation (Galvão Junior et al., 2014).

3.4. Egg hatch assay

The percentages of egg hatch inhibition by the extracts are presented in Table 1. *Opuntia ficus* extracts and fractions significantly ($P < 0.05$) inhibited egg hatching with a dose-dependent effect.

3.5. Larval development assay

The CCE, CFE and fractions, with the exception of HFC and DFC (Table 2), were effective at inhibiting larval development, even at the lowest evaluated concentration.

3.6. Larval migration inhibition assay

The treatment of infective gastrointestinal nematode larvae with CCE and CFE, as well as levamisole, led to a significant reduction in the number of larvae recovered by the migration test compared to the negative control ($P < 0.05$). The assays revealed a dose-dependent behavior for the crude extracts (Table 3).

4. Discussion

The use of plants and their extracts has been an adopted alternative for the treatment of ovine gastrointestinal parasites in several places around the world (Rajeswari, 2014). Our study clearly showed that *O. ficus* extracts were effective against eggs and larvae of *H. contortus* based on the assay results. The crude extracts CCE and CFE possess several classes of phytoconstituents that were responsible for the anthelmintic activity demonstrated.

The AFC and AFF-containing flavonoids, saponins and tannins were the fractions with the greatest activity in all of the *in vitro* assays. These substances are known for anthelmintic properties that act synergistically to inhibit eggs from hatching (Botura et al., 2013). The less polar fractions were the least active with the exception of HFF, whose action can be related to the presence of terpenoid pigments in the fruits.

The CCE and CFE showed significant inhibition of larval migration with a dose-dependent response. Saponins and tannins in these extracts may act in conjunction; the saponins interact with cell membranes, resulting in destabilization and subsequent increased cell permeability that eases the action of tannins on intracellular proteins of the parasite (D'Addabbo et al., 2011).

Numerous biological properties of the *O. ficus* fruit has been associated with the presence of betalains (Stintzing and Carle, 2005; Gonçalves et al., 2015); however, the literature does not contain any mention of the anthelmintic action of these substances. The results of the phytochemical screening for cladodes and fruits are in accordance with Chiteva and Wairagu (2013), however minor variations can occur due to age, environment conditions and maturation stage of the fruits. Nutritional parameters are also affected by the age of the plant as demonstrated by Galvão Junior et al. (2014) in that younger plants present higher crude protein levels than used in this study.

Many studies have shown the anthelmintic action of plants (Rajeswari, 2014); however, our study is the first to analyses and test *O. ficus* for its anthelmintic activity. We demonstrated that *O. ficus* showed ovicidal and larvicidal activity. Furthermore, studies conducted by Bispo et al. (2007) showed that the inclusion of up to 56% of *O. ficus* in ovine feed does not cause toxicity or deleterious effects on the animals' performance, which further supports the use of the *O. ficus*.

In conclusion, our results provide support for conducting *in vivo* assays to verify the anthelmintic activity of this plant and for using edible plants in parasite control.

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3. Isolamento de compostos da *Piper cubeba* e atividades biológicas

Os frutos da *Piper cubeba* são usados como condimento e também na medicina popular da Indonésia para o tratamento da gonorreia, disenteria, sífilis, dor abdominal, diarreia, enterite e asma. Os frutos são ricos em lignanas e este foi o fato que nos chamou atenção, pois as propriedades biológicas atribuídas aos frutos poderiam ter relação justamente com a presença desses compostos. As lignanas apresentam uma grande variedade estrutural e de propriedades biológicas que as tornam potenciais alvos para o desenvolvimento de novos fármacos.

Dentre as várias lignanas dos frutos da *P. cubeba* extraímos 10 delas com as quais trabalhamos nos últimos 10 anos, com maior ênfase para a cubebina, diidrocubebina, hinoquinina, *O*-etilcubebina, *O*-metilcubebina e iateína. Nos próximos tópicos comento alguns artigos que publicamos com as lignanas da *P. cubeba*.

3.1. COX Inhibition Profiles and Molecular Docking Studies of the Lignan Hinokinin and Some Synthetic Derivatives

Em um de nossos estudos sobre as lignanas da *P. cubeba* idealizamos 500 estruturas análogas desses compostos para a realização de estudos *in sílico* com as enzimas COX-1 e COX-2 a fim de selecionar dentre esses compostos aqueles com comportamento semelhante a anti-inflamatórios seletivos COX-2. Nos ensaios preliminares utilizando o programa GOLD, somente 10 estruturas apresentaram bons resultados, porém apenas 3 delas foram selecionadas, de início, para um estudo mais aprofundado. O trabalho que comento contem estudos *in sílico* com esses 3 compostos utilizando docking consensual com três diferentes programas de modelagem molecular e ensaios *in vitro* com as enzimas COX-1 e 2.

A partir do extrato hidroetanólico da *P. cubeba* foi extraída a hinoquinina, a partir da qual foram obtidas a diidrocubebina e a dinitrohinoquinina. O docking consensual usando como alvo biológico as enzimas COX-1 e 2, os três compostos selecionados e os padrões de comparação (rofecoxibe, celecoxibe e meloxicam) forneceu resultados que indicam similaridade de comportamento entre dinitrohinoquinina e o rofecoxibe. Nos ensaios *in vitro* a dinitrohinoquinina foi cerca de 13 vezes mais seletiva para a COX-2 do que para a COX-1.

De acordo com os resultados obtidos no modelo *in vitro* utilizado aliado aos resultados do estudo *in sílico* pode se concluir que a dinitrohinoquinina apresenta características interessantes e pode ser um potencial alvo para o desenvolvimento de fármacos seletivos para COX-2.

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COX Inhibition Profiles and Molecular Docking Studies of the Lignan Hinokinin and Some Synthetic Derivatives

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Abstract: Encouraged by the anti-inflammatory activity of hinokinin *in vivo*, which is also observed for the analogues dinitrohinokinin and diidrocubebin, herein we used *in vitro* and *in silico* methods to assess their selectivity profiles and predict their binding modes with Cyclooxygenases (COX-1 and 2). The *in vitro* assays demonstrated dinitrohinokinin is about 13 times more selective for COX-2 than for COX-1, a similar profile observed for the drugs celecoxib (selective index ≈9) and meloxicam (selective index ≈11). Predictions of the binding modes suggested dinitrohinokinin interacts with COX-2 very similarly to rofecoxib, exploring residues at the hydrophilic pocket of the enzyme that accessible to ligands only in this isoform. This lignan also interacts with COX-1 in a similar mode to meloxicam, blocking the access of the substrate to the catalytic cleft. Therefore, dinitrohinokinin is a promising lead for the design of selective COX-2 inhibitors.

Keywords: Oxirreductases · Inflammation · Molecular Docking · Lignans · Cyclooxygenases

Cyclooxygenases (COXs) (EC 1.14) are endogenous enzymes that catalyze the first step in the conversion of arachidonic acid into prostaglandins, leukotrienes and thromboxanes. The amino acid residues that compose their active sites and closely adjacent areas in the isoforms of COX (COX-1 and 2) are nearly identical, except for the exchange of Ile^{434}, Ile^{523} and His^{513} in COX-1 by, respectively, valine, valine and arginine in COX-2. COX-1 is predominantly a constitutive enzyme, which is associated with the homeostasis of many tissues including the gastrointestinal tract (gastric cytoprotection), kidneys (maintenance of the renal plasma flow and the filtration rate under systemic conditions of vasoconstriction) and platelets (platelet aggregation). The COX-2 isoenzyme is tissue specific, being widely induced by inflammatory agents and expressed in many types of cancer, in the hippocampus and in the female reproductive tissue.[1]

As COXs play a role in the inflammatory processes, they were targeted for the development of non-steroidal anti-inflammatory drugs (NSAIDs). However, while classic NSAIDs inhibit COXs in a non-selective way, promoting undesirable side effects due to COX-1 inhibition (e.g. gastric irritation and ulcers), the chronical use of fully selective COX-2 inhibitors is associated with increased risks for cardiovascular diseases. Therefore, current researches focus on the search for compounds that inhibit COX-2 more efficiently than COX-1, hindering the gastric side effects due to the strong inhibition of COX-1 and the cardiovascular effects resulting from the very marked inhibition of COX-2.[1–3]

Nature has been a source of inspiration for the design of COX inhibitors and, although several natural products were assayed for anti-inflammatory activities *in vivo*, little is known about their mechanism of action. Among these compounds is hinokinin (**1**) (Figure 1), a lignan found in *Piper cubeba* that exhibits analgesic and anti-inflammatory activities *in vivo*, as well as some of its synthetic derivatives, dinitrohinokinin (**2**) and diidrocubebin (**3**).[4] In addition to their anti-inflammatory actions, these lignans have interesting structures since **1** and **2** enclose cores that resemble the structures of rofecoxib (**4**) and celecoxib (**5**), selective COX-2 inhibitors (Figure 1).

Structurally, the coxibs display two aromatic rings, one with hydrogen acceptor groups, and a central heterocycle with hydrogen acceptor substituents. [1,2,5] For meloxicam (**6**), which is also a COX-2 selective NSAID, an amide links a heterocycle to a bicycle system containing *H* acceptor groups.

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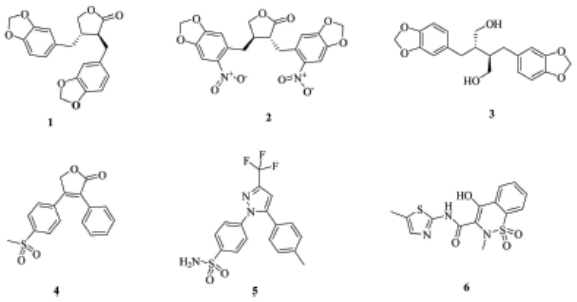

Figure 1. Structures of hinokinin (**1**), dinitrohinokinin (**2**), dihydrocubebin (**3**), rofecoxib (**4**), celecoxib (**5**) and meloxicam (**6**).

Herein we hypothesized the lignans **1** and **2** could display similar binding modes to **4** and **5**. We also studied **3**, where the lactone of **1** and **2** was reduced to a less conformationally restricted butanedioic group. Such comparison would permit assessing if the replacement of the central ring by a linker where carbons freely rotate around the axis of the σ bond would alter the *in vitro* COX-1/2 inhibition profile, which would also reflect its divergent binding modes with the COXs. In this context, we assessed the inhibition affinities of **1**–**3** against COX-1/2 and evaluated their selectivity profiles. To predict their binding modes with COXs, consensus molecular docking studies were also performed using rofecoxib (**4**), celecoxib (**5**) and meloxicam (**6**) as reference ligands.

***In Vitro* Assays for COX-1/2 Inhibition and Selectivity Profiles.** The COX-1/2 inhibition affinities of lignans **1**–**3** were assessed using the immunoenzymatic assay (EIA) method, with the samples tested in triplicates (N = 3). The IC_{50} (half-maximal inhibitory concentration) and selectivity indices were calculated, respectively, from experimental data and the rate of $IC_{50COX\text{-}1}/IC_{50COX\text{-}2}$ (Table 1).

Table 1. *In vitro* inhibition profiles for COX-1/2 and the corresponding selectivity indices calculated for 1, 2, 3, 4,[6] 5[6] and 6*.[6]

Compound	IC_{50} (μM) COX-1	COX-2	Selectivity Index COX-1/COX-2
1	105.54	28.95	3.65
2	157.56	11.66	13.51
3	44.24	32.71	1.35
4	63	0.31	>50.00
5	1.2	0.34	9.33
6	5.7	0.23	11.00

*Values for the positive controls are reported in the supplementary material.

The tested compounds displayed affinities for COX-1 in the 40–160 micromolar range, following the inhibition order: diidrocubebin (**3**) > hinokinin (**1**) > dinitrohinokinin (**2**). Concerning COX-2 inhibition, binding affinities were in the range of 10–35 micromolar and the order was dinitrohinokinin (**2**) > diidrocubebin (**3**) > hinokinin (**1**). Therefore, dinitrohinokinin was the most selective lignan, with a inhibition profile very similar to celecoxib (**5**) and meloxicam (**6**), which are clinically useful NSAIDs.[6]

According to Warner et al.,[6] selectivity indices ranging from 5 to 50 are associated with compounds that markedly inhibit more the COX-2 isoform than COX-1, and represents the ideal range for the development of safe and selective COX-2 inhibitors. Selectivity indices below of 5 are found for non-selective inhibitors, which bind to both isoforms of the enzyme (e.g. indomethacin). The strong inhibition of COX-1 will then affect the biosynthesis of prostanoids related to several homeostatic processes, such as gastric cytoprotection and coagulation.[1,7–10]

Selective indices above of 50 are indicatives of a high degree of selectivity for COX-2, such as observed for rofecoxib (**4**).[6] However, a strong inhibition of COX-2 (with a minimal or insignificant inhibition of COX-1) will constantly trigger platelet aggregation to, ultimately, cause atherosclerosis, then increasing the risks for cardiovascular events such as myocardial infarction and stroke.[7–10] Therefore, one may consider hinokinin (**1**) and diidrocubebin (**3**) as non-selective COX inhibitors, while dinitrohinokinin (**2**) has a COX inhibition profile similar to the safest COX-2 selective NSAIDs.

Studies of the Binding Modes at the Catalytic Site of COX-1/2. To gain a better insight concerning the *in vitro* results of **1**–**3**, molecular docking studies were performed for the two COX isoforms using a consensus *docking* strategy.[11–15] Results for the lignans were analysed and compared to those obtained for NSAIDs **4**–**6**. Meloxicam (**6**), despite of being a selective inhibitor of COX-2, display a different mode of inhibition than the coxibs, since it does not interact the hydrophilic pocket of COX-2. As a result, it was also selected as a reference compound.[16–18]

The choice for consensus docking allows a better reliability concerning the study of the binding modes since individual docking software commonly fails in accurately describing protein-ligand interactions. This typically occurs because their scoring functions are designed to work with a large set of target proteins and not to a particular protein or class of specific proteins (*e.g.* soluble enzymes *versus* membrane receptors).[19–22] Therefore, the individual limitations of each software or algorithm can be minimised by replacing the final result with an average of the multiple scoring functions.[23]

AutoDock 4.2,[11] GOLD 5.3[12–14] and Maestro 10.1[15] were then used to perform the consensus analyses of the binding modes. Their sampling algorithms, although specific, are all considered as stochastic methods, where random searches in the conformational space are performed to find the best pose for the ligands.[23] Both AutoDock and GOLD use search algorithms based on theories originally postulated in the fields of natural genetics and biological evolution,[11–14] while Maestro selects the most energetically favourable

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conformations using a Monte Carlo-based sampling algorithm.[15] As described for the sampling algorithms, the scoring functions of these programs are also different, which helps to improve the reliability of their predictions.

The accuracy of the docking programs regarding the precise generation of the binding poses was validated by *redocking*,[23] where a root mean square deviation (RMSD) value of 2.0 Å was established as the threshold.[24] The *redocking* results for the celecoxib-COX-1 and rofecoxib-COX-2 complexes are described in the supplementary material. According to these results, the poses calculated by the docking programs were able to describe binding modes similar to those observed in the COX-ligand crystallographic complexes. For the analyses of the intermolecular interactions, only poses considered as equivalent from the three software packages were chosen, which were then ranked for selection using the consensual rank-by-rank average method.[24]

Poses with RMSD $\leq$ 2.0 Å were assumed as equivalent while 2.0 Å $<$ RMSD $\leq$ 3.0 Å indicated a partial equivalence.[24–27] These criteria were established by the work of Vieth et al.[26] while assessing the selectivity/efficiency of docking algorithms. However, although an RMSD $\leq$ 2.0 is widely used to suggest the success of a docking method in reproducing a known binding mode, such generalizations have inherent biases since they are highly dependent on the number of rotatable bonds of the ligand. Consequently, docking programs usually find troubles in the correct positioning of very flexible ligands.[28] RMSD values from the poses of 1-6 for COX-1 and COX-2 are displayed in the Tables 2 and 3, respectively.

Table 2. *RMSD values of **1**, **2**, **3**, **4**, **5** and **6** for COX-1. AutoDock (AD), GOLD (G) and Maestro (M).

Compound	AD/G	AD/M	G/M
1	2.04	1.95	0.54
2	1.29	3.00	3.00
3	2.57	2.88	0.56
4	0.63	0.75	0.36
5	0.58	1.01	0.99
6	0.89	1.36	0.81

*RMSD – Root Mean Square Deviation.

As observed, all compounds displayed RMSD values within the threshold stablished for the analysis, except by the poses of compound 2 for COX-2 (Table 3) generated by the Maestro 10.1 software.[24–27] Therefore, this particular docking output was discarded for the analyses. Scores from the three software packages for the poses chosen for analysis of interactions with both COX-1 and COX-2 are reported in the supplementary material.

Regarding the analysis of interactions from the poses considered as equivalent or partially equivalent at the COX-1 complexes, it is interesting to note all compounds can be

Table 3. *RMSD values of **1**, **2**, **3**, **4**, **5** and **6** for COX-2. AutoDock (AD), GOLD (G) and Maestro (M).

Compound	AD/G	AD/M	G/M
1	1.60	1.07	1.51
2	0.77	6.87[a]	7.05[a]
3	1.98	0.71	2.01
4	0.46	0.79	0.63
5	1.30	0.79	1.01
6	1.32	2.54	2.73

*RMSD – Root Mean Square Deviation, [a]RMSD values above the stablished threshold for the analyses of the intermolecular interactions using consensus poses.

categorized in only two different fitting patterns at the protein-binding site. Compounds **4** and **5** (Figures 2-D and 2-E) fill the whole active site, interacting with residues positioned both at the mouth (entrance) of the catalytic site (Val^{116}, Arg^{120}, Val^{349}, Leu^{352}, Ser^{353}, Tyr^{355} and Leu^{359}) and at the roof (top) (Phe^{381}, Leu^{384}, Tyr^{385}, Trp^{387} and Met^{522}), besides of the differences in their interaction modes.

Compound **6** and lignans **1**–**3** (Figures 2-A, 2-B, 2-C and 2-F) interact only with residues close the mouth of the active site (Val^{116}, Arg^{120}, Val^{349}, Leu^{352}, Ser^{353}, Tyr^{355} and Leu^{359}). These compounds are predicted inhibiting the activity of COX-1 by blocking the entrance of the active site, then preventing its substrate, arachidonic acid, from reaching the catalytic cleft of the enzyme.

Unlike the observed for COX-1, all compounds filled the COX-2 binding site in a very similar arrangement (Figure 3-A through 3-F). Comparisons between the poses from **4** (Figure 3-D) and **2** (Figure 3-B) suggest these compounds display very similar binding modes, since both perform a classic hydrogen bond with His^{90}, a non-classical hydrogen bond with Ser^{530} and hydrophobic interaction with the Ala^{527} residue. Concerning Arg^{513}, a residue at the bottom of the hydrophilic pocket that plays a fundamental role in the selectivity of some coxibs for COX-2,[17,18] the consensus poses of **4** suggest an hydrogen bond between its methylsulfone group and the guanidine group of the amino acid residue.

Alternatively, compound **2** displays an hydrogen bond with the backbone of Phe^{518}, which is also a residue from the hydrophilic pocket of COX-2 and, accordingly, is relevant for the selectivity for COX-2.[17,18] Comparisons between the poses of **2** and **5** (Figure 3-E), highlight that both display classical hydrogen bonding with the His^{90} residue and hydrophobic interactions with Ser^{353}, Val^{523} and Ala^{527}.

For **1** (Figure 3-A), it is suggested the existence of classical hydrogen bonding with the guanidine group of Arg^{513} as well as hydrophobic interactions with Ser^{353}, Val^{523} and Ala^{527}, similarly as described for **4**. Compound **3** (Figure 3-C), in spite of displaying a similar binding mode to meloxicam (**6**) concerning the absence of interactions with the residues from the hydrophilic pocket of COX-2, also

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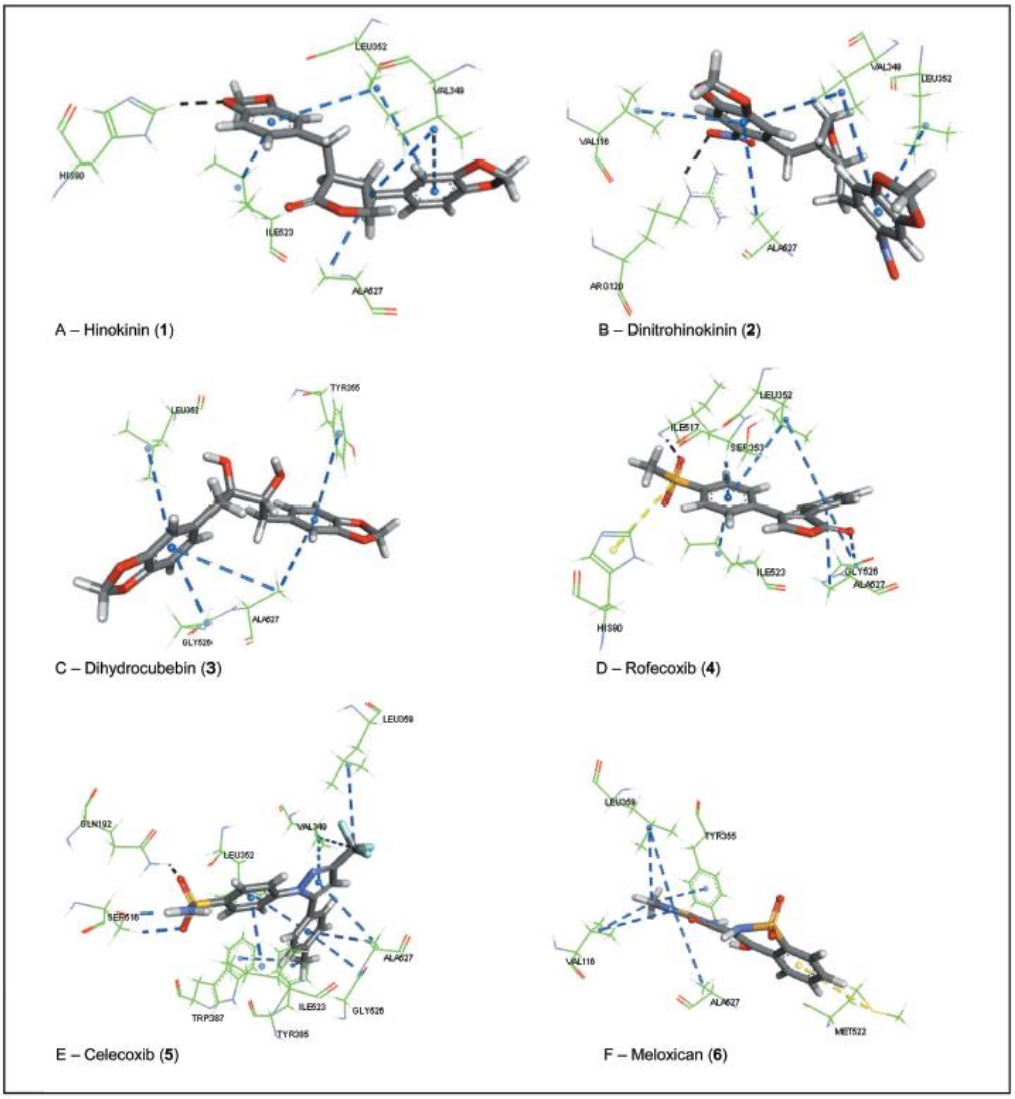

Figure 2. Binding modes of 1(A), 2(B), 3(C), 4(D), 5(E) and 6(F) with COX-1, where black, yelow and blue dashed lines represent hydrogen bonding, π-sulfur and hydrophobic interactions, respectively.

performs fewer/weaker interactions than the reference NSAID.

Therefore, concerning COX-2, compounds **1** and **2** qualitatively displayed similar binding modes to the coxibs **4** and **5**. However, it is important to highlight that **2** is predicted to interact more intensively with the COX-2 hydrophilic pocket than **1** and, accordingly, display a closer fitting pattern to **4**. As previsouly stated, both compounds have groups interacting with His^{90}, but while **4** display an additional interaction with Arg^{513}, the corresponding one in **2** would be with Phe^{518}.

The concerted analysis of the *in vitro* and *in silico* results for lignans suggest that, although compound **1** interacts with the hydrophilic pocket of COX-2, as highlighted by the docking simulations, such interactions are not substantial enough to confer a high degree of selectivity, as demonstrated by the *in vitro* assays. For compound **3**, the absence of interactions with the hydrophilic pocket of COX-2 in association with fewer/ weaker interactions than the reference ligand used for the study of the binding modes (**6**), results in a nearly complete absence of selectivity. Therefore, the lactone ring *per se* does not significantly

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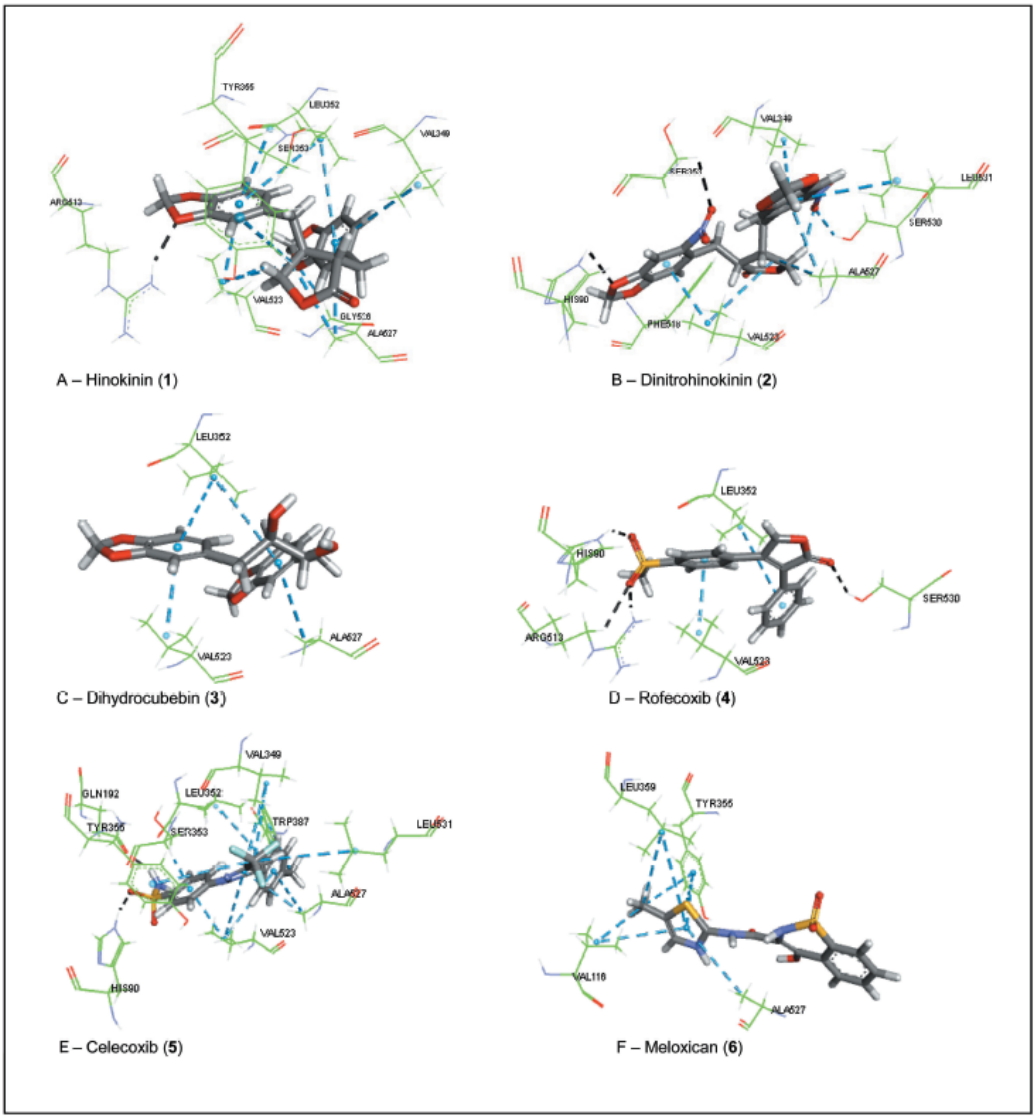

Figure 3. Binding modes of 1(A), 2(B), 3(C), 4(D), 5(E) and 6(F) with COX-2, where black and blue dashed lines represent hydrogen bonding and hydrophobic interactions, respectively.

affect the selectivity by the COXs, considering its replacement by a linker with an equivalent length.

Compound **2** achieved a selective index comparable to the ones from celecoxib (**5**) and meloxicam (**6**).[6] However, in spite of a very similar binding mode to the rofecoxib (**4**) at the catalityc cleft of COX-2, this compound was predicted to interact with residues near or at the mouth of COX-1, as it was also observed for **1**, **3** and even **6**. In fact, bulkier substituents (as the 1,3-dioxolane substituents in lignans when compared to the corresponding groups of the coxibs) tend to impact more on the interaction with COX-1 because of its narrower access to the catalytic cleft in comparison to COX-2.[1]

Therefore, lignan **2** may be considered as a promising compound for the design of selective COX-2 drugs. As we have demonstrated herein, even minor modifications, as the insertion of the nitro groups at the C6/C6′ for the design of **2**, can yield lignans with substantial selectivity for the COX-2 isoenzyme.

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Experimental Section and Computational Methods

Isolation and Semisynthesis of Lignans. According to the methods reported by Da Silva et al.,[4] 1 was extracted from the *Piper cubeba* fruit and, after subsequent teratment with nitric acid, 3 was isolated. The reduction of 1 with lithium aluminum hydride in THF provided (after purification by silica gel column chromatography in hexane:ethylacetate, 1:1) dihydrocubebin (2), as previusly reported by Laurentiz et al.[29] All compounds displayed purity >95%, as determined by HPLC and NMR analyses and are in agreement with data published in the literature. (supplmentary material).

***In Vitro* Assay for Cyclooxygenase Inhibition.** The anti-inflammatory assays were performed using the COX-1 and COX- 2 (catalogue #560101) screening kits from Cayaman Chemical's ACETM according to manufacturer's instructions and a previously reported method,[30] using triplicates in five concentrations (100, 10, 1, 0.1 and 0.001 μg/mL). Compounds 1, 2 and 3 and standard compounds were inially dissolved in DMSO and, subsequenlty, in the buffer. The IC50 values were determined by the sigmoidal dose-response curves (GramPadPrism 5.0). The standard inhibitor for COX-1 was indomethacin (purity ≥ 99%, TLC; Sigma®) and celecoxib (purity ≥ 98%, HPLC; Sigma®) for COX-2.

***In Silico* Methods.** Consensus docking studies[11–15] for the COX-1 and COX-2 complexes with compounds 1–6 were performed using the AutoDock 4.2,[11] GOLD 5.1[12–14] and Maestro 10.1[15] software packages. Ligand binding sites were calculated using Discovery Studio 2016 software.

The crystallographic structure of the rofecoxib in complex with the human COX-2 (PDB ID 5KIR) and the homology-modelled structure of COX-1 in complex with celecoxib were used for the analyses. The model of human COX-1 was built using the COX-1 from sheep (≈91% of identity) as the 3-D template (PDB ID 3KK6;), using the web servers MUSCLE and SWISSMODEL. Model validation was performed using the QMEAN scoring function value (−0.74) and the Ramachandran plot (99.8% of residues in allowed regions).[31,32] The Q-Mean (Qualitative Model Energy ANalysis) is a scoring function that describes the major geometrical aspects of a single protein structure and, consequently, reflects the general reliability of the model.[31] The Ramachandran plot relays on the principle that, due to steric hindrance, the main chain of a polypeptide typically assumes preferred, energetically favorable conformations. Deviations from these ideal conformations (expressed as allowed regions in the plot) are used as indicators of potential errors in the model.[32] For the *docking* simulations, proteins were prepared excluding water molecules and ligands other than the reference NSAIDs, followed by the addition of polar hydrogen atoms. For each compound, the best consensus arrangements were selected for the analysis of the poses using the Discovery Studio 2016 software package.

Conflict of Interest

None declared.

Acknowledgements

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3.2. In vitro anthelmintic activity of the crude hydroalcoholic extracts of Piper cubeba fruits and isolated natural products against gastrointestinal nematode in sheep

O objetivo deste trabalho foi a avaliação anti-helmíntica tanto do extrato dos frutos quanto das lignanas isoladas. As lignanas e o extrato foram escolhidos para essa avaliação devido às atividades antiparasitárias que apresentaram contra outros parasitas em estudos anteriores publicados pelo nosso grupo de pesquisa. Neste trabalho, comprovamos a atividade anti-helmíntica *in vitro* tanto do extrato quanto das lignanas. Os resultados dos ensaios *in vitro* foram essenciais para o desenvolvimento do ensaio *in vivo* que culminou na solicitação de patente junto ao INPI intitulada “Produto Compreendendo Extrato de *Piper Cubeba* e seu Uso, 2019” em parceria com pesquisadores da UNESP-Dracena.

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Research paper

In vitro anthelmintic activity of the crude hydroalcoholic extract of *Piper cubeba* fruits and isolated natural products against gastrointestinal nematodes in sheep

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ABSTRACT

This study describes the *in vitro* anthelmintic activity of a hydroalcoholic extract from the fruit of *Piper cubeba* and its major isolated components against the eggs and larvae of gastrointestinal nematodes obtained from naturally-infected ovines. *In vitro* anthelmintic activity was evaluated using the egg hatch test (EHT), larval development test (LDT) and L3 migration inhibition test (LMT). The extract showed ovicidal and larvicidal activity, with an EC_{50} of 200 µg/mL and 83.00 µg/mL in the EHT and LDT, respectively. The extract inhibited 100% of larval migration at the lowest tested concentration (95 µg/mL). The crude extract was purified using successive silica gel chromatographic columns, which revealed the lignans hinokinin, cubebin and dihydrocubebin as the major compounds that were present, which were then used in *in vitro* tests. Cubebin, dihydrocubebin and hinokinin showed higher activity than the crude extract, with an EC_{50} for ovicidal activity of 150.00 µg/mL, 186.70 µg/mL and 68.38 µg/mL, respectively. In the LDT, cubebin presented an EC_{50} of 14.89 µg/mL and dihydrocubebin of 30.75 µg/mL. Hinokinin inhibited 100% the larval development at all concentrations evaluated. In the LMT, dihydrocubebin inhibited 100% the larval migration in all concentrations evaluated while cubebin and hinokinin showed EC_{50} values of 0.89 µg/mL and 0.34 µg/mL, respectively. *P. cubeba* extract is rich in several classes of active compounds, but here we demonstrate that the described anthelmintic activity may be related to the presence of these lignans, which are present in larger concentrations than other components of the extract. Our results demonstrate for first time the anthelmintic activity against gastrointestinal nematodes in sheep for this class of special metabolites that are present in *P. cubeba* fruit. However, future detailed studies are needed to evaluate the effectiveness of *P. cubeba* fruits extract and active lignans in *in vivo* tests.

1. Introduction

The administration of synthetic anthelmintics has for decades helped to control parasitic infections of gastrointestinal nematodes (GINs) in small ruminants. However, the indiscriminate use of synthetic anthelmintics without epidemiological criteria has selected resistant parasites, further aggravating the problem of parasitosis (Jabbar et al., 2006; Demessie et al., 2016). *Haemonchus contortus* is the most prevalent and pathogenic nematode found in small ruminants in the tropics (Besier et al., 2016); it is the main cause of loss of sheep due to its hematophagous behaviour, causing severe anaemia in animals, which at high levels of infection causes death.

In recent decades, plants have been used as a promising source of target molecules for development of new drugs to treat cancer, pain and parasitic infections. Several research groups around the world have carried out studies on the use of plants and extracts for the treatment of

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gastrointestinal parasites in sheep. These studies have demonstrated the importance of this research area as an alternative in the control of the infection (Qi et al., 2015; Féboli et al., 2016; Katiki et al., 2017; Oliveira et al., 2017; Abidia et al., 2018). Studies examining plant extracts, plant essential oils and condensed tannins have been carried out to evaluate their *in vitro* anthelmintic activity against *H. contortus* (Katiki et al., 2013; Qi et al., 2015; Féboli et al., 2016 Katiki et al., 2017; Mengistu et al., 2015; Oliveira et al., 2017). The anthelmintic activity of these plants is generally associated with the presence of tannins (Katiki et al., 2017; Mengistu et al., 2015; Oliveira et al., 2017). Among these tannins, the main emphasis has been on condensed tannins due to their occurrence in forages used to feed sheep and their effect on the various life stages of nematodes (Alonso-Díaz et al., 2008; Chan-Pérez et al., 2016). However, some studies have also been conducted using non-tanniferous plants, such as *Cymbopogon citratus*, whose anthelmintic properties are related to essential oil components (Macedo et al., 2015), or plants used in ethnoveterinary practices such as *Cissus quadrangularis,* used in livestock against helminthosis in Ethiopia, with flavonoids as the active compounds (Zenebe et al., 2017).

In addition to the plants described above, *Piper cubeba* is non-tanniferous plant of Asian origin whose fruit is used in Asian cuisine and Indian traditional medicine to relieve gastric pain, enteritis, diarrhoea and inflammation, and to treat acute jaundice (Chopra et al., 1956). *P. cubeba* fruit contains an oil that is rich in terpenes, with sabinene and eucalyptol being of the highest proportion (Magalhães et al., 2012). Moreover, *P. cubeba* fruit hydroalcoholic extract presents about 24 lignans with highly varied chemical structures (Elfahmi Ruslan et al., 2007). Despite the structural diversity of the components present in the *P. cubeba* extract, the biological activities of this extract are generally attributed to the lignans cubebin and hinokinin, which are the compounds at the highest concentrations (Lima et al., 2018).

The biological properties attributed to *P. cubeba* fruit extracts, both in terms of its essential oil and isolated compounds, are analgesic, anti-inflammatory (Silva et al., 2005), antimicrobial (Silva et al., 2007; Laurentiz et al., 2015), antioxidant (AlSaid et al., 2015) and anti-parasitic (Magalhães et al., 2012; Esperandim et al., 2013a). Due to these properties and its low toxicity (Graidist et al., 2015), *P. cubeba* fruit appears to be a promising candidate for evaluation against gastrointestinal nematodes in sheep. Therefore, the aim of this study was to evaluate the *in vitro* anthelmintic activity of the hydroalcoholic extract and isolated compounds from *P. cubeba* fruit against gastrointestinal nematodes eggs and larvae obtained from the faeces of naturally infected sheep using the egg hatch test (EHT), larval development test (LDT) and L3 migration inhibition test (LMT).

2. Materials and methods

2.1. Plant materials and isolation of major compounds

The dried fruits of the *P. cubeba* were purchased from Floral Seed Company, Dehradun, India. The powdered *P. cubeba* fruits were exhaustively extracted by maceration for five days with 70% aqueous ethanol (500 g seed for 2 L ethanol). The extract was filtered and concentrated under vacuum to furnish the crude hydroalcoholic extract (PCE), which was fractionated by the partition between the phases of hexane and methanol/water (9:1) (Laurentiz et al., 2015). The crude methanol/water fraction (50 g) was submitted to silica gel column chromatography Bajpai et al. (2016). Elution with increasing proportions of hexane, hexane/ethyl acetate and ethyl acetate yielded 16 fractions (200 mL), of which six, with similar chromatographic profile, were pooled, concentrated under vacuum and again submitted to silica gel column chromatography using a gradient crescent of the mixture of hexane/ethyl acetate. This latter chromatography sequentially presented three compounds as major products. Compound 1 was eluted with hexane-EtOAc (9:1), compound 2 was elueted with hexane-EtOAc (7:3) and compound 3 was elueted with hexane-EtOAc (1:1). These compounds were compared with known lignan standards by thin layer chromatography (TLC) and submitted for analysis by ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR). The PCE and the isolated compounds were kept frozen into use and after diluted in 0.5% DMSO to obtain the concentrations used in the *in vitro* tests.

2.2. NMR data

^{1}H and ^{13}C NMR analyses for structural determination of isolated compounds were performed using a Bruker ARX 500 spectrometer (Bruker-Germany). Samples for the analyses were prepared by dissolving 10 mg of each isolated compound in 0.5 mL of $CDCl_3$. Proton chemical shifts in $CDCl_3$ are associated with the middle of the residual singlet (δ =7.28 ppm). Carbon chemical shifts were reported in parts per million (δ) relative to $CDCl_3$ (77 ppm), and J (coupling constant) values were reported in hertz. The splitting patterns of protons are described as s (singlet), brs (broad singlet), d (doublet), dd (doublet of doublets), t (triplet) and m (multiplet) (Silverstein et al., 2005).

2.3. Recovery and preparation of eggs

The faeces used in *in vitro* tests were obtained from sheep naturally infected with gastrointestinal nematodes, being 95% *H. contortus* and 5% *Trichostrongylus* sp, the nematode species were determined according to Wyk & Mayhew (2013). The faeces samples were obtained directly from the rectum of a donor animal. The eggs were recovered with the sequential use of sieves, according to the method described by Coles et al. (1992), with some modifications. The eggs retained in the last sieve were washed with distilled water, transferred to Falcon tubes (50 mL) and centrifuged at 2054 x g for 5 min. Then, the supernatant was removed and a saturated NaCl solution was added to resuspend the sedimented eggs. After centrifugation under the same conditions, the supernatant was transferred to 25 µm sieves; the eggs were again washed with distilled water and transferred to another tube. The concentration of eggs in this tube was estimated by counting the number of eggs in 50 µL aliquots (5 counts) using the McMaster slide technique and the suspension was diluted to achieve a concentration of 100 eggs/100 µL.

2.4. Egg hatch test

The eggs suspension (100 eggs/100 µL), and 100 µL of PCE at different concentrations (190-12000 g/mL) or lignans (75-1250 µg/mL) were incubated for 48 h at 28 °C in 24-well plate. The eggs and L1 were counted under an inverted microscope. Albendazole (12.5 µg/mL) was used as a positive control, while 0.5% DMSO was used as a negative control (Coles et al. (1992). Five replicates for the controls and for each concentration of the PCE and lignans were performed.

2.5. Larval development test

This test was carried out according to the method described by Lacey et al. (1995), with some modifications. The egg suspension (100 µL/100 eggs), 90 µL of nutritive media (1 g of yeast in 90 mL of normal saline and 10 mL of Earle's balanced salt) and 10 µL of amphotericin (Sigma-Aldrich) (25 µg/mL) were added to each well of a 24-well plate. The plates were incubated for 48 h under humidified conditions at 28 °C, after which 200 µL of PCE or lignans at same concentrations described for the EHT were then added to each well. Ivermectin (10 µg/mL) and 0.5% DMSO were used as a positive and negative control, respectively. Five replicates were carried out for each concentration and for the controls. The plates were incubated for 5 days. The number of L1/L2 and L3 in each well were counted under an inverted microscope at 40x magnification.

2.6. Larval migration test

Infective L3 larvae were obtained by faeces coproculture and collected by sedimentation using Baermann's devices (Van Wyk and Mayhew, 2013). This material was washed three times with PBS and transferred to a Falcon tube (Epprendorf ®) reservoir. The number of L3 in the reservoir was counted under an inverted microscope at 40x magnification through 10% aliquots (6-well plate, 6 counts) and then the suspension in the tube was diluted to reach a concentration of 500 L3 /mL. LMT was performed with live L3 stage larvae (1000/tube) which were added to Falcon (Epprendorf ®) tubes containing either 2 mL of the negative control (PBS; pH 7.2), an anthelmintic control (levamisole at 1.25 mg/mL), PCE or lignans at concentrations as described for the EHT. After incubation for 3 h at 28 °C, the L3 in each tube were washed with PBS and centrifuged (2054 x g) three times. The tubes were capped with 25 µm steel mesh and placed on a Petri dish. After 3 h of incubation, the number of larvae that migrated through the mesh was counted under an inverted microscope at 40x magnification, based on aliquots of 10% (5 counts) (Rabel et al., 1994).

2.7. Scanning electron microscopy (SEM) processing of L3 stage larvae

The larvae (L3 stage) for SEM analysis were recovered from the negative control and lignans (150 µg/mL) treatments in the larval migration test (five L3 for treatment). The L3 were individually preserved in 2% glutaraldehyde solution in phosphate buffer (0.1 M, pH = 7.4) and refrigerated at 4 °C until analysis. The fixed larvae were washed in phosphate buffer (0.1 M, pH = 7.4) and dehydrated in a graded acetone series (15%, 30%, 50%, 70%, 95% and 100%). The dehydrated larvae were dried by critical point drying with Leica EM CPD300 (Leica-Germany) and coated with gold for 2 min at 10 Å min^{-1}. Parasites were then observed with an EVO LS15 scanning electron microscope (ZEISS-Germany) at an accelerating voltage of 15 kV.

2.8. Statistical analysis

The PCE and lignans concentrations used in the statistical analysis were the initial concentrations divided in half due to the final dilution performed during the *in vitro* tests. Comparisons of mean percentages of hatching inhibition, larval development inhibition and larval migration inhibition at different concentrations with the controls were performed by one-way ANOVA followed by Tukey's test ($p < 0.05$) using Sisvar version 5.6 program (Universidade Federal de Lavras, MG, Brazil). The results were expressed as means ± S.E. EC_{50} (the effective concentrations to inhibit 50% of larvae hatching, larval development and larval migration of L3), R^2, hill slope (HS) and EC_{95} (the effective concentrations to inhibit 95% of larvae hatching, larval development and larval migration of L3) were calculated using a nonlinear regression analysis with 95% confidence (log(agonist) *vs.* response-variable slope), using the Graphpad Prism version 8.0 (Graphpad software).

3. Results

3.1. Plant materials, compound isolation and NMR identification

After extraction with ethanol, 500 g of the powered *P. cubeba* fruit furnished 75 g of PCE (15% yield). Sequential silica gel column chromatography of the crude methanol/water fraction from PCE (50 g) with a mixture of solvent in a crescent polarity (hexane /ethyl acetate, dichloromethane) produced three major compounds with mass of 1.04 g (compound **1**), 1.15 g (compound 2) and 1.00 g (compound 3). Results of the NMR analysis (Table 1) confirmed the structures of compounds **1**, **2** and **3** as those of hinokinin (HNK), cubebin (CB) and dihydrocubebin (DHC), respectively, (Fig. 1). The NMR data obtained for these compounds are in agreement with literature data (Silva et al., 2005; Laurentiz et al., 2015).

3.2. Egg hatch test

The percentages of egg hatch inhibition are presented in Table S1 and S2 (Supplementary material). PCE, in the highest evaluated concentrations, inhibited egg hatching by 100% (Table S1), showing ovicidal activity similar to the albendazole control (6.25 µg/mL); it had an EC_{50} of 200.0 µg/mL with 95% confidence interval (95% CI) range from 190.0 to 210.0, $R^2 = 0.99$ and HS = 1.2. HNK was the most efficient lignan to inhibit egg hatchability, with EC_{50} of 68.38 µg/mL (95% CI 58.00–85.70, $R^2 = 0.98$ and HS = 0.62). CB showed lower activity than HNK, with EC_{50} = 150.0 µg/mL (95% CI 143.9–172.0, $R^2 = 0.98$, HS = 0.87), however the lignan less active was the DHC with EC_{50} = 186.7 µg/mL (95% CI 173.2–201.2, $R^2 = 0.98$, HS = 0.90) (Table 2). The values of HS and R^2 (Table 2) indicate that the PCE had an effect dose-dependent higher than the lignans. EC_{95} value for PCE was 2300 µg/mL (95% CI 2059–2570), for CB, DHC and HNK could not be determined because the evaluated concentrations did not reach inhibitions percentage (Table S2) values necessary for the accurate EC_{95} calculations.

3.3. Larval development test

PCE inhibited the development of L1 to L3, even at the lowest evaluated concentration (Table S1), with an EC_{50} of 83.00 µg/mL (95% CI 60.50–114.10), $R^2 = 0.89$ and HS = 0.54. CB showed EC_{50} value of 14.89 µg/mL (95% CI 10.87–20.38), $R^2 = 0.93$ and HS = 3.03, while DHC with EC_{50} of 30.75 µg/mL (95% CI 26.86–35.2), $R^2 = 0.95$ and HS = 1.25 (Table 2) was the lignan less active. EC_{50} value for HNK could not be determined, because it exhibited 100% of activity at all evaluated concentration. DHC showing higher dose-dependent effect when compared to PCE (Table 2). Although HS for CB have been 3.03 this values cannot be used to evaluate dose-dependent effect, because only in the concentration of 35.00 µg/mL there was percentage of inhibition different of 100% (Table S2). Among the EC_{95} values determined, CB showed EC_{50} = 39.48 µg/mL (95% CI 37.48–41.59) while for DHC and PCE the EC_{95} were 320.00 µg/mL (95% CI 233.2–437.3) and 20,300 µg/mL (95% CI 9,610–42,880), respectively, (Table 2). PCE was the least active with the highest confidence interval (95% CI). Overall, larvae were more sensitive to PCE and lignans than eggs and HNK was the most active (Table S2).

3.4. Larval migration test

PCE and lignans significantly inhibited larval migration ($P < 0.05$). PCE showed 100% efficacy, in all concentrations, with activity similar to the positive control (Table S1). The EC_{50} values for CB and HNK were respectively, 0.89 µg/mL (95% CI 0.55.60-1.43) and 0.34 µg/mL (95% CI 0.09–1.02). The R^2 values of 0.9 for CB and 0.84 for HNK together with HS values (0.19 and 0.20, respectively for CB and HNK) indicate a lower dose-dependent effect of these compounds on LMT than on EHT and LDT. The percentage of inhibition for PCE and DHC at the lowest concentration (35 µg/mL) did not reach values below 50% for obtaining of accurate EC_{50} calculations, so their EC_{50} could not be determined (Table 2). DHC showed EC_{95} = 240 µg/mL (95% CI 145.0–384.4), for other lignans and PCE the EC_{95} could not be determined, because the evaluated concentrations did not reach inhibitions percentage values necessary for the accurate EC_{95} calculations (Table S1 and S2).

3.5. Larval migration test and scanning electron microscopy (SEM)

Structural changes induced in the L3 isolated from LMT performed with the lignans were assessed using SEM (Fig. 2A–D). The main changes observed between control (Fig. 2A) and treated L3 involved mainly the surface of the body (cuticle) (Fig. 2B–D). L3 cuticle was injured, with internal content exposure and loss of cylindrical shape mainly after treatment with HNK and DHC (Fig. 2C and D).

Table 1
^{1}H and ^{13}C NMR chemical shifts δ (ppm), multiplicities and coupling constants J for hinokinin (**1**), cubebin (**2**) and dihydrocubebin (**3**) in $CDCl_3$.

	δ_H (multiplicity, H number and J)	δ_C
Hinokinin (**1**)	6.8-6.4 (m, H arom), 5.9 (br.s, 4H), 4.15 (dd, 1H, J = 7.1 Hz and J = 9.3 Hz), 3.85 (dd, 1H, J = 7.1 Hz and J = 9.1 Hz), 3.0 (dd, 1H, J = 5.1 Hz and J = 14.2 Hz), 2.85 (dd, H, J = 7.3 Hz and J = 14.2 Hz), 2.6 (d, 2H, J = 7.1 Hz), 2,55 (m, 1H), 2.45(d, 1H, J = 8.6 Hz), 2.4 (m, 1H).	178.4, 147.9, 147.8, 146.5, 146.4, 131.6, 131.3, 122.2, 121.55, 109.4, 108.8, 108.4, 108.3, 101.0, 71.2, 46.4, 41.3, 38.4, 34.8.
Cubebin (**2**)	6.8-6.5 m (6H arom), 6.6 (s, 4H), 5.2 (s, 1H), 4.1 (dd, 1H, J = 7.1 Hz and J = 14.4 Hz), 4.0 (dd, 1H, J = 7.1 Hz and J = 8.6 Hz), 3.8 (dd, 1H, J = 7.8 Hz and J = 8.4 Hz), 3.6 (dd, 1H, J = 7.1 Hz and J = 8.4 Hz), 2.7-2.2 (m, 4H), 2.0 (m, 1H).	148.0, 147.9, 143.6, 146.1, 134.9, 134.5, 122.1, 121.8, 109.7, 109.5, 108.6, 108.5, 101.3, 101.2, 99.2, 72.6, 53.5, 46.2, 39.6, 34.0.
Dihydrocubebin (**3**)	6.6 (m, 6H), 5.8 (s, 4H), 3.7 (dd, 2H, J = 1.3 Hz and J = 11.3 Hz), 3.4 (dd, 2H, J = 4.1 Hz and J = 11.3 Hz), 3.2 (s, 2H), 2.7 (d, 1H, J = 8.7 Hz), 2.65 (d, 1H, J = 8.7 Hz), 2.56 (d, 1H, J = 5.7 Hz), 2.51 (d, 1H, J = 5.7 Hz), 1.75 (m, 2H).	147.6, 145.7, 134.3, 121.9, 109.3, 108.1, 100.8, 60.2, 44.3, 35.9.

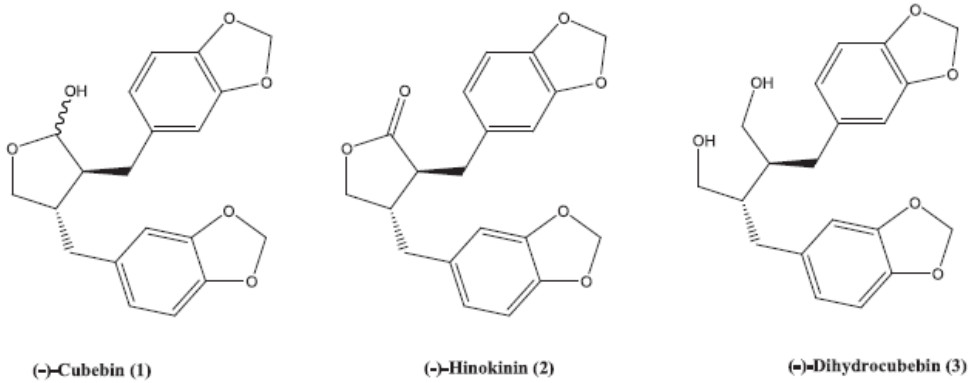
4. Discussion

In the present study, we investigated the *in vitro* anthelmintic effects of PCE and its isolated compounds against gastrointestinal nematodes from sheep. The lignans CB, HNK and DHC were isolated from PCE as the major constituents. The *in vitro* evaluation showed that PCE presented activity against eggs and larvae of the parasite, at all evaluated concentrations. In addition, the lignans CB, HNK and DHC when evaluated separately showed significant *in vitro* anthelminthic activity, superior to that presented by the crude extract, mainly in relation to their ovicidal action.

Studies on anthelmintic activity of other plant of the *Piper* genus were described by Gaínza et al. (2016) which evaluated the effects of the essential oil of *P. aduncum*, against eggs and larvae of *H. contortus*. The authors obtained results lower than those described in our study, with ED_{50} values of 5.72 mg/mL and 0.1 mg/mL, respectively, for the EHT and LDT. *P. aduncum* essential oil contains no lignans and anthelmintic activity has been attributed to its main constituent, dillapiole, which is a phenylpropanoic compound. In another study carried out by Carvalho et al. (2012), the methanol extract of *P. tuberculum* was evaluated against GINs and presented excellent ovicidal activity with an ED_{50} of 0.031 mg/mL. However, this activity was attributed to a set of piperamide compounds and not the lignans. Other plants of the genus *Piper* have been reported to have anthelmintic properties against several types of parasites (Adate et al., 2012; Koorse et al., 2018; Paul et al., 2018). However, our study is the first to report the activity of *P. cubeba* on the eggs and larvae of gastrointestinal nematodes of sheep and to relate this activity to the presence of individual lignans in the compounds found in *P. cubeba*. There are no previous reports on the *in vitro* evaluation of lignans against this class of parasite. The promising activity we have presented for these lignans raises an interest in studying the mechanism by which they act in the different stages of the life cycle of parasite. It is clear that they are able to cross and damage the cuticle of eggs and larvae, preventing hatching, larval development and motility.

Although effective against all stages of the life cycle of the parasite that were evaluated, both PCE and lignans were most active against larvae. Differences in the structure of the egg membrane and the cuticle of larvae may interfere with the anthelmintic activity of the compounds evaluated and alter their mechanisms of action. The nematode cuticle is an extracellular protein complex with trace amounts of lipid and carbohydrate that can vary between developmental stages (Mansfield et al., 1992; Riou et al., 2005). These differences may have contributed to higher PCE and lignan efficiency in larvae than in eggs, however is not the only factor for be considerate. Just like in our result, Oliveira et al. (2017) also found results with higher values of EC_{50} for EHT than for LDT in the *in vitro* anthelmintic evaluation of eight plant extracts from Brazilian savanna. However Araújo Filho et al. (2018) found higher values of EC_{50} for LDT than EHT in the *in vitro* anthelmintic evaluation of the *Eucalyptus citriodora* essential oil. The results of these two studies demonstrate that the greater or lesser ovicidal or larvicidal activity of a compound (plant extract or essential oil) depends not only on the morphological differences between eggs or larvae, but also on their chemical nature.

Lignans caused serious lesions in the integument of larvae that led to death, as observed in SEM studies, especially in relation to treatment with HNK (Fig. 2C) and dihydrocubebin (Fig. 2D). SEM assesses the interaction of compounds with the helminth cuticle and has been used to demonstrate direct effects of compounds with potential anthelmintic effects (Martínez-Ortíz-de Montelhano et al., 2013; Andre et al., 2016). The effect of the lignans on the cuticle of the larvae was different from that obtained by Engstrom et al. (2019) when evaluating the effect of tannins obtained from various plant sources. According to the authors, the damage to the cuticle of the larvae caused by tannins was small and isolated, while with lignans we observed that the lesions were over the entire body of the parasite. The loss of motility of the L3 after treatment with lignans occurred due to cuticle damage and cylindrical form alterations, and not due to paralysis as occurs with ivermectin (Laing et al., 2017). The cuticle provides the worms their shape. It is also involved in their motility and in the exchanges with the parasite



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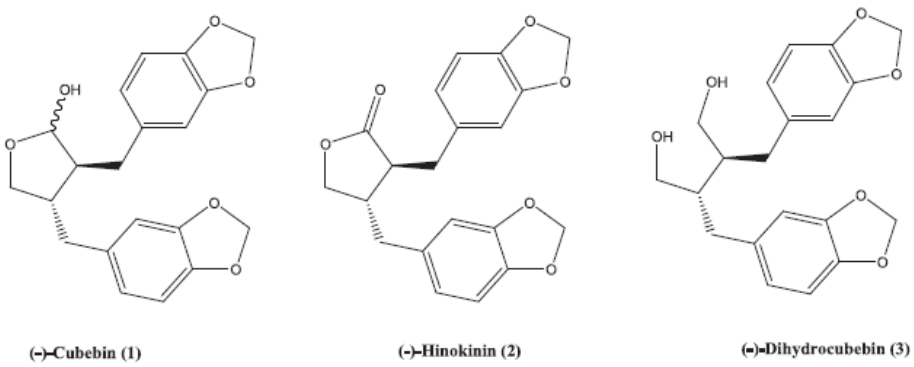

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.108932.

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4. Sínteses de lignanas e de compostos análogos

Começo agora a comentar alguns dos trabalhos que realizei relacionados à síntese de lignanas e de compostos análogos.

4.1. Trypanocidal structure–activity relationship for cis- and trans-methylpluviatolide

O primeiro trabalho que descrevo tem relação não só com a síntese, mas também com o estudo sobre a esteroquímica das lignanas. Lignanas dibenzilbutirolactônicas naturais geralmente possuem estereoquímica *trans* em relação aos hidrogênios ligados aos carbonos α e β do anel lactônico devido à maior estabilidade do estereoisômero *trans*. Esse artigo que apresento agora traz justamente o efeito da esteroquímica e da pureza enantiomérica sobre as propriedades biológicas da lignana metilpluviatolideo, um composto com ação tripanocida. Neste artigo foram sintetizadas as misturas racêmicas do *cis* e *trans* metilpluviatolideo, as quais foram submetidas a ensaios tripanocida. O racemato *trans* apresentou atividade tripanocida, porém o racemato *cis* foi completamente inativo. O racemato *trans* então foi submetido à separação por cromatografia quiral onde os enantiômeros (+) e (-) foram separados e novamente submetidos aos ensaios tripanocida. O enantiômero (+) foi inativo e o (-) apresentou maior atividade tripanocida do que a mistura racêmica, em doses equivalentes, o que comprova a importância da estereoquímica visto que o enantiômero (+) além de ser inativo ainda atua como um possível antagonista do enantiômero (-).

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Trypanocidal structure–activity relationship for *cis*- and *trans*-methylpluviatolide

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ABSTRACT

The trypanocidal activity of racemic mixtures of *cis*- and *trans*-methylpluviatolides was evaluated *in vitro* against trypomastigote forms of two strains of *Trypanosoma cruzi*, and in the enzymatic assay of *T. cruzi* gGAPDH. The cytotoxicity of the compounds was assessed by the MTT method using LLC-MK2 cells. The effect of the compounds on peroxide and NO production were also investigated. The mixture of the *trans* stereoisomers displayed trypanocidal activity (IC_{50} ~89.3 μM). Therefore, it was separated by chiral HPLC, furnishing the (+) and (−)-enantiomers. Only the (−)-enantiomer was active against the parasite (IC_{50} ~18.7 μM). Despite being inactive, the (+)-enantiomer acted as an antagonistic competitor. *Trans*-methylpluviatolide displayed low toxicity for LLC-MK_2 cells, with an IC_{50} of 6.53 mM. Furthermore, methylpluviatolide neither inhibited gGAPDH activity nor hindered peroxide and NO production at the evaluated concentrations.

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1. Introduction

The lignan class of compounds presents a conspicuous chemical diversity, which varies substantially with respect to their enantiomeric composition. Natural lignans usually occur as either predominantly one enantiomer or as enantiomeric mixtures of various enantiomeric compounds (% e.e'. values) (Umezawa et al, 1997a).

In the last decade, the search for antiprotozoal compounds from natural sources has intensified (Rocha et al., 2005; Da Silva Filho et al., 2004; Caniato and Puricelli, 2003). This search has been mainly in plants, which continue to be a major source of biologically active metabolites that may provide lead compounds for the development of new drugs.

The enantiomeric properties of lignans can be summarized into four statements. First, dibenzylbutyrolactone lignans can be optically pure (>99% e.e.), while furofuran and furan lignans can be found as a mixture of both enantiomers of varying enantiomeric composition (Umezawa et al., 1997a). Secondly, most dibenzylbutyrolactone lignans are levorotatory, except for the lignans present in *Thymelaeaceae* plants and *Selaginella doederleinii*, which are dextrorotatory (Chen et al., 2001; Lin et al., 1994; Umezawa and Shimada, 1996; Umezawa et al., 1997a; Xu et al., 2001). Third, the predominant enantiomers of furofuran, furan, and dibenzylbutane lignans vary among plant species, and they differ even within the organs of a single plant species (*Arctium lappa*) (Umezawa et al., 1991; Umezawa et al., 1992; Umezawa et al., 1997b). Fourth, the absolute configurations of the predominant enantiomers of various lignans isolated from a single plant specimen are sometimes different.

In nature, it is possible to find two enantiomeric forms of the same lignan, but these forms bear different stereochemistry, depending on the plant species in which it occurs. In the meantime, only one of the lactone lignan enantiomers, (+)- or (−), has been found to occur in the same plant. Therefore, biological assays carried out with either plant extracts containing lignan lactones or its isolated lignans generally allow the evaluation of only one enantiomer, making it impossible to determine whether the other enantiomer displays activity.

Although lignan lactones are present in numerous plants used in popular medicine (Bianchi et al., 1968; Broomhead and Dewick, 1990; Cho et al., 1999), their trypanocidal activity against *Trypanosoma cruzi* has only recently been discovered by Bastos et al. (1999), who has investigated the trypanocidal activity of the *Zanthoxyllum naranjillo* lignans, and discovered one of the most powerful compounds regarding trypanocidal activity, (-)-methylpluviatolide (**1**) (Bastos et al., 1999; Silva et al., 2003), (Fig. 1).

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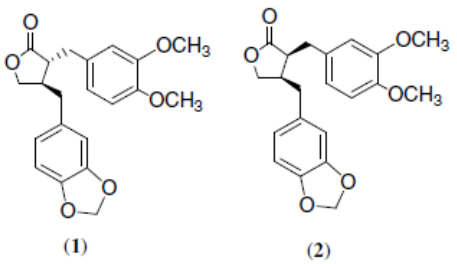


Fig. 1. Structural formulas of *trans*-methylpluviatolide (**1**) and *cis*-methylpluviatolide (**2**).

Lignans, one of the longest known classes of natural products, have attracted much interest over the years on account of their broad range of biological activities, such as antileishmanial (Barata et al., 2000), antimalarial (Zhang et al., 2001), antitumor (Bastos et al., 1996), and anti-inflammatory (Souza et al., 2004) activities, among others. Recently, our group reported the significant trypanocidal and antileishmanial activities of dibenzylbutyrolactone lignans (Souza et al., 2005; Royo et al., 2003; Bastos et al., 1999). Such results aroused the interest within our research group to study the effect of the stereochemistry and the absolute configuration of methylpluviatolide on its trypanocidal activity. To this end, methylpluviatolide was synthesized in its *trans* (**1**) and *cis* (**2**) racemic forms (Fig. 1), allowing us to evaluate the trypanocidal activity not only of a mixture of these two stereoisomers, but also of the pure enantiomers, which were separated by chiral HPLC.

2. Results and discussion

The results obtained against *T. cruzi* show that the racemic *cis*-stereoisomer (**2**) is inactive, while the racemic *trans*-stereoisomer (**1**) displays trypanocidal activity, with an IC_{50} ~89.3 μM. Furthermore, the latter stereoisomer (**1**) presents low toxicity for LLC-MK_2 cells, with an IC_{50} ~6.53 mM. (Fig. 2).

Our result is different from that obtained for the pure (−)-*trans*-methylpluviatolide (**1**) by Bastos et al. (1999), who reported 100% activity at a concentration of 50 μg and an IC_{50} value of 1.3 μg mL^{-1}. This result indicates that the *trans* isomer of this compound should display higher trypanocidal activity. However, it should be pointed out that the obtained results were undertaken for the racemic mixture of *cis* and *trans*-methylpluviatolide isomers. The (+/−)-*cis* (**1**) displayed the following percentage of lysis: 8.0 μM (1.4 ± 1.2), 32.0 μM (8.7 ± 2.7), 128.0 μM (16.8 ± 3.9) and 256 μM (32.2 ± 4.7), with an IC_{50} (μM mL^{-1}) of 641.0. The (+/−)-*trans* (**2**) displayed the following percentage of lysis: 8.0 μM (24.6 ± 1.9), 32.0 μM (39.2 ± 1.0), 128.0 μM (55.0 ± 5.6) and 256 μM (67.9 ± 0.0), with an IC_{50} (μM mL^{-1}) of 89.3.

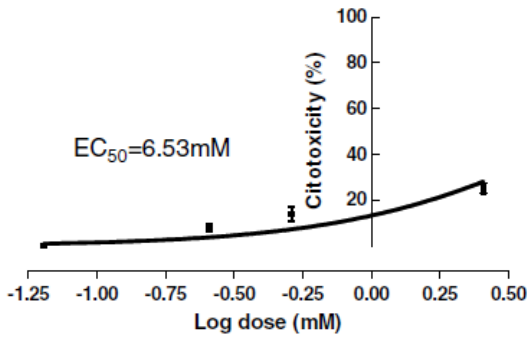


Fig. 2. Cytotoxic potential of (−)-methylpluviatolide (**1**) for LLC-MK2 cells.

Although the concentrations used by Bastos et al. (1999) differed from ours, the fact that the racemic mixture of the *trans* stereoisomer evaluated this time displayed lower activity than the pure compound evaluated before might indicate that the (+)-*trans* enantiomer should compete for the active sites in the parasite and might act as an antagonist in relation to the (−)-*trans* enantiomer.

On the basis of these results, a separation of the *trans*-stereoisomer from the racemic mixture was undertaken by chiral HPLC using an analytical Chiracel OJ (4.6 × 250 mm) column, aiming to evaluate the trypanocidal activity of each enantiomer separately. The chromatogram gave a well resolved peak separation, allowing the isolation of both enantiomers. Therefore, it was possible to compare the trypanocidal activity of both isomers, since in our previous work only the (−) isomer from *Zanthoxylum narangillo* was isolated (Bastos et al., 1999). The results showed that the (+) – *trans*-methylpluviatolide (**1**) displayed the following percentage of lysis: 8.0 μM (5.3 ± 2.5), 32.0 μM (7.6 ± 3.4) and 128.0 μM (9.9 ± 2.5), with IC_{50} (μM mL^{-1}) of 1.3 × 10^6. The (−) – *trans*- methylpluviatolide (**1**) displayed the following percentage of lysis: 8.0 μM (40.6 ± 3.6), 32.0 μM (52.3 ± 4.3) and 128.0 μM (79.7 ± 0.0), with IC_{50} (μM mL^{-1}) of 18.7.

The results show that the (+)-enantiomer is completely inactive, whereas the (−)-enantiomer displayed good activity, with an IC_{50} (μM) = 18.7 and 79.7% lyses, at concentrations lower than those used by Bastos et al. (1999), which corresponded to 50 μg/mL, IC_{50} = 1.3 μg and 100% lyses. These results indicate that, despite being completely inactive, the (+)-enantiomer of (**1**) blocks the action of the (−)-enantiomer when they are present in a racemic mixture. It should be taken into consideration that the (+)-enantiomer might bind to the active sites as a competitive antagonist, which may be confirmed by comparison of the IC_{50} value (μM) of the racemic mixture with that of the (−)-enantiomer itself.

Some metabolic routes were evaluated, such as the methylpluviatolide mechanism of action. In this regard, trypanosomatids are highly dependent on glycolysis for ATP production (Opperdoes, 1987; Opperdoes and Michels, 2001). The enzyme glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) catalyzes the oxidative phosphorylation of D-glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate in the presence of NAD^+ and inorganic phosphate, and can be one of the preferred steps for inhibition (Bakker et al., 1999; Bakker et al., 2000). However, the obtained results demonstrated that methylpluviatolide does not exert inhibitory activity on this enzyme, displaying 0% inhibition at 100 μM.

The effect of methylpluviatolide (**1**) on nitric oxide and H_2O_2 production is shown in Figs. 3 and 4, respectively. The trypanocidal activity of several types of natural and synthetic compounds has been partly attributed to reactive oxygen species generated by the reduction of drugs (Uchiyama et al., 2005). Regarding the obtained results, the production of NO by cells was significantly lower when treated with methylpluviatolide (Figs. 3 and 4). Likewise, the literature reports that benzonidazole, a drug used for the treatment of Chagas disease, also inhibits the production of NO by modifying the balance between pro and anti-inflammatory mediators, with important consequences for the development of the disease (Piaggio et al., 2001; Stoppani, 1999). Fig. 4 shows that methylpluviatolide displays a weak effect in enhancing the peroxide production by *T. cruzi*. Some drugs, such as nifurtimox and gentian violet, act in the parasite by increasing the production of free radicals (Raether and Hanel, 2003).

Moreover, *T. cruzi* is more sensitive to free radicals, as it has a deficient antioxidant mechanism in comparison with host cells. This is especially the case for hydrogen peroxide, as *T. cruzi* does not produce catalase, an antioxidant enzyme responsible for the conversion of hydrogen peroxide into water and molecular oxygen

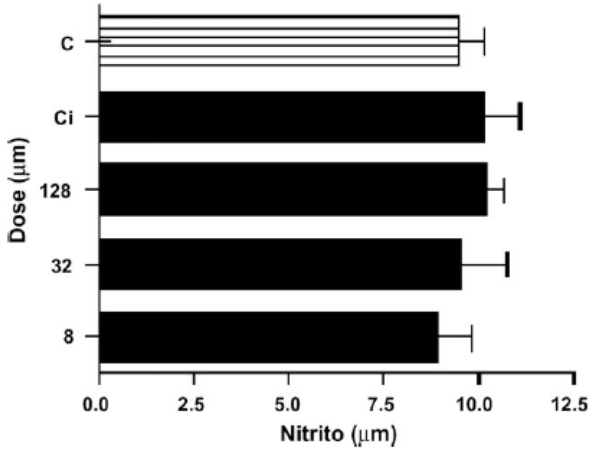


Fig. 3. Nitric oxide production of mouse peritoneal macrophages treated with methylpluviatolide (**1**). (C: cells control without *T. cruzi* infection; Ci: infected cells control).

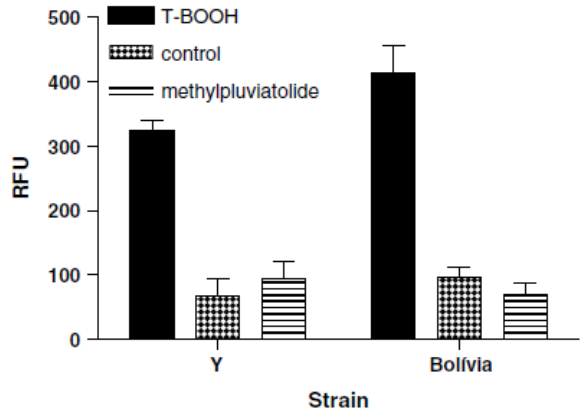


Fig. 4. Effects of methylpluviatolide (**1**) on H_2O_2 produced by *T. cruzi*. (RFU: relative fluorescence unit; T-BOOH: *t*-butyl hydroperoxide).

(Turrens and Mccord, 2006). Therefore, we could conclude that the trypanocidal mechanism of action of this class of compounds might not be related to the increase in the production of peroxides.

Recently, we have described the racemic 7′-oxi-methylpluviatolide, racemic 7′-hydroxi-methylpluviatolide and racemic 7′-oxi-6-nitro-methylpluviatolide as potential respiratory chain complex I inhibitors, a finding of possible relevance due to either the toxicological or therapeutical importance of these compounds (Saraiva et al., 2005).

Therefore, other metabolic routes should be evaluated to elucidate the mechanisms of action of (−)-methylpluviatolide.

3. Conclusion

In conclusion, it is possible to state that the *trans*-stereochemistry is the one responsible for the trypanocidal activity of methylpluviatolide (**1**), probably because it allows the correct binding of this compound to the active sites on the parasite, which is not possible with the *cis*-stereoisomer. As for the absolute configuration of the *trans*-stereoisomers, the inactive (+)-enantiomer probably acts as an antagonist, hindering the binding of the (−)-enantiomer to the active sites on the parasite, therefore decreasing the trypanocidal activity of methylpluviatolide when it is present as a racemic mixture.

4. Experimental

4.1. Reagents, solvents and compound identification procedures

Sulfanilamide, L-glutamine, tetrazolium salt, Triton x, $NaHCO_3$, 20,70-dichlorodihydrofluorescein diacetate (H_2DCFDA), *t*-butyl hydroperoxide and Hepes-KOH were purchased from Sigma–Aldrich Co., St. Louis, MO, USA. Dimethyl sulfoxide (DMSO) isopropanol and sucrose were purchased from Merck Co., whereas RPMI-1640 and fetal calf serum were obtained from GIBCO. Penicillin and streptomycin were acquired from Invitrogen. Optical rotations were measured at $k = 589$ nm on a Schmidt–Haensch polartronic HH8 polarimeter (Berlin, Germany) using a 1.0 cell. A high-performance liquid chromatography (HPLC) apparatus equipped with an LC-10ADVP pump, an SPD-M10AVP diode array detector and a DGU-14A degasser (Shimadzu, Kyoto, Japan) was used for both isolation and purity determination of the methylpluviatolide isomers, using an analytical Chiracel OJ (4.6 × 250 mm) column at a mobile phase flow rate (EtoH *n*-hexane: 65:35, v/v) of 0.9 mL/min and sample volume injection of 20 μL. The solvents used were of chromatographic grade.

4.2. Preparation of compounds

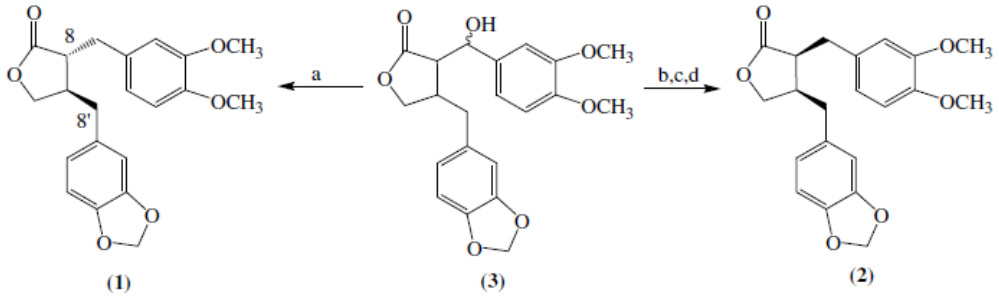
Cis and *trans*-methylpluviatolide were prepared from intermediate **3**, which was obtained by a procedure described by Landais et al. (1991) and Charlton and Chee (1997). Hydrogenolysis of **3** under the conditions described in Scheme 1 furnished the *trans*-steroisomer (**1**) in 80% yield. Acetylation of **3** followed by an elimination reaction with DBU produced a benzylidenebenzyl lignan, which furnished the *cis*-steroisomer (**2**) in 78% yield after hydrogenation (scheme 1).

Coupling constants of 15.2 Hz and 3.8 Hz between protons 8 and 8′ confirmed the *trans*- and *cis*-geometries for stereoisomers **1** and **2**, respectively.

The *trans*-methylpluviatolide racemic mixture was separated by chiral HPLC, and the fraction corresponding to each peak was collected and analyzed in the polarimeter for determination of the specific rotation of each enantiomer. The peak with a retention time of 36.20 min was identified as (+)-methylpluviatolide (**1**) $[\alpha]_D^{25}$ +20.8 (*c* 1.51, $CHCl_3$), and the peak with a retention time of 43.70 min was identified as (−)-methylpluviatolide (**1**) $[\alpha]_D^{25}$ -20.8 (*c* 1.51, $CHCl_3$).

4.3. Parasites and culture procedure

Trypomastigote forms of the Y strain of *T. cruzi* were used for the trypanocidal assay and were cultivated in the $LLMCK_2$ cell lineage (Norval, 1979). The cells were cultivated in RPMI-1640 medium (GIBCO), supplemented with 2 mM L-glutamine, 10 mM $NaHCO_3$, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5% inactivated fetal calf serum. The culture was kept at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. Blood samples containing trypomastigote forms were added to the culture cell at a ratio of 3:1, the supernatant was removed after 24 h, and the medium was replaced. After 7 days under the same culture condition, the supernatant was removed and centrifuged, furnishing free trypomastigote forms of the parasite for the bioassay. The free trypomastigote forms were transferred to a 96-well microtitre plate, each well was filled with 1×10^6 trypomastigote forms of the parasite, and each test compound was added to the wells. After 24-h incubation, activity was evaluated by using a colorimetric technique at 595 nm with the addition of a tetrazolium salt [MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], as described by Muelas-Serrano et al. (2000). Negative (solvent plus medium) and positive (gentian violet at 250 μg/



(1) (3) (2)

Reaction condition: (a) H_2, 4 atm, Pd/C, EtOH, $HClO_4$, 60 h, rt; (b) THF, Ac_2O, Et_3N, DMAP, 2h, rt; (c) DBU, CH_2Cl_2, 5h, rt; (d) H_2, 4 atm, Pd/C, EtOH, 4h, rt.

Scheme 1.

mL) controls were also run in parallel. All the assays were undertaken in triplicate.

4.4. Cytotoxicity assay

The cytotoxic action of the compound was evaluated by the MTT method as described by Sieuwerts et al. (1995). In a 96-well microtitre plate, LLC-MK2 cells (5×10^5 cells/well) were cultivated in RPMI-1640 medium supplemented as described above. The compound was evaluated at concentrations of 64, 256, 512, and 2560 μM for 24 h, at 37 °C, under a 5% CO_2 and 95% humidity atmosphere. After this period, the cells were incubated with MTT (5 mg/mL) for 240 min, and the reaction was interrupted by addition of acidified isopropanol. A microtitre plate reader (TECAN-SUNRISE) was used to obtain the results (570 nm). The negative (cells and solvent) and positive (cells treated with Triton X-100 20% solution) cytotoxicity controls were run in parallel. All assays were carried out in triplicate.

The formula % cytotoxicity = $[1 - (Y - M_d)/(N - M)] \times 100$ was used to calculate the cytotoxicity percentage, where Y is the mean optical density of wells with cells and different concentrations of compound; M_d is the mean optical density of wells with medium and different concentrations of compound; M is the mean optical density of wells with medium only; and N is the mean optical density of wells with cells and no compound (negative control). The level of 50% cellular cytotoxicity (CC_{50}) was obtained by a sigmoidal dose-response curve. Stock solutions were prepared by dissolving the compound in pure dimethylsulfoxide (DMSO), and aliquots of the stock solution were added to cultures to give a final concentration of DMSO lower than 0.2%.

4.5. Enzymatic inhibition studies

The enzymatic activities of the *T. cruzi* gGAPDH enzyme have been determined according to a previously reported procedure by Vieira et al. (2001), using the forward reaction. The percentage of remaining activity was calculated by comparison with an inhibitor-free control experiment.

4.6. Production of H_2O_2 and other peroxides

This activity was evaluated by quantification of the oxidation product of H_2DCFDA, a dichlorofluorescein compound (Carthcart et al., 1983). To this end, trypomastigote forms of *Y* and Bolivian strain of *T. cruzi* were cultured in the LLC-MK2 cell line (Hull et al., 1962) and suspended (1×10^6 parasites/ml) in a medium containing 125 mM sucrose, 65 mM KCl, and 10 mM Hepes-KOH, pH 7.2. Then, methypluviatolide (**1**) was added to the medium at a concentration of 32 μM, followed by addition of 5 μM H_2DCFDA, and the system was incubated at room temperature for 35 min. The parasites were then removed by centrifugation (8000*g*, 4 °C, 10 min), and the fluorescence of the supernatant was determined at 485 nm as described by Fang and Beattie (2003). *t*-Butyl hydroperoxide and a parasite-containing solution of 10% DMSO were used as positive and negative controls, respectively. The assays were performed in triplicate.

4.7. Nitric oxide measurement

The effects on the NO production by mouse peripheral macrophages were evaluated using the method previously reported by Giorgio et al. (1998). Briefly, peritoneal exudate cells were collected from the peritoneal cavities of male balb/c mice, previously injected intraperitoneally with 3% thioglycolate medium 96 h before, and washed with 6 mL of ice-cold phosphate-buffered saline (PBS). Cells were cultured in 96-well-flat-bottomed plates at 5×10^5 cells/well. The cultures were infected with *T. cruzi* at a 10:1 parasite:cell ratio. The methylpluviatolide was added to the medium at a concentration of 8, 32, and 128 μM. Cultures were incubated at 37 °C in 5% CO_2 for 24 h. Nitric oxide was measured in the supernatants by using the Griess reaction (Green et al., 1982). The Griess reagent was prepared by mixing equal volumes of 1% sulfanilamide in 5% H_2PO_4 and 0.1% naphthylethylene diamine dihydrochloride. Equal amounts of culture supernatant and Griess reagent were combined, and incubated for 10 min at room temperature. Absorbance measured at 540 nm was compared to a sodium nitrite standard curve and data were expressed in μM.

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4.2. Convenient synthesis of ketal derivatives from cubebin using amberlite as heterogeneous catalyst

O artigo apresentado a seguir descreve a síntese parcial de derivados da (-)-cubebina. A vantagem em se utilizar um produto natural como material de partida é que a esteroquímica já está definida e se não houver modificação nos centros assimétricos ela permanece inalterada. A modificação estrutural em produtos naturais pode fornecer derivados com propriedades biológicas potencializadas em relação ao material de partida e, por isso tem grande importância na área de produtos naturais, assim como foi a obtenção do etoposídeo e do tenoposideo a partir da podofilotoxina. Desta forma, neste trabalho o objetivo foi obter derivados semissintéticos da cubebina onde a modificação estrutural na cubebina ocorreu na hidroxila do lactol a qual foi substituída por um grupamento éter.

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CONVENIENT SYNTHESIS OF KETAL DERIVATIVES FROM CUBEBIN USING AMBERLITE AS HETEROGENEOUS CATALYST

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Marcio Luis Andrade e Silva,[2] and Paulo Marcos Donate[3]**

Eleven ketal derivatives were prepared from the natural product cubebin using Amberlite IR-120AR as heterogeneous catalyst and ROH as the source of the alkyl group.

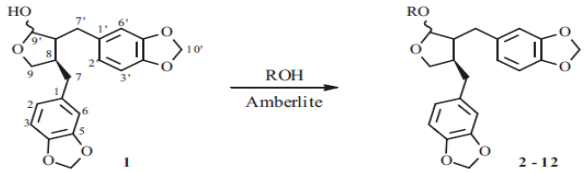
Keywords: cubebin, ketal derivatives, heterogeneous catalysis, Amberlite.

Cubebin [3,4-bis(benzo[d][1,3]dioxol-5-ylmethyl)-tetrahydrofuran-2-ol)] **1** is a dibenzylbutyrolactolic lignan that occurs in the stems, leaves, fruits, and roots of many plant species; it is particularly more widely distributed among the Asteraceae and Piperaceae [1–3]. Over the last decade, researchers have discovered that cubebin (**1**) displays new, important biological activities [4–18].

Studies on the analgesic, anti-inflammatory, antimicrobial, and trypanocidal actions of cubebin (**1**) have demonstrated that converting the lactol group into lactone or ketal provides derivatives with higher biological potential as compared with the starting material [10–14]. However, few cubebin derivatives have been obtained so far. Therefore, to conduct a broader spectrum of studies on the biological properties of these derivatives, it is necessary to synthesize a larger number of compounds.

When it comes to obtain ketal derivatives from cubebin, the *O*-alkylation promoted by hydride/alkoxide and alkyl halides has been the most often employed route [10, 12–14]. However, this methodology has several limitations: alkyl halides are toxic and expensive, anhydrous medium is mandatory, hydride or alkaline metals in excess are necessary, and secondary products arise during the synthesis.

Amberlite IR-120AR is an inexpensive and commercially available resin that has emerged as an efficient reagent for various chemical transformations, including ketal formation [19–21]. Cadote et al. used Amberlite IR-120 and methanol to obtain methyl ketals in sugars [19], albeit in low yields. We have adapted this method to synthesize 11 ketal derivatives of cubebin (**1**) in excellent yields using Amberlite IR-120 as a heterogeneous catalyst and alcohol (R-OH) as the source of alkyl groups and solvent at temperatures below 120°C.



1 **2 - 12**

2: R = CH_3; **3:** R = CH_2CH_3; **4:** R = CH_2=$CHCH_2$; **5:** R = $CH(CH_3)_2$
6: R = CH_2Ph; **7:** R = $C(CH_3)_3$; **8:** R = CF_3CH_2; **9:** R = CH≡CCH_2
10: R = cyclohexyl; **11:** R = $CH_3OCH_2CH_2$; **12:** R = $CH_3(CH_2)_4$

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TABLE 1. Optimization of the Reaction Conditions for the Synthesis of Ketal **2**,* %

Amberlite, mg	Reaction temperature, °C		
	30	80	120
0	0	0	0
10	40	70	18
20	50	95	30
30	50	90	29

*Product yield after 2 h of reaction.

TABLE 2. Synthesis of Ketals **2**–**12** from Cubebin (**1**)

ROH	Temperature, °C	Reaction time, h	Ketal	Yield, %
Methyl alcohol	80	2	**2**	95
Ethyl alcohol	80	2	**3**	93
Allyl alcohol	80	2	**4**	93
Isopropyl alcohol	80	3	**5**	90
Benzyl alcohol	100	24	**6**	70
tert-Butyl alcohol	80	3	**7**	90
Trifluoroethanol	80	3	**8**	80
Propargyl alcohol	80	3	**9**	80
Cyclohexyl alcohol	100	24	**10**	75
Methyl glycol	80	3	**11**	95
Amyl alcohol	80	3	**12**	95

Reaction conditions: cubebin (100 mg, 0.28 mmol), ROH (3 mL), Amberlite IR-120AR (20 mg).

To establish the optimum amount of resin and best experimental conditions, we performed several tests using methanol (3 mL) and cubebin (100 mg) while varying the amount of resin and the reaction temperature. We investigated the model reaction with four different amounts of Amberlite IR-120AR (0, 10, 20, and 30 mg) and at three temperatures (30, 80, and 120°C). We monitored the reactions by thin-layer chromatography every 15 min. Table 1 contains the results of the optimization tests.

In the absence of the catalyst, we did not detect the desired product at the evaluated temperatures. The reaction furnished the desired product for all amounts of Amberlite IR-120AR; however, 20 mg of the resin at a temperature of 80°C (cubebin–resin, 8:2) gave the best yield (95%) after 2 h.

A temperature of 30°C afforded lower yields; the resin began to decompose at 120°C, as seen from the appearance of a dark brown coloration and lower product yield. We used temperatures below120°C to synthesize the other ketals.

After optimization, we applied the reaction conditions to synthesize over 10 ketal derivatives from cubebin (**1**) using alcohols of different structures (Table 2).

The reaction times were proportional to the size of each "R" group of the alcohols (R-OH) because of the steric effect. Compounds with larger "R" group took longer to react and furnished lower product yields. The reactions with benzyl alcohol and cyclohexyl alcohol completely ended only after 24 h. For most of the alcohols, the reaction time ranged from 2 to 3 h. The size of the "R" group also affected the product yields: the yields decreased with increasing "R" size. Alcohols with smaller "R" groups furnished product yields between 80 and 95%; benzyl alcohol and cyclohexyl alcohol furnished 70 and 75% product yields, respectively. TLC analysis did not show any secondary product, but we recovered the starting materials after the synthesis of compounds **6** and **10**, during which we used benzyl alcohol and cyclohexyl alcohol, respectively. We confirmed the structures of compounds **2**–**12** by MS, PMR, and ^{13}C NMR. Because we extracted cubebin (**1**) from *Piper cubeba* [12], we obtained compounds **2**–**12** in the epimeric form at an epimer A–epimer B ratio of 60:40. Various signals overlapped in the PMR and ^{13}C NMR spectra. The ^{13}C NMR data presented here are mostly relative to epimer A.

EXPERIMENTAL

Amberlite IR-120AR was purchased from Carlo Erba Company. (–)-Cubebin (**1**) was isolated from the seeds of *Piper cubeba* as previously described in the literature [12]. PMR and ^{13}C NMR spectra were recorded on a Bruker DPX-400 spectrometer ($CDCl_3$ and $Si(CH_3)_4$ were used as solvent and internal standard, respectively). CG-MS was conducted on a Shimadzu GC-MS QP2010 Plus automatic injector AOC-20i spectrometer at 70 eV. Elemental analyses were performed on a Thermoquest CE EA/1110 CHNS-O analyzer. The reactions were monitored using TLC on silica gel 60G F_{254} aluminum sheets (Merck) using hexane–ethyl acetate (9:1) as eluent.

Compounds were detected by sulfuric vanillin solution with subsequent heating at 100–120°C for 2–3 min. Silica gel (70–230 mesh) was used for column chromatography.

General Procedure for Preparing Ketals 2–12 from Cubebin (1). The mixture containing (–)-cubebin (**1**) (0.100 g, 0.00028 mol), R-OH (3 mL), and resin Amberlite® IR-120AR (20 mg) was stirred under heating (80°C or 100°C) for some hours (Table 2). After the reaction was complete (TLC monitoring), the resin was filtered, the excess R-OH was evaporated under reduced pressure, and the residue was purified by column chromatography on silica gel, using hexane–ethyl acetate (9:1) as eluent.

3,4-Bis(benzo[d][1,3]dioxol-5-ylmethyl)-tetrahydrofuran-2-ol (1). White solid, mp 131–132°C. $C_{20}H_{20}O_6$. PMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 2.20 (5H, m, OH, H-8′, 7′β, 7α, 7β), 2.70–2.00 (2H, m, H-8, 7′α), 3.60 (1H, dd, J = 7.1, 8.4, H-9β, epimer B), 3.80 (1H, dd, J = 7.8, 8.4, H-9β, epimer A), 4.00 (1H, dd, J = 7.1, 8.6, H-9α, epimer B), 4.10 (1H, dd, J = 7.1, 14.4, H-9α, epimer A), 5.20 (1H, br.s, H-9′), 6.60 (4H, br.s, 2H-10, 10′), 6.80–6.05 (6H, m, H-2, 3, 6, 2′, 3′, 6′). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 38.3 (C-7), 39.0 (C-7′), 46.2 (C-8), 53.5 (C-8′), 72.6 (C-9), 99.2 (C-9′), 101.2 (C-10′), 101.3 (C-10), 108.5 (C-6′), 108.6 (C-6), 109.5 (C-3′), 109.7 (C-3), 121.8 (C-2′), 122.1 (C-2), 134.5 (C-1′), 134.9 (C-1), 146.1 (C-5′), 146.6 (C-5), 147.9 (C-4), 148.0 (C-4′). Mass spectrum (EI, 70 eV), *m/z* ($I_{rel.}$, %): 356 ([M]$^+$, 31), 203 (11), 161 (11), 135 (100), 77 (36).

5-((4-(Benzo[d][1,3]dioxol-5-ylmethyl)-5-methoxy-tetrahydrofuran-3-yl)methyl)-benzo[d][1,3]dioxole (2). $C_{21}H_{22}O_6$. PMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 2.70–1.90 (6H, m, H-7′α, 7′β, 7α, 7β, 8, 8′), 3.10 (3H, s, OCH_3, epimer B), 3.20 (3H, s, OCH_3, epimer A), 3.50 (1H, m, H-9β), 3.90 (1H, m, H-9α), 4.55 (1H, d, J = 4.6, H-9′, epimer B), 4.60 (1H, d, J = 1.3, H-9′, epimer A), 5.80 (4H, br.s, 2H-10, 10′), 6.80–6.50 (6H, m, H-2, 3, 6, 2′, 3′, 6′). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 39.1 (C-7′), 39.6 (C-7), 46.1 (C-8), 52.8 (C-8′), 55.1 (OCH_3), 72.4 (C-9), 101.2 (C-10, 10′), 105.8 (C-9′), 108.4 (C-6′), 108.5 (C-6), 109.5 (C-3′), 110.2 (C-3), 121.8 (C-2′), 122.3 (C-2), 132.4 (C-1′), 133.2 (C-1), 144.7 (C-5′), 144.8 (C-5), 146.6 (C-4), 146.7 (C-4′). Mass spectrum (EI, 70 eV), *m/z* (I_{rel}, %): 370 ([M$^+$], 10), 338 (14), 203 (45), 173 (19), 135 (100), 77 (28).

5-((4-(Benzo[d][1,3]dioxol-5-ylmethyl)-5-ethoxy-tetrahydrofuran-3-yl)methyl)-benzo[d][1,3]dioxole (3). $C_{22}H_{24}O_6$. PMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 1.20 (3H, t, CH_3 of ethyl group), 2.0 (2H, m, H-8, 7β), 2.60–2.40 (3H, m, H-7′β, 7α, 8′), 2.70 (1H, m, H-7′α), 3.40 (1H, m, H-9β), 3.65 (2H, m, CH_2 of ethyl group), 4.00 (1H, m, H-9α), 4.70 (1H, d, J = 4.2, H-9′, epimer B), 4.82 (1H, s, H-9′, epimer A), 5.94 (4H, s, 2H-10, 10′), 6.60–6.50 (6H, m, H-2, 3, 6, 2′, 3′, 6′). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 15.7 (CH_3), 39.0 (C-7′), 39.6 (C-7), 46.2 (C-8), 52.7 (C-8′), 63.2 (CH_2), 72.4 (C-9), 101.2 (C-10, 10′), 108.3 (C-9′), 108.4 (C-6′), 108.9 (C-6), 109.3 (C-3′), 109.6 (C-3), 121.8 (C-2′), 122.1 (C-2), 133.9 (C-1′), 134.7 (C-1), 146.1 (C-5′), 146.2 (C-5), 147.9 (C-4), 148.0 (C-4′). Mass spectrum (EI, 70 eV), *m/z* (I_{rel}, %): 384 ([M]$^+$, 10), 338 (23), 161 (38), 135 (100), 77 (28).

5-((5-(Allyloxy)4-(benzo[d][1,3]dioxol-5-ylmethyl)-tetrahydrofuran-3-yl)methyl)-benzo[d][1,3]dioxole (4). $C_{23}H_{24}O_6$. PMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 2.10 (1H, m, H-8), 2.80–2.20 (5H, m, H-8′, 7α, 7β, 7′α, 7′β), 3.58 (1H, t, J = 7.0, H-9β, epimer B), 3.65 (1H, t, J = 8.3, H-9β, epimer A), 3.90 (1H, m, methylenic H), 4.00 (1H, dd, J = 7.8, 16.1, H-9α), 4.50 (1H, m, methylenic H), 4.81 (1H, d, J = 4.6, H-9′, epimer B), 4.85 (1H, s, H-9′, epimer A), 5.15 (1H, m, H–vinylic CH_2=, epimers A and B), 5.22 (1H, dd, J = 1.5, 17.4, H–vinylic CH_2=, epimer A), 5.30 (1H, dd, J = 1.3, 17.4, H–vinylic CH_2=, epimer B), 5.90 (1H, m, H–vinylic CH=), 5.90 (4H, br.s, 2H-10, 10′), 6.60 (6H, m, H-2, 3, 6, 2′, 3′ 6′). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 39.0 (C-7), 39.6 (C-7′), 46.1 (C-8), 52.7 (C-8′), 68.4 (CH_2-O), 72.5 (C-9), 101.1 (C-10′), 101.2 (C-10), 104.1 (C-9′), 108.4 (C-6′), 108.5 (C-6), 109.3 (C-3′), 109.6 (C-3), 116.9 (vinylic CH_2), 121.8 (C-2′), 122.1 (C-2), 133.8 (C-1′), 134.7 (C-1), 135.1 (CH=), 146.2 (C-5′), 146.3 (C-5), 147.9 (C-4), 148.0 (C-4′). Mass spectrum (EI, 70 eV), *m/z* (I_{rel}, %): 396 ([M]$^+$, 5), 338 (20), 161 (31), 135 (100), 77 (20).

5-((4-(Benzo[d][1,3]dioxol-5-ylmethyl)-5-isopropoxy-tetrahydrofuran-3-yl)methyl)-benzo[d][1,3]dioxole (5). $C_{23}H_{26}O_6$. PMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 1.04 (3H, d, J = 6.2, CH_3 of isopropyl group, epimer A), 1.09 (3H, d, J = 6.2, CH_3 of isopropyl group, epimer B), 1.19 (3H, d, J = 6.5, CH_3 of isopropyl group, epimer A), 1.22 (3H, d, J = 6.2, CH_3 of

isopropyl group, epimer B), 2.1 (2H, m, H-8, 7α), 2.8–2.4 (4H, m, H-8′, 7′β, 7β, 7′α), 3.56 (1H, t, J = 7.0, H-9β, epimer B), 3.65 (1H, t, J = 8.1, H-9β, epimer A), 3.80 (1H, m, CH of isopropyl group), 4.00 (1H, m, H-9α, epimers A and B), 4.89 (1H, d, J = 4.7, H-9′, epimer B), 4.91 (1H, s, H-9′, epimer A), 5.90 (4H, br.s, 2H-10, 10′), 6.60 (6H, m, H-2, 3, 6, 2′, 3′ 6′). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 22.0 (CH_3), 24.2 (CH_3), 38.9 (C-7), 39.4 (C-7′), 46.3 (C-8), 52.8 (C-8′), 69.3 (CH of isopropyl group), 72.2 (C-9), 101.2 (C-10, 10′), 107.3 (C-9′), 108.3 (C-6′), 108.4 (C-6), 109.3 (C-3′), 109.6 (C-3), 121.8 (C-2′), 122.1 (C-2), 133.9 (C-1′), 134.8 (C-1), 146.1 (C-5′), 146.2 (C-5), 147.9 (C-4), 148.0 (C-4′). Mass spectrum (EI, 70 eV), *m/z* (I_{rel}, %): 398 ([M]$^+$, 5), 338 (15), 161 (32), 135 (100), 77 (21).

5-((4-(Benzo[d][1,3]dioxol-5-ylmethyl)-5-benzyloxy-tetrahydrofuran-3-yl)methyl)-benzo[d][1,3]dioxole (6). $C_{27}H_{26}O_6$. PMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 2.20–2.00 (2H, m, H-7β, 8), 2.30 (1H, dd, J = 7.8, 13.5, H-7′β), 2.70–2.50 (3H, m, H-8′, 7α, 7′α), 3.60 (1H, t, J = 9.9, H-9β), 3.92 (1H, t, J = 9.9, H-9α), 4.30 (1H, d, J = 12.20, CH_2 of benzyl group), 4.60 (1H, d, J = 12.20, CH_2 of benzyl group), 4.80 (1H, d, J = 1.5, H-9′, epimers A and B), 5.80 (4H, br.s, 2H-10, 10′), 6.50 (6H, m, H-2, 3, 6, 2′, 3′, 6′), 7.20 (5H, m, Ar-H of benzyl group). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 38.6 (C-7), 39.04 (C-7′), 45.9 (C-8), 52.5 (C-8′), 69.0 (CH_2 of benzyl group), 72.2 (C-9), 100.8 (C-10′), 100.9 (C-10), 103.9 (C-9′), 108.1 (C-6′), 108.2 (C-6), 108.9 (C-3′), 109.2 (C-3), 121.4 (C-2′), 121.8 (C-2), 128.7, 127.9, 127.5 (arom. C of benzyl group), 133.4 (C-1′), 134.2 (C-1), 138.1 (arom. C of benzyl group), 145.7 (C-5′), 145.9 (C-5), 147.5 (C-4), 147.6 (C-4′). Mass spectrum (EI, 70 eV), *m/z* (I_{rel}, %): 338 ([M$^+$], 108, 5), 203 (19), 135 (73), 81 (100).

5-((4-(Benzo[d][1,3]dioxol-5-ylmethyl)-5-*tert*-butoxy-tetrahydrofuran-3-yl)methyl)-benzo[d][1,3]dioxole (7). $C_{24}H_{28}O_6$. PMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 1.16 (9H, s, $3CH_3$ of *tert*-butyl group, epimer B), 1.28 (9H, s, $3CH_3$ of *tert*-butyl group, epimer A), 2.80–2.00 (6H, m, H-8, 8′, 7α, 7β, 7′α, 7′β, epimers A and B), 4.10–3.4 (2H, m, H-9α, 9β, epimers A and B), 5.2 (1H, br.s, H-9′, epimers A and B), 5.90 (4H, br.s, 2H-10, 10′), 6.60 (6H, m, H-2, 3, 6, 2′, 3′, 6′). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 31.6 (CH_3), 38.7 (C-7), 39.4 (C-7′), 46.1 (C-8), 52.7 (C-8′), 69.6 (C of *tert*-butyl group), 72.4 (C-9), 101.2 (C-10, 10′), 104.7 (C-9′), 108.4 (C-6′), 108.6 (C-6), 109.3 (C-3′), 109.7 (C-3), 121.8 (C-2′), 122.2 (C-2), 133.9 (C-1′), 134.7 (C-1), 146.2 (C-5′), 146.3 (C-5), 147.9 (C-4), 148.0 (C-4′). Mass spectrum (EI, 70 eV), *m/z* (I_{rel}, %): 412 ([M]$^+$, 5), 338 (19), 161 (31), 135 (100), 77 (31).

5-((4-(Benzo[d][1,3]dioxol-5-ylmethyl)-5-(2,2,2,-trifluoroethoxy)-tetrahydrofuran-3-yl)methyl)-benzo[d][1,3]dioxole (8). $C_{22}H_{21}O_6$. PMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 2.00 (1H, m, H-8, epimers A and B), 2.5 (2H, m, H-7β, 7′β epimers, A and B), 2.60 (1H, m, H-8′, epimers A and B), 2.70 (2H, m, H-7α, 7′α), 3.7 (2H, br.d, J = 7.3, CH_2-CF_3, epimers A and B), 3.80 (1H, dd, J = 9.9, 16.6, H-9β, epimers A and B), 4.10 (1H, dd, J = 8.3, 16.6, H-9α, epimers A and B), 4.85 (1H, d, J = 4.6, H-9′, epimer B), 4.9 (1H, br.s, H-9′, epimer A), 6.00 (4H, br.s, 2H-10, 10′), 6.70 (6H, m, H-2, 3, 6, 2′, 3′, 6′). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 38.4 (C-7), 39.2 (C-7′), 45.2 (C-8), 51.9 (C-8′), 62.8 ($\underline{C}H_2$-CF_3), 72.6 (C-9), 100.9 (C-10, 10′), 104.2 (C-9′), 108.6 (C-6′), 108.9 (C-6), 109.1 (C-3′), 109.2 (C-3), 121.6 (C-2′), 121.8 (C-2), 132.8 (CF_3), 133.9 (C-1′), 134.2 (C-1), 145.8 (C-5′), 145.9 (C-5), 147.5 (C-4), 147.6 (C-4′). Mass spectrum (EI, 70 eV), *m/z* (I_{rel}, %): 438 ([M]$^+$, 30), 161 (30), 135 (100), 77 (29).

5-((4-(Benzo[d][1,3]dioxol-5-ylmethyl)-5-(prop-2-ynyloxy)-tetrahydrofuran-3-yl)methyl)-benzo[d][1,3]dioxole (9). $C_{23}H_{22}O_6$. PMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 2.15 (2H, m, H-8′, 8), 2.45 (3H, m, H-7′β, 7α, 7β), 2.53 (1H, d, J = 7.8, HC≡), 2.7 (1H, m, H-7α′), 3.65 (1H, t, J = 8.3, H-9β), 4.00 (1H, dd, J = 8.3, 16.0, H-9α), 4.15 (1H, d, J = 2.3, O-CH_2-C, epimer B), 4.20 (1H, dd, J = 2.3, 7.8, O-CH_2-C, epimer A), 4.24 (1H, dd, J = 2.3, 7.8, O-CH_2-C, epimer A), 4.28 (1H, d, J = 2.3, -O-CH_2-C, epimer B), 4.95 (1H, d, J = 4.6, H-9′, epimer B), 5.90 (4H, br.s, 2H-10, 10′), 6.60 (6H, m, H-2, 3, 6, 2′, 3′, 6′). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 33.8 (C-7), 39.6 (C-7′), 46.1 (C-8), 52.6 (C-8′), 54.3 ($\underline{C}H_2$–C≡), 72.8 (C-9), 74.6 (HC≡), 79.8 (C≡), 100.9 (C-10, 10′), 107.2 (C-9′), 108.1 (C-6′), 108.1 (C-6), 108.9 (C-3′), 109.3 (C-3), 121.5 (C-2′), 121.8 (C-2), 133.2 (C-1′), 134.2 (C-1), 145.8 (C-5′), 145.9 (C-5), 147.5 (C-4), 147.6 (C-4′). Mass spectrum (EI, 70 eV), *m/z* (I_{rel}, %): 394 ([M]$^+$, 12), 338 (12), 203 (47), 135 (100), 77 (24).

5-((4-(Benzo[d][1,3]dioxol-5-ylmethyl)-5-cyclohexyloxy-tetrahydrofuran-3-yl)methyl)-benzo[d][1,3]dioxole (10). $C_{26}H_{30}O_6$. PMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 1.2 (6H, m, $3CH_2$ of cyclohexyl group), 1.70 (4H, m, $2CH_2$ of cyclohexyl group), 2.1 (1H, m, H-8), 2.75–2.40 (5H, m, H-7′α, 7′β, 7α, 7β, 8′), 3.30 (1H, m, CH of cyclohexyl group), 3.55 (1H, dd, J = 6.6, 8.6, H-9β, epimer B), 3.65 (1H, t, J = 8.1, H-9β, epimer A), 3.94 (1H, dd, J = 7.3, 8.6, H-9α), 3.98 (1H, t, J = 8.3, H-9α, epimer B), 4.93 (1H, d, J = 4.6, H-9′, epimer B), 4.94 (1H, d, J = 1.8, H-9′, epimer A), 5.90 (4H, s, 2H-10, 10′), 6.60 (6H, m, H-2, 3, 6, 2′, 3′, 6′). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 23.5, 24.3, 25.7, 33.9, 34.0 (CH_2 of cyclohexyl group), 38.5 (C-7), 39.6 (C-7′), 43.8 (C-8), 52.3 (C-8′), 71.8 (C-9), 74.8 (CH of cyclohexyl group), 100.8 (C-10, 10′), 106.7 (C-9′), 107.9 (C-6′), 108.1 (C-6), 108.9 (C-3′), 109.2 (C-3), 121.4 (C-2′), 121.8 (C-2), 134.4 (C-1′), 134.6 (C-1), 145.7 (C-5′), 145.8 (C-5), 147.4 (C-4), 147.5 (C-4′). Mass spectrum (EI, 70 eV), *m/z* (I_{rel}, %): 438 ([M]$^+$, 5), 338 (14), 161 (33), 135 (100).

5-((4-(Benzo[d][1,3]dioxol-5-ylmethyl)-5-(2-methoxyethoxy)-tetrahydrofuran-3-yl)methyl)-benzo[d][1,3]dioxole(11). $C_{23}H_{26}O_7$. PMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 2.20–2.00 (2H, m, H-8, 7′β), 2.5 (3H, m, H-7, 8′), 2.7 (1H, m, H-7′α), 3.38 (3H, s, OCH_3, epimer A), 3.39 (3H, s, OCH_3, epimer B), 3.60–3.50 (4H, m, CH_2–CH_2), 3.70 (1H, m, H-9β), 4.00 (1H, m, H-9α), 4.80 (1H, d, J = 4.5, H-9′, epimer B), 4.85 (1H, d, J = 1.5, H-9′, epimer A), 5.90 (4H, s, 2H-10, 10′), 6.60 (6H, m, H-2, 3, 6, 2′, 3′, 6′). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 38.5 (C-7), 39.1 (C-7′), 43.2 (C-8), 52.1 (C-8′), 59.0 (OCH_3), 66.3 ($OCH_2\underline{C}H_2OCH_3$), 71.9 (C-9), 72.0 ($O\underline{C}H_2CH_2OCH_3$), 100.8 (C-10, 10′), 104.5 (C-9′), 108.0 (C-6′), 108.1 (C-6), 108.9 (C-3′), 109.2 (C-3), 121.4 (C-2′), 121.8 (C-2), 133.4 (C-1′), 134.3 (C-1), 145.7 (C-5′), 145.8 (C-5), 147.5 (C-4), 147.6 (C-4′). Mass spectrum (EI, 70 eV), *m/z* (I_{rel}, %): 414 ([M]$^+$, 7), 338 (16.6), 203 (7.1), 161 (38), 135 (100).

5-((4-(Benzo[d][1,3]dioxol-5-ylmethyl)-5-pentyloxy-tetrahydrofuran-3-yl)methyl)-benzo[d][1,3]dioxole (12). $C_{25}H_{30}O_6$. PMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 2.10–2.00 (2H, m, H-8, 7′β), 2.8–2.4 (4H, m, H-7′α, 7α, 7β, 8′), 3.28 (1H, m, CH_2-O of amyl group), 3.60 (2H, m, H-9), 4.00 (1H, m, CH_2-O of amyl group), 4.75 (1H, d, J = 4.5, H-9′, epimer B), 4.79 (1H, d, J = 1.5, H-9′, epimer A), 5.90 (4H, s, 2H-10, 10′), 6.60 (6H, m, H-2, 3, 6, 2′, 3′, 6′). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 14.0 (CH_3 of amyl group), 22.5 (CH_2 of amyl), 28.5 (CH_2 of amyl), 29.3 (CH_2 of amyl), 38.6 (C-7), 39.1 (C-7′), 45.7 (C-8), 52.3 (C-8′), 67.5 (OCH_2 of amyl group), 71.8 (C-9), 100.8 (C-10, 10′), 108.0 (C-9′), 108.1 (C-6′), 108.7 (C-6), 108.9 (C-3′), 109.2 (C-3), 121.4 (C-2′), 121.8 (C-2), 133.5 (C-1′), 134.3 (C-1), 145.4 (C-5′), 145.8 (C-5), 147.5 (C-4), 147.6 (C-4′). Mass spectrum (EI, 70 eV), *m/z* (I_{rel}, %): 426 ([M]$^+$, 10), 338 (19), 203 (19), 161 (40), 135 (100).

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4.3. Synthesis of 4-azo-butenolides

Neste artigo sintetizamos 4-azo-butenolidas de forma mais rápida e com maior rendimento do que os descritos na literatura. Esses compostos são intermediários para a síntese de diidroquinolinas e de outros compostos heterocíclicos.

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Synthesis of 4-azo-butenolides

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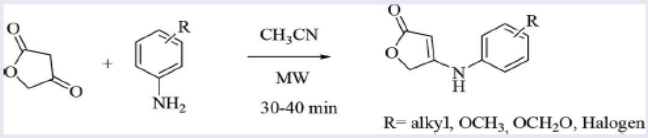
ABSTRACT

An efficient, catalyst-free, microwave-assisted approach has been developed for the synthesis of 4-azo-butyenolides derivatives by condensing tetronic acid with various anilines. This approach exhibited good functional group compatibility and produced the desired products in good to excellent yields in just 30–40 min. This approach can be seen as a better alternative to protocols with long reaction times used for the synthesis of these compounds, which are synthons for the obtaining of quinolines.

GRAPHICAL ABSTRACT



ARTICLE HISTORY
Received 2 May 2017

KEYWORDS
Microwave irradiation; quinolines synthons; short reaction time; substrate scope

Introduction

Heterocyclic natural or synthetic compounds are an important class of molecules for the development of new drugs.[1–3] Several of these compounds containing the quinoline nucleus display innumerous biological properties and are used as drugs against malaria, cardiovascular diseases, allergies, and vasodilators.[4–15] The search for new drugs more potent and with less side effects requires chemical strategies of synthesis that facilitate the obtaining of intermediates for the construction of heterocycles in a quick way and in smaller reaction times, speeding up the process of the synthesis until the biological evaluation.[16]

4-Azo-butenolides are synthons for the synthesis of quinolines, dihydroquinolines, lactones, and a large amount of derivatives of great pharmacological interest.[17–20] Despite the importance of these compounds, the syntheses described in the literature use reaction conditions that involve long reaction times and expensive purification processes.[20] These conditions make it difficult to obtain these intermediates for the construction of more complex molecules in quantity and structural variety for biological evaluation. In this work, a new method for the synthesis of 4-azo-butenolide derivatives from reaction between tetronic acid (1) and anilines promoted by microwave is described.

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Supplemental data (full experimental detail, ^{1}H and ^{13}C NMR spectra) can be accessed on the publisher's website.
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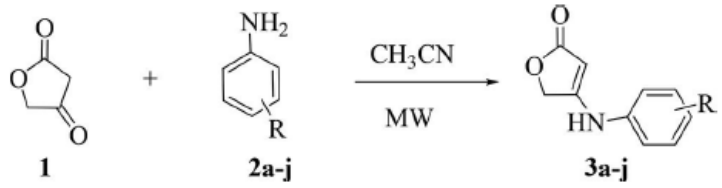
Results and discussion

First, the reaction between equimolar quantity of tetronic acid (**1**) and 4-bromoaniline (**2a**) was used as a model for the evaluation of better solvent and temperature for the synthesis (Scheme 1). Dioxane, water, ethanol, acetonitrile, and trifluoroacetic acid (Table 1) were evaluated as a solvent at room temperature and under microwave heating. Results are summarized in Table 1. The product **3a** was purified and its structure was confirmed by ^{1}H NMR and ^{13}C NMR.

Table 1 shows that in the reaction with dioxane has obtained good yield at room temperature (entries 1, Table 1), but with a reaction time of 144 h. Under microwave heating at 200 W, the reaction time decreases, however, the yield also decreases with the formation of by-products as seen by thin-layer chromatography (TLC) (entry 2, Table 1). Microwave heating of dioxane occurs due to the polarity of the starting materials. Water, ethanol, and trifluoracetic acid at room temperature furnished the product **3a** in yield varying between 60 at 70% with reaction time of 72 h. Under microwave irradiation, these solvents provided the product **3a** in shorter reaction times in yields similar to those obtained at room temperature but with by-product formation. Acetonitrile at room temperature furnished the product **3a** in better yield in the time of 48 h and 98% of yield after 30 min under microwave heating. In protic solvents, used part of the aniline is ionized, while in acetonitrile no ionization and nucleophilic character of nitrogen are maintained. In dioxane also,



Scheme 1. Reaction between tetronic acid (1) and benzaldehyde derivatives (2a–j) promoted by microwave.

Table 1. Results obtained to the reaction between tetronic acid (1) and Br-aniline (2a) in different solvents.

Entry	Solvent	Temperature[b]	Time (h)	Yield[a] (%)
1	DX	r.t.	144	86
2		MW	2	68
3	H_2O	r.t.	72	60
4		MW	0.5	65
5	EtOH	r.t.	72	68
6		MW	0.6	60
7	TFA	r.t.	72	70
8		MW	0.6	60
9	CH_3CN	r.t.	48	75
10		MW	0.5	98

[a]Isolated product.
[b]MW, microwave reflux temperature of the solvent, 200 W, open vessel.
TFA, trifluoroacetic acid.

Table 2. Results obtained by reaction between tetronic acid (1) and anilines derivatives (2a–j) promoted by MW.

Entry	Aniline	Product	Yield[a,b] (%)
1	4-Br* (2a)	3a	98
2	3,4 (OC_2H_4O) (2b)	3b	97
3	4-(OCH_3) (2c)	3c	90
4	3-(OCH_3) (2d)	3d	74
5	4-Cl (2e)	3e	84
6	4-$CH(CH_3)_2$ (2f)	3f	84
7	3,4 (OCH_2O) (2g)	3g	96
8	3,4-(OCH_3)$_2$ (2h)	3h	93
9	3,4,5-(OCH_3)$_3$ (2i)	3i	89
10	4-CF_3 (2j)	3j	88

[a]Isolated yields.
[b]MW, microwave reflux, 200 W, open vessel.

there is no ionization, however, the starting materials are insoluble, therefore, the reaction time is longer.[21]

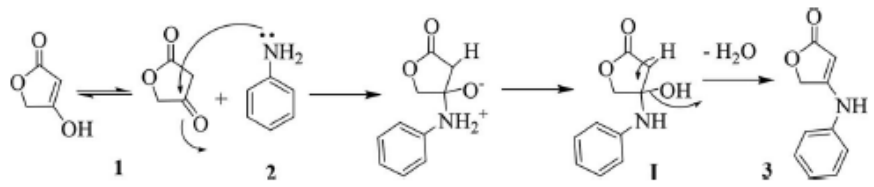
Based on these results, it was established a time of 30–40 min using acetonitrile as a solvent under microwave heating for the other reactions performed.

After optimization of the reaction conditions, other anilines (2b–j) were examined for the synthesis of 4-azo-butenolides derivatives (3b–j) (Table 2).

The results in Table 2 show that by the use of CH_3CN/MW in a reaction time of 30–40 min, it was possible to obtain 4-azo-butenolide derivatives (3b–j) with high yields (84–91%). Large yield differences were observed for aniline 2d which furnished the product 3d in yield of 74%. The inductively electron-withdrawing methoxy group in the position 3 of the aniline 2d removes electron density from nitrogen, making it less nucleophilic.[22]

The nucleophilic attack of the nitrogen in the carbonyl of the tetronic acid (1) furnishes the intermediate I (Scheme 2). The MW heating causes an increase in molecular kinetic energy turning faster the reaction[23–26] and the acetonitrile (Lewis base) promotes the remoting of proton α-carbonyl and subsequent loss of H_2O with formation of the product 3.

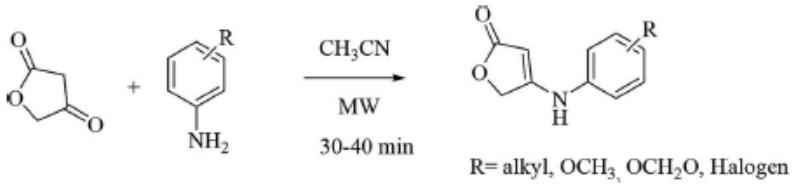
In the literature methodologies describing the synthesis of 4-azo-butenolides derivatives,[27–31] however, most of them use anhydrous and toxic solvents such as benzene, low yields, long reaction times, and chromatographic purification methods. Our methodology has the advantage not only of the higher yield but also of the shortest reaction time and the higher purity of the products that do not require chromatographic purification.



Scheme 2. Mechanistic proposal for reaction between tetronic acid and aniline.

Experimental

General procedure for the synthesis of 4-azo-butenolides 3a



A mixture of tetronic acid (1.0 mmol) and anilines (1.2 mmol) in acetonitrile (2 mL) was taken in a reaction flask equipped with a small magnetic stirring bar, and the reflux condenser. The mixture was then irradiated in a microwave reactor for 40 min (reflux temperature of the solvent) at a power of 200 W. Completion of the reaction was monitored by TLC (20% ethyl acetate in hexane). The reaction mixture was then cooled to room temperature and the solvent was removed on rota-evaporator. The precipitated crude product was washed with mixture of hexane–ethyl acetate (8:2) and dried under vacuum to give **3a** (31.1 mg, 98%) as a yellow solid; mp 239–241 °C.

It was obtained as brown solid having mp 247–249 °C in 98% yield. ^{1}H NMR (400 MHz, DMSO-d6): δ (ppm) 9.83 (s, 1H, NH), 7.53 (d, 2H arom, $J = 8.8$ Hz), 7.16 (d, 2H arom, $J = 8.8$ Hz), 5.36 (s, 1H), 4.87 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d6): δ (ppm) 174.80, 162.32, 139.52, 132.18, 120.47, 114.58, 84.47, 68.04. HRMS (ESI^{+}): m/z $[M + H]^{+}$ calcd for $C_{10}H_9NBrO_2$: 253.9816; found: 253.9810.

Conclusion

Finally, we can conclude that the use of acetonitrile and MW heating in the reaction between tetronic (**1**) and aniline derivatives (**2a–j**) produces new 4-azo-butenolide derivatives with excellent yields, in a very efficient manner. This reaction condition can be applied using anilines with electron-withdrawing substituents as well as electron-donating substituents at different positions on the aromatic ring.

Acknowledgments

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4.4. Synthesis and antibacterial activity of new lactone 1,4-dihydroquinoline derivatives

O artigo que apresento agora foi o primeiro artigo provenientes de uma série de resultados das reações entre o ácido tetrônico, aldeídos, aminas ou fenóis para a obtenção de compostos com vários núcleos heterocíclicos. Nesse artigo descrevo a síntese total de 1,4-diidroquinolinas, que são derivados heterocíclicos de lignanas ariltetralínicas. O artigo descreve a otimização da metodologia e a obtenção dos derivados de forma mais eficiente e em menor tempo, a partir da reação multicomponentes assistida por micro-ondas. Além da síntese, este e o próximo artigo trazem os resultados da avaliação biológica realizada para esses compostos.

Medicinal Chemistry Research
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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and antibacterial activity of new lactone 1,4-dihydroquinoline derivatives

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Abstract
In this work, a series of lactone 1,4-dihydroquinoline derivatives **4** were efficiently synthesized and characterized by ^{1}H and ^{13}C NMR. The synthesized compounds were evaluated for their in vitro antibacterial activity against the bacterial strains *Porphyromonas gingivalis*, *Prevotella nigrescens*, *Streptococcus mitis*, and *Streptococcus sanguinis* and against *Mycobacterium tuberculosis*, *Mycobacterium avium* and *Mycobacterium kansasii*. The results revealed that the evaluated compounds were more active against Gram negative bacteria. Compounds **4ba**, **4bb**, **4bg**, **4bi**, **4bn**, **4ch**, and **4ci** displayed moderate antibacterial activity against *P. gingivalis*. **4bi** was the most active compound against the three strains of *Mycobacterium*. Based on structure–activity relationship studies, we observed that the presence of a nitro group on the benzylic ring and a methylenedioxy group on the dihydroquinoline ring enhanced the antibacterial activity of the derivatives.

Keywords Azo-heterocyclic compounds · quinoline derivatives · antibacterial activity

Introduction

The combination of pharmacophoric moieties of different bioactive substances can produce a new hybrid molecule with improved biological efficacy and affinity, a modified selectivity profile, and different and/or dual modes of action, and may reduce undesired side effects (Viegas-Junior et al. 2007). The combination of lactone and 1,4-dihydroquinoline rings provides 7-azo synthetic analogs of aryltetralin lignan lactones. Aryltetralin lignan lactones are among the various classes of naturally occurring bioactive molecules that have aroused interest in the area of medicinal chemistry due to their structural diversity and biological properties (Teponno et al. 2016). Several studies have shown that the biological properties of these lignans are closely related to the nature of ring A, their stereochemistry, and substituents on the aromatic rings (Antunez-Mojica et al. 2016). However, little is known about how modifications in the chemical skeleton of ring B can affect these properties. The replacement of C7 with nitrogen can greatly alter these properties, creating new analogs with biological properties that have not yet been described for this class of compounds (Fig. 1). These derivatives present two important pharmacophoric groups, the lactone and dihydroquinoline rings. The lactone ring is present in many compounds with biological properties (Qiu et al. 2016) including antimicrobials (Grabarczyk et al. 2013; Mazur et al. 2016), along with quinoline and dihydroquinoline rings (Meléndez-Gómez and Kouznetsov 2013; Kharb and Kaur 2013; Desai et al. 2017).

New quinoline derivatives have been synthesized and investigated for several biological properties. The position and type of the substituents on the quinoline and dihydroquinoline rings are responsible for the variety of pharmacological activities that these compounds present, including antibacterial (El-Essawy and El-Sayed 2013; Desai et al. 2017), antiplasmodial (Vandekerckhove et al. 2015), antituberculosis (Keri and Patil 2014), antimalarial (Vandekerckhove and D'hooghe 2015) and anticancer

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00044-017-2129-x) contains supplementary material, which is available to authorized users.

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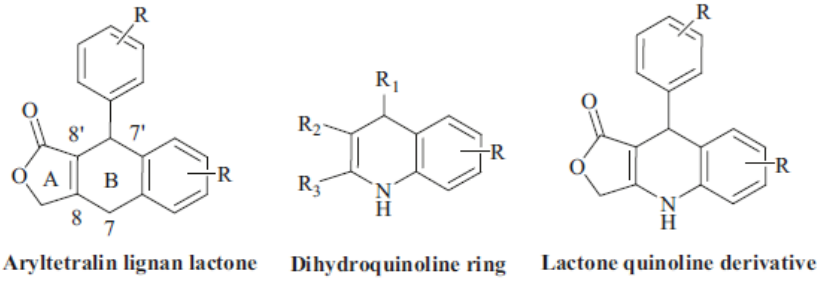
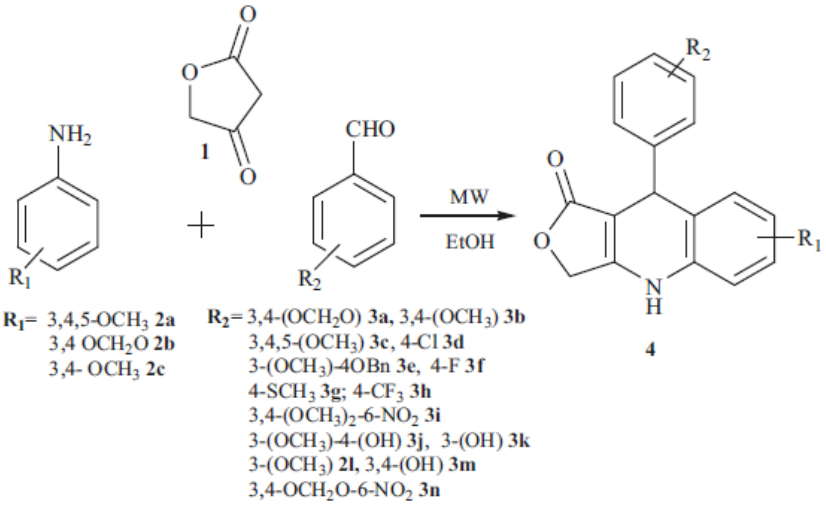


Fig. 1 General structure of dihydroquinoline ring, aryltetralin lignan lactone and lactone quinoline derivative



Scheme 1 Reaction conditions for obtaining derivatives 4

(Spanò et al. 2015). However, there are no reports in the literature on the antimicrobial activity of lactone dihydroquinoline derivatives; therefore we decided to synthesize these compounds and evaluate their antibacterial activities.

Result and Discussion

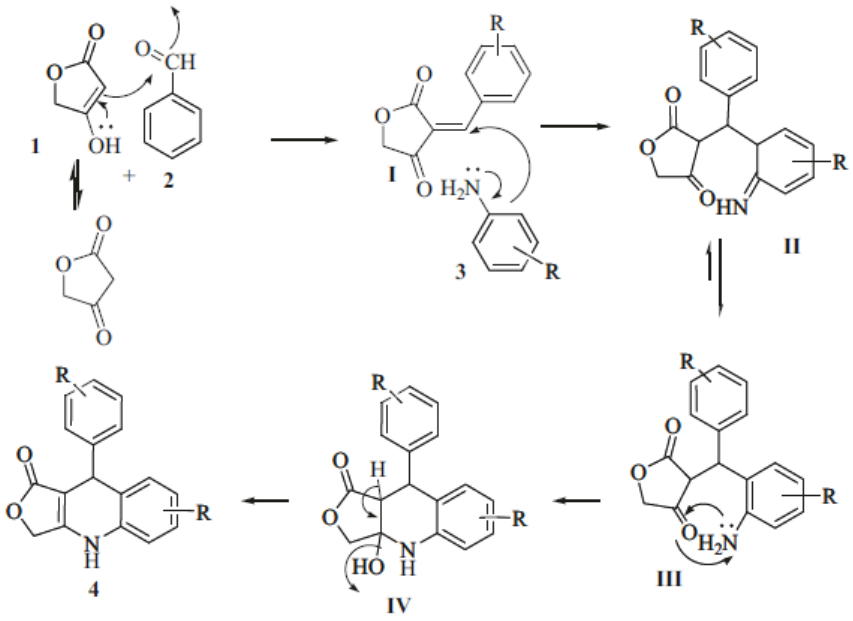
Chemistry

The lactone dihydroquinoline derivatives **4** presented in this paper were prepared via a microwave-assisted reaction between tetronic acid **1**, aniline **2** and aromatic aldehyde **3** in EtOH (Scheme 1). The use of a microwave furnished the derivatives **4** in high yield and with a shorter reaction time than the traditional methodology (Frackenpohl et al. 2009). The reaction was realized with anilines **2a-c** and aromatic aldehydes **3b-n** with electron-withdrawing, as well as electron-donating substituents in different positions on the aromatic ring (Scheme 1).

Substitution of aldehydes with a nitro group (*ortho* position) furnished the derivatives **4** in lower yields, due to the steric hindrance that the nitro group causes on the carbonyl. More pronounced effects on the reaction were observed depending on the substituent present in the aniline. Anilines with electron-withdrawing substituents or without electron-donating substituents in the *meta* position did not react in these conditions. According to the reactional mechanism proposed herein (Scheme 2), the reaction might proceed via sequential condensation, addition, cyclization, and elimination. First, condensation takes place between the tetronic acid **1** with aromatic aldehydes **3** to afford the intermediate **I**. The Michael addition of aniline **2** in **I** then furnishes the intermediate product **II**, which isomerizes to **III**.

In the intermediate **III**, the nucleophilic addition of the nitrogen in the carbonyl leads to the formation of the intermediate **IV**, which then undergoes dehydration to generate the target derivative **4**. In this proposed mechanism, the donating group in the *meta* position in the aniline activates the aromatic ring (*ortho* position at NH_2 group) to attack at intermediate **I**. The proposed mechanism explains the low reactivity of anilines with electron-withdrawing, as well as weakly electron-donating substituents in the *para* or *ortho* positions.

The combination of several anilines and aldehydes furnished 39 lactone dihydroquinoline derivatives, whose structures were confirmed by ^{1}H and ^{13}C NMR spectra. The



Scheme 2 Mechanistic proposal for the formation of 4

spectral analyses were consistent with the structures proposed and are listed in the supplementary material.

Antibacterial activity

The 39 compounds synthesized were evaluated in vitro for antibacterial activity against Gram negative bacteria, Gram positive bacteria and mycobacterium strains by the microplate microdilution method using resazurin as an indicator of microbial activity. The minimum inhibitory concentration (MIC) values of antibacterial activity are shown in Table 1. The evaluated compounds were most active against Gram negative bacteria. *P. gingivalis* was more sensitive to the compounds evaluated than *P. nigrescence*. The substituents of aniline and aldehyde, such as halogen, methoxy, trifluormethyl, hydroxy, nitro, and methylthio were varied to explore the structure–activity relationships between the lactone dihydroquinoline derivatives. The results of the evaluation indicated that in the dihydroquinoline ring methylenedioxy and dimethoxy substituents were more favorable for antibacterial activity than trimethoxy substituents. In the benzylic ring, methylenedioxy, dimethoxy, methylthio, trifluormethyl, and nitro substituents favored antibacterial activity. Compounds **4ba**, **4bb**, **4bg**, **4bi**, **4bn**, **4ch**, and **4ci** showed good antibacterial activity against *P. gingivalis*. Only compounds **4ab**, **4ae** were active against *P. nigrescence*. None of the compounds evaluated were active against Gram positive bacteria. Compound **4bi** was the most active against the three strains of *Mycobacterium*. Overall, the most active compounds were **4bi** and **4bn**, which possess nitro and electron-donating groups attached to the benzylic ring and a methylenedioxy group attached to the dihydroquinoline ring. Nitro-organic compounds are reduced by microbial systems and reduced derivatives were shown to be reactive and to bind to DNA and to proteins (Shahid et al. 2016). However, the antibacterial effect of compounds **4bi** and **4bn** depend not only on the nitro group, but also on their overall structure and the type of bacteria evaluated.

Conclusion

Thirty-nine lactone dihydroquinoline derivatives were synthesized and their antibacterial activities against anaerobic bacteria, aerobic bacteria, and mycobacterium were assayed using the microplate microdilution method. The presence of halogen or hydroxyl groups did not enhance their antibacterial activity. The most active compounds were **4bi** and **4bn**, which possess nitro and electron-donating groups attached to the benzylic ring and a methylenedioxy group attached to the dihydroquinoline ring. Therefore, for this class of compounds the presence of these substituents was essential for antibacterial activity against *P. gingivalis* and *Mycobacterium*.

Materials and Methods

Chemistry

All chemicals and solvents were purchased from commercial sources and were used as received without further purification. Microwave irradiation was carried out with a

Table 1 In vitro antibacterial activity (MIC μg/mL)

Compounds	G-negative		G-positive		*Mycobacterium*		
	P. gingivalis	*P. nigrescens*	*S. mitis*	*S. sanguinis*	*Tuberculosis*	*Avium*	*kansasii*
4aa	>200	>200	>200	>200	>2000	>2000	>2000
4ab	200	100	>200	>200	>2000	>2000	>2000
4ac	>200	200	>200	>200	500	>2000	>2000
4ad	>200	>200	>200	>200	>2000	>2000	>2000
4ae	>200	100	200	>200	>2000	>2000	500
4af	200	>200	>200	>200	>2000	>2000	>2000
4ag	>200	>200	>200	>200	>2000	>2000	>2000
4ah	>200	>200	>200	>200	>2000	>2000	>2000
4ai	>200	>200	>200	>200	>2000	>2000	>2000
4aj	>200	>200	>200	>200	>2000	>2000	>2000
4am	>200	>200	>200	>200	>2000	>2000	>2000
4an	>200	>200	>200	>200	>2000	>2000	>2000
4ba	100	>200	200	200	2000	2000	1000
4bb	50	>200	>200	>200	>2000	>2000	>2000
4bc	200	>200	>200	>200	>2000	>2000	>2000
4bd	200	>200	>200	>200	>2000	>2000	>2000
4be	>200	>200	>200	>200	>2000	>2000	>2000
4bf	>200	>200	200	>200	>2000	>2000	>2000
4bg	100	>200	>200	>200	>2000	>2000	>2000
4bh	>200	>200	>200	>200	>2000	>2000	>2000
4bi	25	>200	>200	200	250	250	125
4bj	>200	>200	>200	>200	>2000	>2000	>2000
4bn	12,5	>200	>200	>200	>2000	>2000	>2000
4ca	>200	>200	>200	>200	>2000	>2000	>2000
4cb	>200	>200	>200	>200	>2000	>2000	>2000
4cc	>200	>200	>200	>200	>2000	>2000	>2000
4cd	>200	>200	>200	>200	>2000	>2000	>2000
4ce	200	>200	>200	>200	>2000	>2000	>2000
4cf	>200	>200	>200	>200	>2000	>2000	500
4cg	>200	>200	>200	>200	>2000	>2000	>2000
4ch	25	>200	>200	>200	>2000	>2000	>2000
4ci	100	>200	>200	>200	>2000	>2000	>2000
4cn	>200	>200	>200	>200	>2000	>2000	>2000
4cm	>200	>200	>200	>200	>2000	>2000	>2000
Isoniazid	—	—	—	—	0.06	>1	1
Chlorhexidine	0.922	0.922	3.68	3.68	—	—	—

—: not tested

Reactor Discover Reflux (CEM Corporation, 300 W). Reactions were monitored using thin-layer chromatography (TLC) plates coated with 0.2 mm silica gel 60 F254 (Merck, Germany). TLC plates were visualized using ultraviolet (UV) irradiation (254 nm). The products were washed with hexane:ethyl acetate (8:2) for purification. Both ^{1}H and ^{13}C NMR spectra were determined on a Bruker ARX 400 spectrometer in DMSO-d_6. Proton chemical shifts in DMSO-d_6 are related to the middle of the residual multiplet ($\delta = 2.50$). Carbon chemical shifts were reported in parts per million (δ) relative to DMSO- d_6 (39.5 p.p.m.), and J (coupling constant) values were reported in hertz. The splitting patterns of protons are described as s (singlet), d (doublet), dd (doublet of doublets), t (triplet) and m (multiplet). All melting points were determined on Melting Point B-540 Buchi apparatus and are uncorrected. The high-resolution mass spectral data for new compounds were obtained using Bruker Daltonics—micrOTOF-Q, fitted with an ESI operating in the positive ion mode.

Table 1 In vitro antibacterial activity (MIC µg/mL)

Compounds	G-negative		G-positive		*Mycobacterium*		
	P. gingivalis	*P. nigrescens*	*S. mitis*	*S. sanguinis*	*Tuberculosis*	*Avium*	*kansasii*
4aa	>200	>200	>200	>200	>2000	>2000	>2000
4ab	200	100	>200	>200	>2000	>2000	>2000
4ac	>200	200	>200	>200	500	>2000	>2000
4ad	>200	>200	>200	>200	>2000	>2000	>2000
4ae	>200	100	200	>200	>2000	>2000	500
4af	200	>200	>200	>200	>2000	>2000	>2000
4ag	>200	>200	>200	>200	>2000	>2000	>2000
4ah	>200	>200	>200	>200	>2000	>2000	>2000
4ai	>200	>200	>200	>200	>2000	>2000	>2000
4aj	>200	>200	>200	>200	>2000	>2000	>2000
4am	>200	>200	>200	>200	>2000	>2000	>2000
4an	>200	>200	>200	>200	>2000	>2000	>2000
4ba	100	>200	200	200	2000	2000	1000
4bb	50	>200	>200	>200	>2000	>2000	>2000
4bc	200	>200	>200	>200	>2000	>2000	>2000
4bd	200	>200	>200	>200	>2000	>2000	>2000
4be	>200	>200	>200	>200	>2000	>2000	>2000
4bf	>200	>200	200	>200	>2000	>2000	>2000
4bg	100	>200	>200	>200	>2000	>2000	>2000
4bh	>200	>200	>200	>200	>2000	>2000	>2000
4bi	25	>200	>200	200	250	250	125
4bj	>200	>200	>200	>200	>2000	>2000	>2000
4bn	12,5	>200	>200	>200	>2000	>2000	>2000
4ca	>200	>200	>200	>200	>2000	>2000	>2000
4cb	>200	>200	>200	>200	>2000	>2000	>2000
4cc	>200	>200	>200	>200	>2000	>2000	>2000
4cd	>200	>200	>200	>200	>2000	>2000	>2000
4ce	200	>200	>200	>200	>2000	>2000	>2000
4cf	>200	>200	>200	>200	>2000	>2000	500
4cg	>200	>200	>200	>200	>2000	>2000	>2000
4ch	25	>200	>200	>200	>2000	>2000	>2000
4ci	100	>200	>200	>200	>2000	>2000	>2000
4cn	>200	>200	>200	>200	>2000	>2000	>2000
4cm	>200	>200	>200	>200	>2000	>2000	>2000
Isoniazid	—	—	—	—	0.06	>1	1
Chlorhexidine	0.922	0.922	3.68	3.68	—	—	—

—: not tested

Reactor Discover Reflux (CEM Corporation, 300 W). Reactions were monitored using thin-layer chromatography (TLC) plates coated with 0.2 mm silica gel 60 F254 (Merck, Germany). TLC plates were visualized using ultraviolet (UV) irradiation (254 nm). The products were washed with hexane:ethyl acetate (8:2) for purification. Both ^{1}H and ^{13}C NMR spectra were determined on a Bruker ARX 400 spectrometer in DMSO-d_6. Proton chemical shifts in DMSO-d_6 are related to the middle of the residual multiplet ($\delta = 2.50$). Carbon chemical shifts were reported in parts per million (δ) relative to DMSO- d_6 (39.5 p.p.m.), and J (coupling constant) values were reported in hertz. The splitting patterns of protons are described as s (singlet), d (doublet), dd (doublet of doublets), t (triplet) and m (multiplet). All melting points were determined on Melting Point B-540 Buchi apparatus and are uncorrected. The high-resolution mass spectral data for new compounds were obtained using Bruker Daltonics—micrOTOF-Q, fitted with an ESI operating in the positive ion mode.

General experimental procedure and characterization of synthesized derivatives 4

The mixture of tetronic acid **1** (1.0 mmol), aldehyde **2** (1.0 mmol), and aniline **3** (1.0 mmol) in 2 mL of EtOH was irradiated at a power of 200 W in an open vessel in a microwave reactor (reflux temperature of the solvent). The reaction was monitored using TLC every 3 min and after 15 min the reaction was cooled, the solvent was removed under vacuum and the solid obtained was washed with hexane-acetate (8:2).

9-(benzo[d][1,3]dioxol-5-yl)-6,7,8-trimethoxy-4,9-dihydrofuro[3,4-b]quinolin-1(3H)-one (4aa)

Yellow solid, yield 92%, mp 271–273 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.93 (s, 1H, NH), 6.77 (d, 1H, $J = 7.9$ Hz), 6.63 (d, 1H, $J = 1.7$ Hz), 6.54 (dd, 1H, $J = 7.9$ and 1.7 Hz), 6.38 (s, 1H), 5.94 (d, 1H, $J = 0.9$ Hz), 5.93 (d, 1H,, $J = 0.9$ Hz), 4.90 (d, 1H, $J = 15.7$ Hz), 4.88 (s, 1H), 4.79 (d, 1H, $J = 15.7$ Hz), 3.79 (s, 3H), 3.64 (s, 3H), 3.42 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 171.94, 157.59, 152.77, 151.66, 146.84, 145.27, 141.11, 137.47, 132.99, 120.37, 110.05, 108.00, 107.69, 95.92, 95.48, 64.84, 60.31, 60.013, 55.64, 34.92.

9-(3,4-dimethoxyphenyl)-6,7,8-trimethoxy-4,9-dihydrofuro[3,4-*b*]quinolin-1(*3H*)-one (4ab)

White solid, yield 91%, mp 258–260 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.89 (s, 1H, NH), 6.80 (d, 1H, $J = 8.2$ Hz), 6.80 (d, 1H, $J = 1.8$ Hz), 6.51 (dd, 1H, $J = 1.8$ and 8.2 Hz), 6.38 (s, 1H), 4.90 (s, 1H), 4.88 (d, 1H, $J = 15.7$ Hz), 4.78 (d, 1H, $J = 15.7$ Hz), 3.78 (s, 3H), 3.68 (s, 3H), 3.67 (s, 3H), 3.63 (s, 3H), 3.39 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 172.12, 157.58, 152.70, 151.67, 148.10, 147.08, 139.68, 137.52, 133.02, 119.42, 111.76, 111.66, 10.13, 99.11, 95.53, 64.87, 64.83, 60.32, 59.98, 55.69, 55.46, 34.70.

6,7,8-trimethoxy-9-(3,4,5-trimethoxyphenyl)-4,9-dihydrofuro[3,4-b]quinolin-1(3H)-one (4ac)

Yellow solid, yield 89%, mp 229–231 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.91 (s, 1H, NH), 6.40 (s, 3H), 4.97 (s, 1H), 4.92 (d, 1H, $J = 15.6$ Hz), 4.79 (d, 1H, $J = 15.6$ Hz), 3.80 (s, 3H), 3.67 (s, 3H), 3.65 (s, 3H), 3.60 (s, 3H), 3.47 (s, 3H), 3.46 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 172.06, 157.87, 152.78, 152.40, 151.73, 142.48, 136.45, 135.88, 133.15, 109.53, 104.85, 95.72, 95.50, 64.83, 60.32, 60.02, 59.91, 55.74, 55.66, 35.33.

9-(4-chlorophenyl)-6,7,8-trimethoxy-4,9-dihydrofuro[3,4-b]quinolin-1(3*H*)-one (4ad)

Yellow solid, yield 81%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.99 (s, 1H, NH), 7.31 (d, 2H, $J = 8.4$ Hz), 7.13 (d, 2H, $J = 8.4$ Hz), 6.39 (s, 1H), 4.96 (s, 1H), 4.90 (d, 1H, $J = 15.7$ Hz), 4.81 (d, 1H, $J = 15.7$ Hz), 3.79 (s, 3H), 3.63 (s, 3H), 3.38 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 171.86, 157.79, 152.97, 151.66, 145.76, 137.46, 132.97, 130.46, 129.34, 127.91, 109.47, 95.51, 95.37, 64.92, 60.30, 59.94, 55.63, 34.95. HRMS (ESI$^+$): m/z $[M + Na]^+$ calculated for $C_{20}H_{18}ClNO_5Na$: 410.0764; found: 410.0755.

9-(4-(benzyloxy)-3-methoxyphenyl)-6,7,8-trimethoxy-4,9-dihydrofuro[3,4-b]quinolin-1(3*H*)-one (4ae)

White solid, yield 85%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.89 (s, 1H, NH), 7.43–7.28 (m, 5H), 6.88 (d, 1H, $J = 8.3$ Hz), 6.83 (d, 1H, $J = 2.0$ Hz), 6.49 (dd, 1H, $J = 8.3$ and 2.0 Hz), 6.38 (s, 1H), 5.00 (s, 1H), 4.89 (d, 1H, $J = 16.0$ Hz), 4.79 (s, 1H, $J = 16.0$ Hz), 3.79 (s, 3H), 3.71 (s, 3H, 3.64 (s, 3H), 3.38 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 172.03, 157.55, 152.70, 151.69, 148.53, 146.01, 140.18, 137.48, 137.23, 133.06, 128.31, 127.70, 119.38, 113.56, 112.01, 110.07, 96.03, 95.47, 69.97, 64.81, 60.30, 59.94, 55.65, 55.53, 34.78. HRMS (ESI$^+$): m/z $[M + H]^+$ calculated for $C_{28}H_{28}NO_7$: 490.1857; found: 490.1849.

9-(4-fluorophenyl)-6,7,8-trimethoxy-4,9-dihydrofuro[3,4-b]quinolin-1(3*H*)-one (4af)

White solid, yield 82%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.96 (s, 1H, NH), 7.17–7.11 (m, 2H), 7.10–7.03 (m, 2H), 6.39 (s, 1H), 4.96 (s, 1H), 4.89 (d, 1H, $J = 15.7$ Hz), 4.81 (d, 1H, $J = 15.7$ Hz), 3.79 (s, 3H), 3.63 (s, 3H), 3.36 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 171.93, 161.69, 159.28, 157.70, 152.89, 151.66, 143.11, 143.08, 137.49, 132.95, 129.26, 129.18, 114.71, 114.50, 109.82, 95.67, 95.51, 64.89, 60.29, 59.91, 55.65, 34.71. HRMS (ESI$^+$): m/z $[M + H]^+$ calculated for $C_{20}H_{19}FNO_5$: 372.1241; found: 372.1244.

6,7,8-trimethoxy-9-(4-(methylthio)phenyl)-4,9-dihydrofuro[3,4-b]quinolin-1(3*H*)-one (4ag)

White solid, yield 81%, mp 221–223 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.94 (s, 1H, NH), 7.14 (d, 2H, $J = 8.4$ Hz), 7.06 (d, 2H, $J = 8.4$ Hz), 6.39 (s, 1H), 4.91 (s, 1H), 4.89 (d, 1H, $J = 15.8$ Hz), 4.80 (d, 1H, $J = 15.8$ Hz), 3.79 (s, 3H), 3.63 (s, 3H), 3.39 (s, 3H), 2.41 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 171.91, 157.64,

9-(benzo[d][1,3]dioxol-5-yl)-6,9-dihydro-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-8(5*H*)-one (4ba)

White solid, yield 86%, mp 288–289 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.87 (s, 1H, NH), 6.78 (d, 1H, $J = 8.0$ Hz), 6.73 (s, 1H), 6.65 (d, 1H, $J = 8.0$ Hz), 5.95 (brs, 1H), 5.94 (brs, 1H), 5.93 (brs, 1H), 5.89 (brs, 1H), 4.94 (d, 1H, $J = 15.76$ Hz), 4.83 (d, 1H, $J = 15.7$ Hz), 4.83 (s, 1H) ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 172.56, 158.70, 147.82, 147.00, 146.17, 143.80, 141.75, 130.94, 120.92, 117.27, 110.03, 108.51, 108.40, 101.68, 101.29, 97.79, 94.94, 65.43, 40.09.

9-(3,4-dimethoxyphenyl)-6,9-dihydro-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-8(5H)-one (4bb)

White solid, yield 86%, mp 289–291 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.84 (s, 1H, NH), 6.88 (s, 1H), 6.82 (d, 1H, $J = 8.5$ Hz), 6.62 (brd, 1H), 6.62 (s, 1H), 6.52 (s, 1H), 5.91 (brs, 1H), 5.90 (brs, 1H), 4.96 (d, 1H, $J = 15.7$ Hz), 4.85 (s, 1H), 4,84 (d, 1H, $J = 15.7$ Hz), 3.71 (s, 3H), 3.69 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 172.11, 158.12, 148.44, 147.25, 146.34, 143.16, 139,78, 130.30, 119.41, 116.88, 111.86, 111.47, 109,58, 101.10, 97.19, 94.41, 64.85, 55.47, 55.44, 39.10.

9-(3,4,5-triimethoxy)-6,9-dihydro-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-8(5*H*)-one (4bc)

White solid, yield 90%, mp 271–274 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.86 (s, 1H, NH), 6.67 (s, 1H), 6.53 (s, 1H), 6.48 (s, 2H), 5.95 (d, 1H, $J = 0.7$ Hz), 5.90 (d, 1H, $J = 0.7$ Hz), 4.98 (s, 1H, $J = 15.7$ Hz), 4.85 (s, 1H), 4.84 (d, 1H, $J = 15.7$ Hz), 3.70 (s, 6H), 3.60 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 172.18, 158.41, 152.74, 146.44, 143.22, 142.68, 136.04, 130.25, 116.50, 109.49, 104.82, 101.24, 101.14, 97.28, 94.14, 64.92, 59.86, 55.82, 39.76.

9-(4-chlorophenyl)-6,9-dihydro-[1,3]dioxolo[4,5-g]furo[3,4-*b*]quinolin-8(*5H*)-one (4bd)

White solid, yield 80%, mp 290–292 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.93 (s, 1H, NH), 7.33 (d, 2H, $J = 8.4$ Hz), 7.22 (d, 2H, $J = 8.4$ Hz), 6.58 (s, 1H), 6.54 (s, 1H), 5.97 (brs, 1H), 5.91 (brs, 1H), 4.96 (s, 1H), 4.96 (d, 1H, $J = 15.7$ Hz), 4.86 (d, 1H, $J = 15.7$ Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 171.98, 158.37, 146.61, 145.72, 143.36, 130.90, 130.48, 129.34, 128.24, 116.05, 109.53, 101.22, 97.35, 93.92, 64.97, 38.95.

9-(4-(benzyloxy)-3-methoxyphenyl)- 6,9-dihydro-[1,3]dioxolo[4,5-g]furo[3,4-*b*]quinolin-8(*5H*)-one (4be)

White solid, yield 84%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.85 (s, 1H, NH), 7.45–7.29 (m, 5H), 6.92 (d, 1H, $J = 1.9$ Hz), 6.90 (d, 1H, $J = 8.4$ Hz), 6.63 (s, 1H), 6.59 (dd, 1H, $J = 8.4$ and 1.9 Hz), 6.52 (s, 1H), 5.96 (brs, 1H), 5.90 (brs, 1H), 5.01 (s, 2H), 4.96 (d, 1H, $J = 15.7$ Hz), 4.85 (d, 1H, $J = 15.7$ Hz), 4.86 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 172.12, 158.16, 148.76, 146.36, 146,29, 143.18, 140.17, 137.26, 130.31, 128.36, 127.75, 127.66, 119.36, 116.82, 113.52, 111.73, 109.58, 101.11, 97.20, 94.38, 69.908, 64.86, 55.53, 39.11. HRMS (ESI$^+$): m/z [M + H]$^+$ calculated for $C_{26}H_{22}NO_6$: 444.1440; found: 444.1444.

9-(4-fluorphenyl)-6,9-dihydro-[1,3]dioxolo[4,5-g]furo[3,4-*b*]quinolin-8(*5H*)-one (4bf)

White solid, yield 81%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.90 (s, 1H, NH), 7.24–7.19 (m, 2H), 7.11–7.04 (m, 2H), 6.56 (s, 1H), 6.53 (s, 1H), 5.95 (brs, 1H), 5.90 (brs, 1H), 4.95 (s, 1H), 4.94 (d, 1H, $J = 15.6$ Hz), 4.85 (d, 1H, $J = 15.6$ Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 171.99, 158.24, 146.55, 143.33, 143.08, 143.05, 130.49, 129.29, 129.21, 116.35, 115.04, 114.83, 109.54, 101.19, 97.31, 94.23, 64.93, 38.81. HRMS (ESI$^+$): m/z [M + H]$^+$ calculated for $C_{18}H_{13}FNO_4$: 326.0824; found: 326.0828.

9-(4-(methylthio)phenyl)-6,9-dihydro-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-8(5*H*)-one (4bg)

White solid, yield 81%, mp 277–280 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.89 (s, 1H, NH), 7.18–7.12 (m, 4H), 6.56 (s, 1H), 6.53 (s, 1H), 5.96 (brs, 1H), 5.90 (brs, 1H), 4.94 (d, 1H, $J = 15.6$ Hz), 4.89 (s, 1H), 4.85 (d, 1H, $J = 15.6$ Hz), 2.43 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 172.02, 158.22, 146.47, 143.75, 143.27, 135.71, 130.42, 128.08, 126.08, 116.48, 109.56, 101.16, 97.27, 94.18, 64.91. HRMS (ESI$^+$): m/z [M + K + H_2O − 2H]$^+$ calculated for $C_{19}H_{16}NO_5S$: 408.0307; found: 408.0324.

9-(4-(trifluoromethyl)phenyl)-6,9-dihydro-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-8(5*H*)-one (4bh)

White solid, yield 88%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.98 (s, 1H, NH), 7.64 (d, 2H, $J = 8.2$ Hz), 7.43 (d, 2H, $J = 8.2$ Hz), 6.58 (s, 1H), 6.57 (s, 1H), 5.97 (brs, 1H), 5.91 (brs, 1H), 5.08 (s, 1H), 4.96 (d, 1H, $J = 15.7$ Hz), 4.88 (d, 1H, $J = 15.7$ Hz). ^{13}C NMR (100

MHz, DMSO-d_6): δ (p.p.m.) 171.95, 158.54, 146.75, 143.45, 130.57, 128.35, 125.29, 125.26, 115.53, 109.54, 101.27, 97.45, 93.70, 65.05, 39.47. HRMS (ESI^+): m/z $[M + Na]^+$ calculated for $C_{19}H_{13}F_3NO_4Na$: 398.0611; found: 398.0609.

9-(4,5-dimethoxy-2-nitrophenyl)-6,9-dihydro-[1,3]dioxolo [4,5-g]furo[3,4-b]quinolin-8(5*H*)-one (4bi)

Yellow solid, yield 69%, mp 243–244 °C. ^1H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.96 (s, 1H, NH), 7.43 (s, 1H), 6.71 (s, 1H), 6.57 (s, 1H), 5.97 (brs, 1H), 5.94 (brs, 1H), 5.69 (s, 1H), 4.93 (d, 1H, $J = 15.6$ Hz), 4,84 (d, 1H, $J = 15.6$ Hz), 3.83 (s, 3H), 3.71 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 171.65, 158.39, 152.68, 147.14, 146.87, 143.56, 140.69, 134.45, 130.86, 115.11, 112.95, 108.86, 107,10, 101.34, 97.63, 93.76, 64.99, 56.01, 55.89, 34.32. HRMS (ESI^+): m/z $[M + H - H_2O]^+$ calculated for $C_{20}H_{15}N_2O_7$: 395.0872; found: 395.0869.

9-(4-hydroxy-3-methoxyphenyl)-6,9-dihydro-[1,3]dioxolo [4,5-g]furo[3,4-b]quinolin-8(5*H*)-one (4bj)

White solid, yield 81%, mp 279–281 °C. ^1H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.80 (s, 1H, NH), 8.76 (s, 1H, OH), 6.84 (d, 1H, $J = 1.8$ Hz), 6.64 (d, 1H, $J = 8.0$ Hz), 6.61 (s, 1H), 6.51 (s, 1H), 6.49 (dd, 1H, $J = 1.8$ and 8.0 Hz), 5.95 (brs, 1H), 5.90 (brs, 1H), 4.94 (d, 1H, $J = 15.6$ Hz), 4.83 (d, 1H, $J = 15.6$ Hz), 4.79 (s, 1H), 3.3 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 172.12, 158.02, 147.24, 146.28, 144.97, 143.14, 38.31, 130.30, 119.72, 117.14, 115.33, 111.89, 109.57, 101.06, 97.15, 94.61, 64.81, 55.63, 39.12. HRMS (ESI^+): m/z $[M + Na]^+$ calculated for $C_{19}H_{15}NO_6Na$: 376.0791; found 376.0792

9-(6-nitrobenzo[d][1,3]dioxol-5-yl)-6,9-dihydro-[1,3]dioxolo [4,5-g]furo[3,4-b]quinolin-8(5*H*)-one (4bn)

Yellow solid, yield 71%, mp 225–227 °C. ^1H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 10.00 (s, 1H, NH), 7.47 (s, 1H), 6.71 (s, 1H), 6.58 (s, 2H), 6.16 (d, 1H, $J = 0.8$ Hz), 6.14 (d, 1H, $J = 0.8$ Hz), 5.98 (d, 1H, $J = 0.7$ Hz), 5.94 (d, 1H, $J = 0.7$ Hz), 5.62 (s, 1H), 4.92 (d, 1H, $J = 15.5$ Hz), 4.85 (d, 1H, $J = 15.5$ Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 171.62, 158.38, 151.50, 147.00, 146.18, 143.66, 141.87, 136.94, 130.84, 114.89, 109.64, 108.85, 103.84, 103.16, 101.39, 97.68, 93.72, 68.08, 34.15. HRMS (ESI^+): m/z $[M + H - H_2O]^+$ calculated for $C_{19}H_{11}N_2O_7$: 379.0560; found: 379.0558

9-(benzo[d][1,3]dioxol-5-yl)-6,7-dimethoxy-4,9-dihydrofuro [3,4-b]quinolin-1(3*H*)-one (4ca)

White solid, yield 91%, mp 287–289 °C. ^1H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.85 (s, 1H, NH), 6.79 (d, 1H, $J = 7.9$ Hz), 6.73 (d, 1H, $J = 1.6$ Hz), 6.66 (dd, 1H, $J = 7.9$ and 1.6 Hz), 6.62 (s, 1H), 6.51 (s, 1H), 5.95 (brs, 1H), 5.94 (brs, 1H), 4.95 (d, 1H, $J = 15.5$ Hz), 4.87 (s, 1H), 4.84 (d, 1H, $J = 15.5$ Hz), 3.73 (s, 3H), 3.60 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 172.15, 158.20, 148.16, 147.19, 145.51, 144.92, 141.15, 129.63, 120.35, 115.44, 113.71, 107.97, 107.85, 100.72, 100.34, 99.46, 64.92, 55.84, 55.43, 38.86.

9-(3,4-dimethoxyphenyl)-6,7-dimethoxy-4,9-dihydrofuro [3,4-b]quinolin-1(3*H*)-one (4cb)

Yellow solid, yield 90%, mp 228–230 °C. ^1H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.82 (s, 1H, NH), 6.89 (d, 1H, $J = 1.9$ Hz), 6.1 (d, 1H, $J = 8.6$ Hz), 6.66 (s, 1H), 6.60 (dd, 1H, $J = 8.3$ and 1.9 Hz), 6.56 (s, 1H), 4.95 (d, 1H, $J = 15.6$ Hz), 4.88 (s, 1H), 4.84 (d, 1H, $J = 15.6$ Hz), 3.73 (s, 3H), 3.71 (s, 3H), 3.69 (s, 3H), 3.60 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 172.22, 158.18, 148.40, 148.07, 147.16, 144.85, 139.59, 29.62, 119.35, 115.56, 113.78, 111.71, 111.49, 100.29, 94.48, 64.88, 55.83, 55.44, 55.42, 38.74.

6,7-dimethoxy-9-(3,4,5-trimethoxyphenyl)-4,9-dihydrofuro [3,4-b]quinolin-1(3*H*)-one (4cc)

White solid, yield 92%, mp 236–238 °C. ^1H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.83 (s, 1H, NH), 6.85 (s, 1H), 6.73 (s, 1H), 6.52 (s, 1H), 6.50 (s, 1H), 5.00 (d, 1H, $J =$ 15.5 Hz), 4.89 (s, 1H), 4.85 (d, 1H, $J = 15.5$ Hz), 3.73 (s, 3H), 3.70 (s, 3H), 3.62 (s, 3H), 3.61 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 17.22, 158.51, 15.70, 148.20, 144.87, 142.49, 135.95, 129.63, 115.17, 113.83, 107.62, 104.78, 100.78, 94.11, 64.94, 59.85, 56.08, 55.99, 55.93, 55.8, 55.45, 18.52.

9-(4-chlorophenyl)-6,7-dimethoxy-4,9-dihydrofuro[3,4-b] quinolin-1(3*H*)-one (4 cd)

White solid, yield 81%, mp 265–267 °C. ^1H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.88 (s, 1H, NH), 7.31 (d, 2H, $J = 8.4$ Hz), 7.21 (d, 2H, $J = 8.4$ Hz), 6.58 (s, 1H), 6.52 (s, 1H), 4.97 (s, 1H), 4.94 (d, 1H, $J = 15.7$ Hz), 4.85 (d, 1H, J = 15.7 Hz), 3.73 (s, 3H), 3.57 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 172.04, 158.34, 148.37, 145.63, 145.08, 130.78, 129.79, 129.35, 128.18, 114.78, 113.83,

100.52, 94.09, 64.99, 55.89, 55.48, 38.66. HRMS (ESI^+): m/z $[M + Na]^+$ calculated for $C_{19}H_{17}ClNO_4Na$: 380.0659: found: 380.0661.

9-(4-(benzyloxy)-3-methoxyphenyl)-6,7-dimethoxy-4,9-dihydrofuro[3,4-b]quinolin-1(3*H*)-one (4ce)

White solid, yield 92%, mp 232–234 °C. ^1H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.80 (s, 1H, NH), 7.44–7.28 (m, 5H), 6.92 (d, 1H, $J = 1.9$ Hz), 6.89 (d, 1H, $J = 8.3$ Hz), 6.66 (s, 1H), 6.56 (dd, 1H, $J = 8.3$ and 1.9 Hz), 6.50 (s, 1H), 4.99 (s, 1H), 4.94 (d, 1H, $J = 15.6$ Hz), 4.87 (s, 1H), 4.83 (d, 1H, $J = 15.6$ Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 172.95, 158.19, 148.81, 148.16, 146.27, 144.92, 140.02, 137.27, 129.70, 128.34, 127.73, 127.68, 119.36, 115.56, 113.93, 113.56, 111.89, 100.41, 94.50, 69.98, 64.89, 55.91, 55.61, 55.48, 38.76. HRMS (ESI^+): m/z $[M + Na]^+$ calculated for $C_{27}H_{25}NO_6Na$: 482.1571; found: 482.1578.

9-(4-fluorophenyl)-6,7-dimethoxy-4,9-dihydrofuro[3,4-b]quinolin-1(3*H*)-one (4cf)

White solid, yield 79%, mp 257–258 °C. ^1H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.87 (s, 1H, NH), 7.24–7.20 (m, 2H), 7.11–7.06 (m, 2H), 6.59 (s, 1H), 6.53 (s, 1H), 4.98 (s, 1H), 4.94 (d, 1H, $J = 15.6$ Hz), 4.85 (d, 1H, $J = 15.6$ Hz), 3.73 (s, 3H), 3.58 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 172.08, 158.24, 1478.31, 145.05, 14.98, 142.95, 129.79, 129.28, 129.20, 115.98, 114.98, 114.77, 113.90, 100.50, 94.35, 64.95, 55.90, 55.49, 38.50. HRMS (ESI^+): m/z $[M + H]^+$ calculated for $C_{19}H_{17}FNO_4$: 342.1136; found: 342.1133.

6,7,8-trimethoxy-9-(4-(methylthio)phenyl)-4,9-dihydrofuro[3,4-b]quinolin-1(3*H*)-one (4cg)

White solid, yield 81%, mp 286–287 °C. ^1H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.85 (s, 1H, NH), 7.17–7.12 (m, 4H), 6.58 (s, 1H), 6.52 (s, 1H), 4.93 (d, 1H, $J = 15.7$ Hz), 4.91 (s, 1H), 4.84 (d, 1H, $J = 15.7$ Hz), 3.73 (s, 3H), 3.58 (s, 3H), 2.42 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 172.13, 158.22, 148.25, 145.01, 143.65, 135.55, 129.74, 128.08, 126.03, 115.25, 113.86, 100.47, 94.37, 64.94, 55.89, 55.44, 55.49, 38.74, 14.78. HRMS (ESI^+): m/z $[M + H]^+$ calculated for $C_{20}H_{20}NO_4S$: 370.1106; found: 370.1109.

6,7-dimethoxy-9-(4-(trifluoromethyl)phenyl)-4,9-dihydrofuro[3,4-b]quinolin-1(3*H*)-one (4ch)

White solid, yield 82%, mp 255–256 °C. ^1H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.95 (s, 1H, NH), 7.64 (d, 2H, $J = 8.1$ Hz), 7.43 (d, 2H, $J = 8.1$ Hz), 6.60 (s, 1H), 6.56 (s, 1H), 5.10 (s, 1H), 4.97 (d, 1H, $J = 15.7$ Hz), 4.87 (d, 1H, $J = 15.7$ Hz), 3.75 (s, 3H), 3.58 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 172.02, 158.53, 150.96, 148.49, 145.16, 130.67, 129.88, 128.33, 125.21, 125.18, 114.30, 113.83, 100.61, 93.85, 65.07, 55.88, 55.48, 39.15. HRMS (ESI^+): m/z $[M + H]^+$ calculated for $C_{20}H_{17}F_3NO_4$: 392.1104; found: 392.1105.

9-(4,5-dimethoxy-2-nitrophenyl)-6,7-dimethoxy-4,9-dihydrofuro[3,4-b]quinolin-1(3*H*)-one (4ci)

Yellow solid, yield 77%, mp 249–251 °C. ^1H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.94 (s, 1H, NH), 7.45 (s, 1H), 6.68 (s, 1H), 6.65 (s, 1H), 6.57 (s, 1H), 5.73 (s, 1H), 4.95 (d, 1H, $J = 15.6$ Hz), 4.85 (d, 1H, $J = 15.6$ Hz), 3.83 (s, 3H), 3.76 (s, 3H), 3.70 (s, 3H), 3.57 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 171.77, 158.48, 152.61, 148.53, 147.01, 145.11, 140.69, 134.52, 130.04, 113.93, 112.98, 112.75, 107.00, 100.63, 93.87, 65.04, 55.95, 55.83, 55.69, 55.42, 33.81. HRMS (ESI^+): m/z $[M + H]^+$ calculated for $C_{21}H_{21}N_2O_8$: 429.1290: found: 429.1297.

9-(3,4-dihydroxyphenyl)-6,7-dimethoxy-4,9-dihydrofuro[3,4-b]quinolin-1(3*H*)-one (4 cm)

White solid, yield 80%, mp 244–246 °C. ^1H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.75 (s, 1H, NH), 8.70 (s, 1H, OH), 8.64 (s, 1H, OH), 6.58 (d, 1H, $J = 8.0$ HZ), 6.55 (s, 1H), 6.50 (d, 1H, $J = 2.0$ Hz), 6.49 (s, 1H), 6.46 (dd, 1H, $J = 8.0$ and 2.0 Hz), 4.89 (d, 1H, $J = 15.7$ Hz), 4.81 (d, 1H, $J = 15.7$ Hz), 4.71 (s, 1H), 3.72 (s, 3H), 3.58 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 172.14, 157.76, 148.06, 144.92, 144.89, 143.63, 138.27, 129.70, 118.19, 116.06, 115.10, 115.03, 113.95, 100.35, 94.98, 64.73, 55.87, 55.49, 38.67. HRMS (ESI^+): m/z $[M + H + K]^+$ calculated for $C_{19}H_{18}NO_6K$: 395.0765; found: 395.0761

6,7-dimethoxy-9-(6-nitrobenzo[d][1,3]dioxol-5-yl)-4,9-dihydrofuro[3,4-b]quinolin-1(3*H*)-one (4cn)

Yellow solid, yield 70%, mp 245–246 °C. ^1H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 9.98 (s, 1H, NH), 6.67 (s, 1H), 6.65 (s, 1H), 6.56 (s, 1H), 6.15 (s, 1H), 6.14 (s, 1H), 5.64 (s, 1H), 4.94 (d, 1H, $J = 15.6$ Hz), 4.87 (d, 1H, $J = 15.6$ Hz), 3.75 (s, 3H), 3.58(s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (p.p.m.) 171.74, 158.50, 141.45, 148.63, 146.08, 145.21, 141.84, 137.00, 129.97, 113.75, 112.95, 109.53, 103.88, 103.14, 100.71, 93.78, 65.13, 55.69, 55.45, 33.73. HRMS (ESI^+): m/z $[M + H - H_2O]^+$ calculated for $C_{20}H_{15}N_2O_7$: 395.0878; found: 395.0872.

Biological activity

The antibacterial activity of the derivatives **4** was assessed against *Porphyromonas gingivalis* (ATCC 33277), *Prevotella nigrescens* (ATCC 33563), *Streptococcus mitis* (ATCC 49456), *Streptococcus sanguinis* (ATCC 10556), *Mycobacterium tuberculosis* (ATCC 27294), *Mycobacterium avium* (ATCC 25291), and *Mycobacterium kansasii* (ATCC 12478).

The minimal inhibitory concentration (MIC) of the derivatives **4** was determined using the microdilution broth method in 96-well microplates (Sousa et al. 2015). Samples were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 8000 µg/mL, followed by dilution in tryptic soy broth (TSB) for aerobic microorganisms and Schaedler broth (Difco) supplemented which hemin (5.0 g/mL), and menadione (10.0 g/mL) for anaerobic microorganisms; the sample concentrations tested ranged from 200 to 0.195 µg/mL. The final DMSO content was 4% (v/v), and this solution was used as a negative control. The inoculum was adjusted for each organism to yield a cell concentration of 5×10^5 colony forming units per mL (CFU/mL), according to the Clinical and Laboratory Standards Institute (CLSI 2012a) guidelines (CLSI 2012b). The 96-well microplates containing the aerobic microorganisms were closed with a sterile plate sealer and incubated aerobically at 37 °C for 24 h. The plates containing the anaerobic microorganisms were closed with a sterile plate sealer and incubated for 72 h in an anaerobic chamber, in 5–10% H_2, 10% CO_2, 80–85% N_2 atmosphere, at 36 °C. After that, resazurin (30 µL) in aqueous solution (0.01%) was added to the microplates to indicate microorganism viability for MIC determination. Chlorhexidine dihydrochloride (CHD) was used as a positive control, and the concentrations ranged from 0.115 to 59 µg/mL. Controls were also performed to determine the sterility of the TSB and Schaedler broths, control culture (inoculum), CHD, the derivatives **4**, and control DMSO. The MIC values were determined as the lowest concentration of derivatives **4** capable of inhibiting the growth of the microorganisms.

For *Mycobacterium* spp. the antimicrobial activity of the derivatives **4** was evaluated in vitro using the microplate microdilution technique (Palomino et al. 2002), using resazurin as an indicator of microbial activity (REMA-Resazurin Microtiter Assay), which allowed the determination of the minimum inhibitory concentration (MIC) against the microorganisms evaluated. The compounds were dissolved in dimethyl sulfoxide (DMSO) and serially diluted in Middlebrook 7H9 broth prior to inoculation, the final concentration of DMSO being <0.3%. The inoculum was adjusted to each organism to produce a cellular concentration of 10^8 colony forming units (CFU/mL). The concentrations of the compounds tested ranged from 0.195 to 200 µg/mL for the bacteria and from 31.5 to 2000 µg/mL for *Mycobacterium*. Microplates (96 wells) were incubated at 37 °C for 24 h. Thereafter, 30 µL of aqueous resazurin solution (0.01%) was added to indicate the viability of the microorganisms. The MIC was determined as the lowest concentration of the compound capable of inhibiting the growth of the microorganism. Chlorhexidine was used as the reference antibiotic at concentrations of 0.115–59.0 µg/mL in the bioassays for bacteria and isoniazid at concentrations of 0.015–1.0 µg/mL for *Mycobacterium*. Middlebrook 7H9 broth containing 0.2% DMSO was used as a negative control.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

In vitro leishmanicidal activity of lactone 1,4-dihydroquinoline derivatives against *Leishmania (Leishmania) amazonensis*

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Abstract
A series of lactone 1,4-dihydroquinoline derivatives **4** (**4** and **4aa**–**4cm**) was screened for in vitro antileishmanial activity against the promastigote form of *Leishmania (Leishmania) amazonensis*. Screening results indicate that all of the synthesized compounds significantly reduced the growth of promastigote forms of promastigotes of *L. (L.) amazonensis*. The cytotoxicity of the most active compounds was also measured on peritoneal macrophage cells. Compounds **4ah** and **4bn** showed better activities than other derivatives with IC_{50} values of 6.22 and 9.05 µM, respectively, with selectivity index 22 and 15 times less toxicity to macrophages cells than to parasites, respectively. The experimental data propose that the compounds may be further investigated against amastigote forms and may contribute to the search of new candidates drugs for treatment of cutaneous leishmaniasis.

Keywords Cutaneous leishmaniasis · Dihydroquinoline derivatives · Lactone

Introduction

Leishmaniasis are neglected tropical diseases caused by protozoan parasites of the *Leishmania* genus and transmitted by the bite of female sandfly vectors (Pace 2014). These infections are endemic in 98 countries, with more than 12 million cases, and 350 million people living in an area at risk (Bhutta et al. 2014; Okwor and Uzonna 2016). The expression of leishmaniasis depends on a complex interaction between the type of infecting species and host immune response. Infection may be asymptomatic or may manifest as cutaneous leishmaniasis (CL) that is pleiomorphic in presentation, mucocutaneous leishmaniasis (MCL), or visceral leishmaniasis (VL) that may be lethal if untreated (Desjeux 2004; Pace 2014). In Latin America, *Leishmania (Leishmania) amazonensis* is responsible for a cutaneous diffuse form that in some cases may also result in visceral leishmaniasis (Torres-Guerrero et al. 2017).

The treatment of CL and VL is based on the use of expensive and toxic drugs, and that to a great extent must be administered by the parenteral route. The most used drugs are derivatives of pentavalent antimony; however, in many areas of this disease, parasitic resistance is already widespread (Ponte-Sucre et al. 2017). The second line of treatment is amphotericin B, an antifungal with a broad spectrum of action and that shows a high toxicity in the host (Fernández-García et al. 2017). Its less toxic lipid formulation is extremely expensive and incompatible with treatment in developing countries (Kumar et al. 2009; Pace 2014; Durieu et al. 2016; Fernández-García et al. 2017). Among the other drugs used in leishmaniasis therapy, miltefosine is the first oral treatment against VL, but its teratogenicity excludes the treatment of pregnant women and its slow turnover could promote the emergence of clinical parasite resistance (Singh et al. 2012; Durieu et al. 2016).

Therefore, these factors justify the search for new chemical series in order to find an orally safe and active drug (Loedige 2015; Ashok et al. 2017). The choice of the dihydroquinolinic nucleus associated with the lactonic ring is due to the presence of these pharmacophoric groups in several compounds with important leishmanicidal

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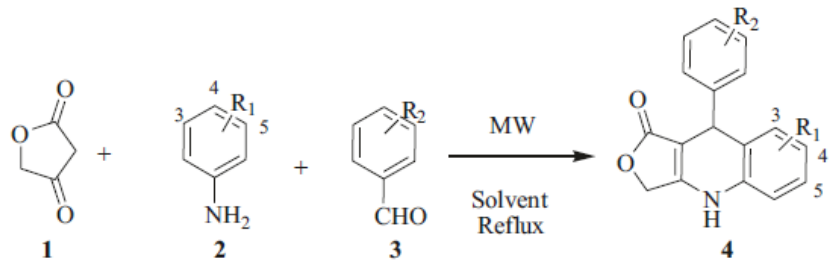
Table 1 Synthesis of dihydroquinoline lactone derivatives **4** and inhibition of the growth of *L. amazonensis* promastigote forms (%)



Entry	Aniline R1	Aldehyde R2	MM	Product	Inhibition (%)
1	**H**	H	261.27	4	64.75 ± 5.75
2	**3,4,5-OCH$_3$ (2a)**	3,4-(OCH$_2$O) (**3a**)	395.36	4aa	40.04 ± 6.68
3		3,4-(OCH$_3$) (**3b**)	411.40	4ab	38.60 ± 4.33
4		3,4,5-OCH$_3$ (**3c**)	441.43	4ac	79.85 ± 2.59
5		4-Cl (**3d**)	385.80	4ad	77.45 ± 5.05
6		4-Bn, 3-OCH$_3$ (**3e**)	487.50	4ae	94.96 ± 3.29
7		4-F (**3f**)	369.34	4af	78.89 ± 5.05
8		4-CH$_3$S (**3g**)	397.44	4ag	92.32 ± 2.72
9		4-CF$_3$ (**3h**)	419.35	4ah	89.92 ± 4.37
10		6-NO$_2$-3,4-(OCH$_3$) (**3i**)	456,40	4ai	50.36 ± 3.29
11		3-OCH$_3$, 4-OH (**3j**)	397.38	4aj	48.68 ± 2.31
12		3,4-OH (**3m**)	383.35	4am	56.49 ± 0.86
13		6-NO$_2$-3,4-(OCH$_2$O) (**3n**)	440.36	4an	100.00 ± 0.00
14	**3,4-(OCH$_2$O) (2b)**	3,4-(OCH$_2$O) (**3a**)	349.29	4ba	61.63 ± 5.40
15		3,4-(OCH$_3$) (**3b**)	365.34	4bb	70.26 ± 11.24
16		3,4,5-OCH$_3$ (**3c**)	395.36	4bc	64.26 ± 2.90
17		4-Cl (**3d**)	369.76	4bd	50.83 ± 2.90
18		4-Bn, 3-OCH$_3$ (**3e**)	441.43	4be	79.67 ± 2.45
19		4-F (**3f**)	323.27	4bf	55.15 ± 2.19
20		4-CH$_3$S (**3g**)	351.38	4bg	16.78 ± 13.97
21		4-CF$_3$ (**3h**)	373.28	4bh	35.97 ± 2.59
22		6-NO$_2$-3,4-(OCH$_3$) (**3i**)	410.33	4bi	100.00 ± 0.00
23		3-OCH$_3$, 4-OH (**3j**)	351.31	4bj	55.13 ± 3.78
24		6-NO$_2$-3,4-(OCH$_2$O) (**3n**)	394.29	4bn	95.99 ± 3.13
25	**3,4-(OCH$_3$) (3c)**	3,4-(OCH$_2$O) (**3a**)	365.34	4ca	73.62 ± 7.06
26		3,4-(OCH$_3$) (**3b**)	381.38	4cb	78.01 ± 2.52
27		3,4,5-OCH$_3$ (**3c**)	411.40	4cc	72.56 ± 4.21
28		4-Cl (**3d**)	355.77	4cd	100.00 ± 0.00
29		4-Bn, 3-OCH$_3$ (**3e**)	457.47	4ce	68.82 ± 5.40
30		4-F (**3f**)	339.32	4cf	74.10 ± 2.15
31		4-CH$_3$S (**3g**)	367.42	4cg	74.10 ± 2.87
32		4-CF$_3$ (**3h**)	389.32	4ch	100.00 ± 0.00
33		6-NO$_2$-3,4-(OCH$_3$) (**3i**)	426.38	4ci	65.23 ± 1.14
34		3,4-OH (**3m**)	353.33	4cm	95.99 ± 2.17
35		6-NO$_2$-3,4-(OCH$_2$O) (**3n**)	441.33	4cn	83.96 ± 5.00

properties (Castaño et al. 2009; Reynolds et al. 2013; Coimbra et al. 2016). As part of our ongoing interest on the antiparasitic activity of compounds, we have evaluated herein the leishmanicidal potential of 35 lactone 1,4-dihydroquinoline derivatives against the parasite *L. (L.) amazonensis* in vitro.

Results and discussion

Chemistry

The 1,4-dihydroquinoline lactone derivatives **4** and **4aa**–**4cm** were obtained in a previous work of our research group from microwave-assisted multicomponent reaction between tetronic acid, aromatic aldehyde, and anilines in ethanol (Table 1). These compounds showed interesting antimicrobial activities against oral bacteria (Laurentiz et al. 2018).

Derivatives **4a**–**4cn** have three different groups of electron-donating substituents on the dihydroquinolinic ring (3,4,5-OCH_3, 3,4-OCH_2O, and 3,4-OCH_3) and on the benzyl ring with electron-donating or electron-withdrawing substituents: OCH_3, OCH_2O, OH, Cl, CF_3, OBn, F, and NO_2 e SCH_3.

Antileishmanicidal activities

A preliminary screening of 35 lactones 1,4-dihydroquinoline derivatives was performed at 100 µM against promastigote forms de *L. (L.) amazonensis* to select the most active compounds at higher concentrations.

Although the clinically relevant form of the parasite is the amastigote form, which shows metabolic differences from the extracellular forms, promastigotes can be used for fast screenings of potential compounds (Tempone et al. 2005; Martín-Montes et al. 2017; Silva et al. 2017). Thus, in this study, we evaluated the effect of dihydroquinoline lactone derivatives against promastigote forms of *L. (L.) amazonesis* in vitro.

The compounds **4aa, 4ab, 4aj, 4bg**, and **4bh** showed a percentage of inhibition of cell growth of less than 50% at 24 h (Table 1). The other compounds showing activity higher than 50% include the unsubstituted compound **4**, however, only those with activity greater than 80% were submitted to additional assays in lower concentrations. Among the compounds with parasite activity above 80%, it was possible to observe that the compounds containing the nitro piperonal group as a substituent on the benzyl ring, independent of the substituent of the quinolinic moiety, are among the most active compounds (**4an**, **4bn**, and **4cn**). Compounds **4ah** and **4ch** containing the CF_3 group were also very active at this concentration.

Table 2 In vitro leishmanicidal activity: IC_{50}, CC_{50}, and selectivity index for dihydroquinolinine lactone derivatives

Compounds	Promastigote forms	Peritoneal macrophages	
	IC_{50} µM[a]	CC_{50} µM[b]	SI
4ae	5.29	6.07	1.13
4ag	32.5	ND	–
4ah	6.22	139.20	22.31
4an	33.40	ND	–
4bi	2.62	13.90	5.03
4bn	9.05	136.27	15.05
4cd	22.09	ND	–
4ch	17.90	ND	–
4cm	73.50	ND	–
4cn	42.90	ND	–
Amphotericin B	0.65	0.49	0.75

ND not determined, *SI* selectivity index

[a]Inhibition concentration 50 (IC_{50}) values were calculated using a nonlinear regression curve

[b]Cytotoxic concentration 50 (CC_{50}) values were calculated using a nonlinear regression curve

[c]SI was calculated from the ratio of CC_{50} values of macrophage and IC_{50} promastigotes

The substitution on phenyl and dihydroquinoline rings had a considerable effect on potency of other derivatives in comparison with unsubstituted compound **4**. All the evaluated compounds inhibited the growth of *L. (L.) amazonensis* promastigote forms to a greater or lesser degree, however, only those compounds that presented reduction on growth inhibition of promastigote forms with a percentage above 80% were evaluated at concentrations of 1.00–100 µM (Table 2).

According to hit-and-lead criteria, a hit compound should demonstrate $IC_{50} < 10$ µM against protozoan parasites and a selectivity index greater than 10-fold between the half-maximal cytotoxic concentration (CC_{50}) for the mammalian cell line and the IC_{50} for parasite (Dardonville et al. 2009; Katsuno et al. 2015). In this study, in promastigote activity assay at lower concentrations, the reported lactones 1,4-dihydroquinoline, displayed the growth of *L. (L.) amazonensis* promastigote forms with IC_{50} value ranges of 2.62–73.50 µM (Table 2). Among the reported compounds, analogs **4ae**, **4ah**, **4bi**, and **4bn** were more potent in inhibiting the *L.(L.) amazonensis* promastigotes with IC_{50} values of 5.29, 6.22, 2.62, and 9.05 µM, respectively. The positive control, amphotericin B, displayed the growth of the *L.(L.) amazonensis* promastigote forms with an IC_{50} value of 0.65 µM.

Among the compounds containing R1 = 3,4,5-OCH_3, **4ae** and **4ah** were more active with IC_{50} values of 5.29 and 6.22 µM, respectively. Compound **4ae** has electron donor

substituents in *para* (OBn) and *meta* (OCH_3) positions, while compound **4ah** has a single electron-withdrawing substituent in *para* (CF_3) position. The change of the OBn group for CF_3 and OCH_3 groups for H resulted in a small loss of activity, but contributed to enhance the selectivity index of **4ah** by almost 20x in relation to **4ae**. The nature of R2 and the degree of substitution for these two compounds mainly affected the selectivity index, showing that the hydrophobic and electronegative character of the CF3 group in addition to their smaller size contributed to the higher selectivity of **4ah** on *L. (L.) amazonensis* promastigote forms. The compounds more active with R1 = OCH_2O were **4bi** and **4bn** that have in common the nitro group connected to the phenyl ring. The presence of two OCH_3 groups on the phenyl ring (R2) contributes to the increase of **4bi** activity in relation to **4bn**, which presents the methylenedioxy group in the same position. These two groups of substituents in R2 have an electron donor character; however, the methylenedioxy group presents a more rigid character because it is a cycle and also has a greater hydrophobic character. Compound **4bi** showed better activity (IC_{50} = 2.62 µM, SI = 5.03), however, it was about three times less selective than **4bn** (IC_{50} = 9.05 µM, SI = 15.05).

The compounds containing the 3,4-dimethoxy substituents on the 1,4-dihydroquinolinic moiety were the least active (**4cd**, **4ch**, **4cm**, **and 4cn** with IC_{50} > 17 µM) independently of a substituent on the phenyl ring, however, these substituents on the dihydroquinolinic ring are not favorable to the leishmanicidal activity for this class of compounds.

Thus, the compounds with IC_{50} values below 10.0 µM (Table 2) are those with 3,4,5-trimethoxy and 3,4-methylenedioxy substituents in the 1,4,-dihydroquinolinic moiety, i.e., these groups are favorable to the action of this class of compounds and, therefore, the choice of the substituent on the benzyl ring is determinant for the increase in leishmanicidal activity.

Regarding macrophage cytotoxicity, the less toxic compounds were **4ah** and **4bn** with CC_{50} values of 139.20 and 136.27 µM, respectively. Compound **4bi** was one of the most toxic, as well as **4ae**, respectively, with CC_{50} values of 13.9 and 6.07 µM. In relation to the selectivity index, **4ah** and **4bn** were, respectively, 22 and 15 times less toxic to macrophage cells than the control amphotericin B that showed SI = 0.75 (Table 2).

The presence of the nitro group allied to the methylenedioxy group on the benzylic ring in **4bn** increased the selectivity index, as evidenced by the much lower selectivity index of the compound **4bi**. The presence of the CF_3 group in **4ah**, smaller and more lipophilic, favored the increase of the selectivity index when compared to **4ae** that has the OCH_3 and OBn groups on the phenyl ring.

Our results demonstrate the importance of the 1,4-dihydroquinolinic nucleus in the study of compounds with antileishmanial properties. Recent works have confirmed the interesting antileishmanial properties of quinoline derivatives (Antinarelli et al. 2015; Herrera et al. 2016; Navneetha et al. 2017). In these works, various quinoline derivatives have been synthesized and showed results that attest to the importance of this pharmacophore nucleus for leishmanicidal activity against the promastigote form. However, few studies report the activity of 1,4-dihydroquinoline derivatives, which demonstrates that the potential of this class of compounds has not yet been adequately explored.

Conclusion

Thirty-five lactone 1,4-dihydroquinoline derivatives were submitted to antileishmanial assay against promastigote forms of *L. (L.) amazonesis* in vitro. Among the evaluated compounds, **4ah** and **4bn** showed promising in vitro antileishmanial activity and low toxicity in macrophage cells. These compounds are interesting targets that emerge among the various classes of compounds already synthesized and evaluated against leishmaniasis. The results obtained with these compounds are an additive justification for further studies of the lactone 1,4-dihydroquinoline derivatives against amastigote forms and may contribute to the search for new molecules as targets for the development of more potent and fewer side-effect drugs.

Materials and methods

Chemistry

A mixture of tetronic acid (1.0 mmol), benzaldehydes (1.0 mmol), and anilines (1.0 mmol) in ethanol (2 mL) was taken in a reaction flask equipped with a small magnetic stirring bar and the reflux condenser. The mixture was then irradiated in a microwave reactor for 15 min (reflux temperature of the solvent) at a power of 200 W. After the reaction was complete (TLC monitoring), the mixture was then cooled to room temperature and the solvent was removed on rotary evaporator. The precipitated crude product was washed with a mixture of hexane-ethyl acetate (8:2) and dried under vacuum. The variation of anilines (**2a–c**) and benzaldehydes (**3a–n**) furnished the lactone 1,4-dihydroquinolines **4aa**–**4cn** in yields ranging from 69 to 92%. Data of characterization of the compounds were described by Laurentiz et al. (2018).

Biological assays

Antileishmanial activity

Cultures of *L. (L.) amazonensis* (MHOM/BR/PH8) promastigote forms were maintained at 25 °C in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Cultilab), 50 units·mL^{-1} penicillin, and 50 mg·mL^{-1} streptomycin (Cultilab).

The initial screening was assessed in vitro by cultivating *L. (L.) amazonensis* promastigotes (1×10^6 parasites mL^{-1}) in a 96-well plate containing RPMI 1640 medium (Gibco) supplemented with 10% FBS (Cultilab), 50 units·mL^{-1} penicillin, and 50 mg·mL^{-1} streptomycin (Cultilab) in the presence of 100 µM of compounds previously dissolved in 100% dimethylsulfoxide (DMSO) (Synth). Cultures were incubated at 25 °C in BOD (Quimis) for 24 h and the leishmanicidal activity was determined by growth inhibition of promastigote forms by counting the total number of live promastigotes in the Neubauer chamber (Global Glass), considering flagellar motility (Azzouz et al. 2005).

The compounds that showed greater than or equal to 80% inhibition of cell growth during 24 h were evaluated at concentrations of 1.00–100 µM. RPMI 1640 medium (Gibco) containing 0.5% DMSO (Synth) (highest concentration) was used as a negative control and amphotericin B (Eurofarma) at concentrations of 0.20–3.37 µM was used as a positive control. Two experiments were performed in triplicate and repeated twice. Determination of 50% inhibition concentration values (IC_{50}) was carried out by GraphPad Prism version 5.0 Windows software (GraphPad Software, USA) using a nonlinear regression model of variable slope.

Macrophages and cytotoxicity assay

Thioglycolate-elicited peritoneal macrophages were obtained from BALB/c mice (weighing 20–25 g) by injection of 500 µL of 3% thioglycolate 3 days prior to peritoneal lavage with 5 mL of ice-cold phosphate- buffered saline (PBS 1 ×). The peritoneal exudate cells were centrifuged at 400 × g for 10 min at 4 °C, the supernatant was removed, and RPMI 1640 (Gibco) medium was supplemented with 10% FBS (Cultilab), 50 units·mL^{-1} penicillin, and 50 mg·mL^{-1} streptomycin (Cultilab) was added to the pellet (macrophages) (Toledo et al. 2014). The experiment was authorized by the University of Franca´s Ethics Committee for Animal Care (approval number: 046/15).

Macrophages (2×10^5 cells/well) were seeded in a 96-well microtiter plate and incubated at 37 °C in a 5% CO_2 atmosphere for 24 h and when the macrophages had developed a monolayer, the cells were exposed with the compounds at concentrations of 1.60 to 200 µM (**4ae, 4ah, 4bi, and 4bn**) or 0.20–3.38 µM (amphotericin B) for 24 h. The morphological changes of treated and untreated cell lines (control) were compared by monitoring, using an inverted microscope (Axio Vert, Carl Zeiss). Cell viability was determined using the vital stain Trypan Blue (Aldrich) at 0.1% concentration in phosphate buffer (Mesa-Valle et al. 1996). The number of dead cells was recorded, and the percentage of viability was determined as [no. of viable cells in the cells treated/no. of viable cells in the cells not treated (negative control)] × 100. RPMI 1640 medium (Gibco) containing 0.5% DMSO (Synth) (highest concentration) was used as a negative control and 25% DMSO was used as a positive control. Two experiments were performed in triplicate and repeated twice. Determination of 50% cytotoxic concentration values (CC_{50}) was carried out by GraphPad Prism version 5.0 Windows software (GraphPad software, USA) using a nonlinear regression model of variable slope.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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5. Conclusões

Nesse texto sistemático o objetivo foi demonstrar, de uma forma sequencial, a evolução das pesquisas desenvolvidas por mim e a contribuição na área de produtos naturais com o estudo de extratos, extração de metabólitos secundários e na obtenção de derivados semissintéticos. Nossa expectativa é contribuir para a descoberta de novas moléculas que possam ser utilizadas como alvos no desenvolvimento de novos fármacos tanto para uso humano quanto para o uso animal, a partir dos extratos de plantas estudados e de alguns dos seus metabólitos isolados. Alguns desses estudos resultaram em patentes e uma delas foi licenciada e está em estudo para comercialização do produto alvo no exterior.

Na parte sintética visamos contribuir também para o desenvolvimento de novas metodologias sintéticas para a obtenção mais eficiente de moléculas conhecidas e inéditas sempre com o foco no desenvolvimento de novos fármacos.

A síntese vinculada ao estudo das propriedades biológicas proporcionou a interação com vários grupos de pesquisa no Brasil e no exterior contribuindo para crescimento e fortalecimento do Grupo de Produtos Naturais e Síntese Orgânica do qual sou coordenadora aqui na FEIS. Dessas interações obtivemos resultados que foram publicados em diversos artigos científicos e outros ainda em fase de redação que são resultados da síntese de compostos heterocíclicos e de suas propriedades biológicas e luminescentes.