SAÚDE E AMBIENTE
V.10 • N.1 • 2025 - Fluxo Contínuo

ISSN Digital: **2316-3798**
ISSN Impresso: **2316-3313**
DOI: **10.17564/2316-3798.2025v10n1p220-238**

INTER FACES CIENTÍFICAS

ENZYME MODULATION AND ANTIMICROBIAL ACTIVITY OF THE POLYHYDROQUINOLINE DERIVATIVE 4-(4-HYDROXY-PHENYL)-2-METHYL-5-OXO-1,4,5,6,7,8-HEXAHYDROQUINOLINE-3-CARBOXYLIC ACID ETHYL ESTER

MODULAÇÃO ENZIMÁTICA E ATIVIDADE ANTIMICROBIANA DO DERIVADO DE POLIHIDROQUINOLINA ÉSTER ETÍLICO DO ÁCIDO 4-(4-HIDROXI-FENIL)-2-METIL-5-OXO-1,4,5,6,7,8-HEXAHIDROQUINOLINA-3-CARBOXÍLICO

MODULACIÓN ENZIMÁTICA Y ACTIVIDAD ANTIMICROBIANA DEL DERIVADO DE POLIHIDROQUINOLINA ÉSTER ETÍLICO DEL ÁCIDO 4-(4-HIDROXI-FENIL)-2-METIL-5-OXO-1,4,5,6,7,8-HEXAHIDROQUINOLINA-3-CARBOXÍLICO

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ABSTRACT

N-heterocyclic compounds are widely used in medicine and pharmacology due to their enormous diversity of biological activities (for example: antimicrobial, anticonvulsant, anti-inflammatory, anticancer, anthelmintic, antihistamine, antihypertensive, and antidepressant). Thus, these compounds are relevant for the synthesis of new drugs. The molecule 4-(4-Hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester was obtained using a multicomponent reaction, and its structure was characterized by Nuclear Magnetic Resonance (NMR). Its biological prospection included modulating action on phospholipases A_2 and proteases, assessment of cytotoxicity and effects on processes associated with hemostasis, and antimicrobial activity. Several doses of the molecule were tested in each test, ranging from 25 to 500 mg. For antimicrobial activity, the molecule exerted significant inhibitions for both *S. aureus* and *P. aeruginosa*. When assessed the effects on the coagulation of citrated human plasma, the molecule previously incubated with *Bothrops moojeni* venom (40 µg) was able to delay plasma coagulation, induced by proteases, in 14 and 13.66 seconds ($p<0.05$) at doses of 125 and 100 mg, respectively. Furthermore, the synthesized compound previously incubated with plasma, with the subsequent addition of venom, was able to prolong the clotting time in all evaluated doses ($p<0.05$). *B. moojeni* venom had its thrombolytic activity reduced to 73, 88, and 82% ($p<0.05$) when incubated with the compound at doses of 125, 100, and 50 mg, respectively. At 25 mg, an increase in thrombolysis was observed, combining the effects of the venom and the compound. The phospholipase activity induced by the venom was significantly reduced after incubation of the venom with the

compound at the highest dose (125 mg). In addition, a protective effect on human erythrocytes was exerted by the compound at all doses evaluated against *B. moojeni* venom (20 µg). Thus, in this manuscript, a relatively simple multicomponent reaction protocol (satisfactory yield – 56%, low cost, and ecologically adequate) was effective in obtaining a new polyhydroquinoline with various biological activities, that can be better explored for future applications in the context of human health.

KEYWORDS

Coagulant activity; anti-inflammatory activity; anti-cytotoxic activity; multicomponent reaction.

RESUMO

Os compostos N-heterocíclicos são amplamente utilizados na medicina e na farmacologia devido à sua enorme diversidade de atividades biológicas (por exemplo: antimicrobiana, anticonvulsivante, anti-inflamatória, anticancerígena, anti-helmíntica, anti-histamínica, anti-hipertensiva e antidepressiva). Assim, estes compostos são relevantes para a síntese de novos fármacos. A molécula éster etílico do ácido 4-(4-Hidroxi-fenil)-2-metil-5-oxo-1,4,5,6,7,8-hexahidroquinolina-3-carboxílico foi obtida utilizando uma reação multicomponente, e sua estrutura foi caracterizada por Ressonância Magnética Nuclear (RMN). Sua prospecção biológica incluiu ação moduladora sobre fosfolipases A_2 e proteases, avaliação de citotoxicidade e efeitos em processos associados à hemostasia e atividade antimicrobiana. Várias doses da molécula foram testadas em cada teste, variando de 25 a 500 mg. Para a atividade antimicrobiana, a molécula exerceu inibições significativas tanto para *S. aureus* quanto para *P. aeruginosa*. Quando avaliados os efeitos na coagulação do plasma humano citratado, a molécula, previamente incubada com veneno de *Bothrops moojeni* (40 µg), foi capaz de retardar a coagulação plasmática induzida por proteases em 14 e 13,66 segundos ($p<005$) nas doses de 125 e 100 mg, respectivamente. Além disso, o composto sintetizado previamente incubado com plasma, com posterior adição de veneno, foi capaz de prolongar o tempo de coagulação em todas as doses avaliadas ($p<0,05$). O veneno de *B. moojeni* teve sua atividade trombolítica reduzida para 73, 88 e 82% ($p<0,05$) quando incubado com o composto nas doses de 125, 100 e 50 mg, respectivamente. Enquanto que à 25 mg foi observado um aumento na trombólise, somando-se os efeitos da peçonha e do composto. A atividade de fosfolipase induzida pelo veneno foi significativamente reduzida após a incubação do veneno com o composto na dose mais elevada (125 mg). Além disso, um efeito protetor sobre os eritrócitos humanos foi exercido pelo composto em todas as doses avaliadas, contra o veneno de *B. moojeni* (20 µg). Assim, neste manuscrito um protocolo de reação multicomponente relativamente simples (rendimento satisfatório – 56%, baixo custo e ecologicamente adequado), foi eficaz para a obtenção de uma nova polihidroquinolina que apresenta diversas atividades biológicas, que podem ser melhor exploradas para futuras aplicações no contexto da saúde humana.

PALAVRAS-CHAVE

Atividade coagulante; atividade anti-inflamatória; atividade anticitotóxica; reação multicomponente.

RESUMEN

Los compuestos N-heterocíclicos se utilizan ampliamente en medicina y farmacología debido a su enorme diversidad de actividades biológicas (por ejemplo: antimicrobianos, anticonvulsivos, antiinflamatorios, anticancerígenos, antihelmínticos, antihistamínicos, antihipertensivos y antidepresivos). Por tanto, estos compuestos son relevantes para la síntesis de nuevos fármacos. La molécula de éster etílico del ácido 4-(4-hidroxi-fenil)-2-metil-5-oxo-1,4,5,6,7,8-hexahidroquinolina-3-carboxílico se obtuvo mediante una reacción multicomponente y su estructura se caracterizó por Resonancia Magnética Nuclear (RMN). Su prospección biológica incluyó la acción moduladora sobre las fosfolipasas A2 y proteasas, evaluación de la citotoxicidad y efectos sobre procesos asociados a la hemostasia y actividad antimicrobiana. En cada ensayo se probaron diferentes dosis de la molécula, que oscilaban entre 25 y 500 mg. En cuanto a la actividad antimicrobiana, la molécula ejerció inhibiciones significativas tanto contra *S. aureus* como contra *P. aeruginosa*. Cuando se evaluaron los efectos sobre la coagulación del plasma humano citratado, la molécula, previamente incubada con veneno de *Bothrops moojeni* (40 µg), fue capaz de retrasar la coagulación plasmática inducida por proteasas en 14 y 13,66 segundos ($p<0,005$) a dosis de 125 y 100 mg, respectivamente. Además, el compuesto sintetizado previamente incubado con plasma, con posterior adición de veneno, fue capaz de prolongar el tiempo de coagulación en todas las dosis evaluadas ($p<0,05$). La actividad trombolítica del veneno de *B. moojeni* se redujo al 73, 88 y 82% ($p<0,05$) cuando se incubó con el compuesto en dosis de 125, 100 y 50 mg, respectivamente. Mientras que a 25 mg se observó un aumento de la trombolisis, sumándose los efectos del veneno y del compuesto. La actividad de la fosfolipasa inducida por el veneno se redujo significativamente después de la incubación del veneno con el compuesto en la dosis más alta (125 mg). Además, el compuesto ejerció un efecto protector sobre los eritrocitos humanos en todas las dosis evaluadas, contra el veneno de *B. moojeni* (20 µg). Así, en este manuscrito, un protocolo de reacción multicomponente relativamente simple (rendimiento satisfactorio – 56%, bajo costo y ecológicamente adecuado) fue eficaz para obtener una nueva polihidroquinolina que presenta varias actividades biológicas, que pueden explorarse mejor para futuras aplicaciones en el contexto de la salud humana.

PALABRAS CLAVE

Actividad coagulante; actividad antiinflamatoria; actividad anticitotóxica; reacción multicomponente.

1 INTRODUCTION

When developing new pharmacological substances, organic synthesis is directly linked to molecules and components previously isolated from natural products (ABDEL-RAZEK *et al.*, 2020; SULEIMAN *et al.*, 2020). Alkaloids, found mainly in plants, are organic nitrogen compounds where one or more nitrogen atoms are present (typically primary, secondary, or tertiary amines) (BRAVO *et al.*, 2010). The presence of nitrogen and/or aromatic rings in molecules is evidenced in several active ingredients used in medicine, pharmacology, and agrochemistry (CORDELL *et al.*, 2001; MICHAEL, 2008; BHAMBHANI *et al.*, 2021).

Cyclic compounds containing one or more nitrogen atoms, also known as N-heterocyclic compounds, are widely used in medicine and pharmacology due to their diverse biological activities (DUARTE *et al.*, 2010; JANA *et al.*, 2020). For example, we can mention the following functions: antimicrobial, anticonvulsant, anti-inflammatory, anticancer, anthelmintic, antihistamine, antihypertensive, and antidepressant (DUA *et al.*, 2011; CHOVANCOVA *et al.*, 2020; NGUYEN *et al.*, 2021). Thus, using these compounds to synthesize a new drug is extremely important due to their different actions.

However, for the industry, the synthesis still faces problems related to the approval of the new drug (probability of 22,900:1 to be approved) and the increased funding needed (MICHILES; BOTSARIS, 2005). In addition, large amounts of catalysts and a variety of products are often used to obtain a low yield of the desired molecules.

Thus, developing a relatively simple protocol that results in high yield, low cost, and is ecologically appropriate for producing molecules that have biological activities already described, has been a challenge for synthetic organic chemists (WILSON; DANISHEFSKY, 2006). Therefore, multicomponent reaction (MCR) is an excellent option since it is easy to perform, has high atomic efficiency, reduced reaction time, results in less environmental impact, and can be used in the development of new drugs (WEBER, 2002; DEKAMIN *et al.*, 2018; NGUYEN *et al.*, 2021).

In this manuscript, the molecule 4-(4-hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester was obtained using a simple, effective, and reliable multicomponent reaction, without the use of solvents or catalysts and its structure was characterized. Once synthesized, the molecule underwent biological evaluation, including its modulatory effects on phospholipases A_2 and proteases, cytotoxicity assessment, and its effects on hemostasis-related processes and antimicrobial activity.

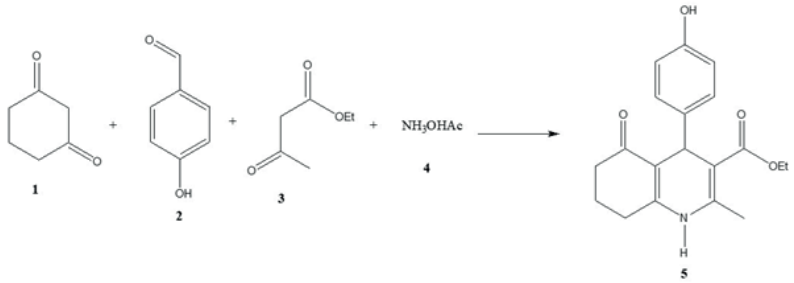
2 MATERIAL AND METHODS

2.1 ORGANIC SYNTHESIS

The multicomponent reaction performed to synthesize the molecule was based on the methodology described by Kumar *et al.* (2008). Catalyst and solvents were not used in the synthesis. The MCR was

performed using the following reagents, as demonstrated in Figure 1: 1,3-cyclohexanedione (2 mmol) **1**, 4-hydroxybenzaldehyde (2 mmol) **2**, ethyl acetoacetate (2 mmol) **3,** and ammonium acetate (3 mmol) **4**.

Figure 1. Multicomponent reaction used to synthesize the molecule 4-(4-hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester.



Source: Elaborated by the authors

2.2 CHARACTERIZATION BY NUCLEAR MAGNETIC RESONANCE (NMR)

The characterization by Nuclear Magnetic Resonance (NMR) was performed at Federal University of São Carlos (UFSCar). A Bruker Avance III – 14.1 Tesla (600.23 MHz for 1H frequency) equipment with an Ultrashield Plus® magnet was used to obtain the NMR spectra. The spectrometer is equipped with a 5 mm TCI (Triple Resonance Inverse) CryoProbe - ^{1}H and ^{13}C nuclei- and cold preamplifiers (temperature at approximately -196 °C). The probe has a Z-Gradient (53G/cm) and an Automatic Tuning and Matching accessory (ATMA).

2.3 ATTENUATED TOTAL REFLECTION- FOURIER TRANSFORM INFRARED (FTIR/ATR) SPECTROSCOPY

A Varian equipment coupled to a diamond ATR accessory (GladiATR - PIKE Technologies) was used to obtain the attenuated total reflection (ATR) infrared spectra (spectral range of 4000 to 400 cm^{-1} and resolution of 2000 to 4 cm^{-1}).

The analysis of the functional groups in the synthesized molecule was performed by Fourier transform infrared spectroscopy (FTIR) at the Chemical Analysis and Prospection Center (CAPQ), located at the Department of Chemistry/UFLA. The IR absorption spectra were obtained with KBr pellets (2.0 mg of the sample was added into 200.0 mg of KBr).

2.4 TEST SOLUTIONS

Solubility tests were performed to obtain samples partially diluted in phosphate-buffered saline - PBS (137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 10 mmol L^{-1} NaH_2PO_4, 2 mmol L^{-1} KH_2PO_4) and decrease the possible interference of dimethyl sulfoxide (DMSO), which was used to dissolve the compound. Screening tests were performed with the synthesized compound to identify the doses that could have their pharmacological effects evaluated, without changing the reaction environment. The doses of the controls used in each assay were also previously determined.

Four (4) doses were defined for the evaluation of the modulatory potential on enzymes that act in hemostatic and anti-inflammatory processes: 25, 50, 100, and 125 mg. For the antimicrobial activities, the following doses were used: 100, 125, 250, and 500 mg.

All tests were performed in triplicates.

2.5 EVALUATION OF THE ANTIMICROBIAL ACTIVITY

Susceptibility testing using the agar diffusion method (antibiogram) and pour-plate technique with serial dilution (broth microdilution) were performed to evaluate the antimicrobial activity of the molecule 4-(4-hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydro-quinoline-3-carboxylic-acid ethyl ester. Both tests were performed following the guidelines set forth by the Clinical and Laboratory Standards Institute (CLSI, 2024).

2.5.1 OBTAINING THE MICROORGANISMS AND INOCULUM

The microorganisms used (*Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853) were obtained from Fundação Oswaldo Cruz (FIOCRUZ, Rio de Janeiro, Brazil), and gently ceded by the Laboratory of Bacteria staff at the Department of Food Sciences of Federal University of Lavras. The stock cultures were stored in a cell freezing medium (100 mL of distilled water, 15 mL of glycerol, 0.3 g of yeast extract, 0.5 g peptone/bacteriological, and 0.5 g of NaCl). To reactivate the strains, 100 µL of the culture was inoculated in tubes containing 10 mL Brain Heart Infusion (BHI) broth at 37°C for 4h under aerobic conditions. The inoculum was standardized by McFarland standards to a cell density of approximately 10^8 CFU mL^{-1}.

2.5.2 ANTIMICROBIAL TESTING

All tests and concentrations were evaluated in triplicate.

In the first technique, an aliquot (standardized at 10^8 CFU mL^{-1}) containing either the culture of *S. aureus* or *P. aeruginosa* was spread in Petri dishes coated with Trypticase Soy Agar (TSA). Afterwards, to perform the antibiogram, disks were placed into the dishes with the synthesized molecule (4-(4-hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydro-quinoline-3-carboxylic-acid ethyl ester) in the doses of 500, 250, 125, and 100 mg. The Petri dishes containing the treatments were incubated at 37 °C for 24h. The antibiotic Chloramphenicol (CLSI, 2024) was used as a positive control (30 µg). To evaluate the activity, inhibition halos were measured around the disks.

For the second technique, a solution containing 500 mg of the compound diluted in 1 mL DMSO was used. 500 µL of this solution were transferred to a tube containing 500 µL of BHI broth, obtaining a final dose of 250 mg (used as the stock solution). The spread plate technique was performed at 0, 24, and 48 hours, with serial dilutions (900 µL of distilled water for each 100 µL of the stock solution) to *obtain an adequate number of colonies (considering the count of isolated colonies and numbers compatible with the growth observed in the control containing only the bacteria in BHI medium). S. aureus* or *P. aeruginosa* in 1000 µL of BHI were used as positive controls (CLSI, 2024).

2.6 OBTAINING HUMAN BIOLOGICAL MATERIAL

Experimental protocols (coagulation, cytotoxicity on erythrocytes, and thrombolytic) that require the collection and use of human blood were previously approved by the Committee for Ethics in Research on Human Beings (COEP) of the Federal University of Lavras (Opinion Number: 3.288.976; CAAE 10991019.4.0000.5148).

Five (5) volunteers between 18 and 35 years old were randomly selected following some pre-determined standards, such as not be using prescription medication or have not been under surgical procedures close to the blood collection period. The volunteers were non-smokers that did not work under chemical or biological risk conditions. In addition, all of them signed an Informed Consent Form (ICF).

2.7 EFFECTS ON THE COAGULATION OF CITRATED HUMAN PLASMA

The coagulation activity was performed according to the methodology described by Mourao *et al*. (1996), which used 200 µL of citrated human plasma stabilized at 37°C. The treatments (compound in the doses of 125, 100, 50, and 25 mg) were applied to the plasma to evaluate their coagulant activity. Time (seconds) was counted until the formation of a rigid clot. *Bothrops moojeni* (40µg) was used as positive control. The compound was also incubated with *B. moojeni* to evaluate its possible interactions with proteases that induce coagulation.

2.8 EFFECTS ON HUMAN BLOOD THROMBI AND VENOM-INDUCED THROMBOLYTIC ACTIVITY

The thrombolytic activity was evaluated on blood clots formed *in vitro* (CINTRA *et al*., 2012). 100 µL of freshly collected blood (without anticoagulant) were placed into each well of a 96-well microplate, which remained for 15 minutes in room temperature to obtain the thrombi. Afterwards, the thrombi were incubated with the compound (125, 100, 50, and 25 mg) for 24 hours at 37°C. *Bothrops moojeni* (20 µg) venom and PBS (30 µL) were used as positive and negative controls, respectively.

The compound was also evaluated as to its potential in modulate the activity of thrombolytic proteases present in the snake venom, and for that purpose, all doses of the compound were incubated for 30 minutes at 37°C with *B. moojeni* venom. The activities were estimated by measuring the volume of liquid released by each thrombus. The values were converted into percentage and the positive control was considered as 100% of activity.

2.9 EFFECTS ON THE ACTIVITY OF PHOSPHOLIPASES A_2

The phospholipase activity was evaluated using an adapted version of the methodology described by Gutiérrez *et al*. (1988). 0.01 mol L^{-1} $CaCl_2$, egg yolk phospholipids (phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine), 0.005% sodium azide, and 1% bacteriological agar dissolved in PBS (cooled to 45 ºC) were mixed to form a medium (pH 7.2). The medium was poured into Petri dishes and, after solidification, the samples were applied to holes made in the gel (~ 0.5 cm diameter).

Pre-incubation of the venom with the synthesized compound (125, 100, 50, and 25 mg) was performed for 30 minutes at 37°C. The anti-inflammatory and enzyme modulating activity of the compound was evaluated using phospholipase A_2 present in *Bothrops moojeni* venom (20µg), which served as a tool to induce the breakdown of phospholipids. The translucent halos, formed around the sites of

sample application on the gel, resulting from phospholipase activity (expressed in millimeters), were evaluated after 18 hours of the plates' stay in the cell culture chamber at 37ºC.

2.10 CYTOTOXIC ACTIVITY ON HUMAN ERYTHROCYTES

This activity was also evaluated using an adapted version of the methodology described by Gutiérrez *et al*. (1988). However, the phospholipids were replaced by human erythrocytes to form the gel (0.01 mol L^{-1} $CaCl_2$, erythrocytes 1:3 v v^{-1} in PBS, 0.005% sodium azide, and 1% bacteriological agar dissolved in PBS - pH 7.2).

To obtain the erythrocytes concentrate, blood collected in tubes containing heparin was immediately centrifuged, and the supernatant discarded. 10 mL of PBS was added to the erythrocytes, which were washed twice more in PBS with centrifugations at 1200 g for 10 minutes.

The compound (125, 100, 50, and 25 mg) were applied into holes made in the gel (~0.5 cm diameter) and evaluated for lysis induction. Then, to verify its anti-cytotoxic potential, the compound was evaluated after pre-incubation with *Bothrops moojeni* venom (20 µg) for 30 minutes at 36 ºC, and then the samples were applied to the gels. The Petri dishes were kept in a cell culture chamber at 36°C for 18 hours. *B. moojeni* venom was used as a positive control. The formation of a translucent halo around the hole characterizes hemolysis, which was measured (millimeters) with a caliper.

2.11 STATISTICAL ANALYSIS

The data obtained were submitted to the Shapiro-Wilk test at a 5% significance level to verify their normality. Dunnett's test was performed for the data that had a normal distribution. A normalization attempt was made using the RANK procedure (PROC RANK) on the data that did not show a normal distribution. Non-normalized data were compared by the Kruskal-Wallis test at a 5% significance level. The statistical software SAS (v. 9.0) was used.

3 RESULTS AND DISCUSSION

3.1 SYNTHESIS AND STRUCTURAL CHARACTERIZATION

The maceration of the reagents led to a yellow solid product, corroborating literature data (KUMAR *et al.*, 2008), which was rinsed with ice-cold distilled water to remove excess unreacted ammonium acetate, and then remained at rest for 24h. For the recrystallization of the product, 20 µL of ethanol at 68°C were added to dissolve the product (solid mass), which turned into a viscous liquid, and then filtered using a filter, kitassate, and vacuum pump, generating the hexahydroquinoline crystals. The yield for the obtained molecule was 56%.

For the molecule 4-(4-hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester, a hydroxyl group is connected at the *para* position in relation to the nitrogen ring. 1H NMR spectrum shows resonances at H 4.83 (H4, 1H, *s*), 4.03 (H12, 2H, *q*, $^3J_{H12\text{-}H13}$ = 7.0 Hz), 2.55-1.70 (H7, H8, and H9, 6H, *m*), 2.30 (H1, 3H, *s*), and 1.18 (H13, 3H, *t*, $^3J_{H13\text{-}H12}$ = 7.0 Hz) compatible with what is

expected for this class of molecules. In the olefinic and aromatic region, two well separated and defined multiplets, integrating for 4 hydrogen atoms, confirm the presence of a disubstituted aromatic ring with *para* pattern: H 6.99-6.96 (H15 and H19, 2H, m) and 6.61-6.59 (H16 and H18, 2H, m) ppm.

In the 13C NMR spectrum, it was possible to identify a signal at C 155.7 (C17) ppm, compatible with sp2 carbon of an aromatic ring connected directly to an oxygen atom (hydroxyl), and a signal at C 138.9 (C14) ppm according to sp2 carbon of non-hydrogenated aromatic ring at a *para* position to the oxygenated carbon. Carbons C15 and C19 resonate at C 128.7 and C16 and C18 at C 114.9. The other peaks observed also corroborate for the structural characterization: C 194.1 (C6), 167.5 (C11), 151.4 (C10), 144.7 (C2), 111.9 (C5), 104, 5 (C3), 59.4 (C12), 50.7 (C7), 37.0 (C4), 35.0 (C7), 26.6 (C9), 21.3 (C8), 18.6 (C1), and 14.6 (C13) ppm.

The IR spectrum for the molecule shows the O-H stretch band of alcohol (phenol) at 3522 cm^{-1}, which is not widened since it is difficult for these molecules to interact with each other by hydrogen bonding due to steric hindrance. The N-H stretch band of a secondary amine is found at 3294 cm^{-1}, C_{sp2}-H stretch at 3067 cm^{-1}, C_{sp3}-H stretch at 2954, ester C=O stretch at 1610 cm^{-1}, as this is a weaker bound carbonyl due to resonance, ketone C=O stretch at 1671 cm^{-1}, aromatic C=C bond stretch at 1478 cm^{-1} and C-O-C (ester) bond stretch bands at 1181 and 1136 cm^{-1}. The band at 842 cm^{-1} probably refers to out-of-plane bending of an aromatic ring with *para* substitution pattern.

3.2 BIOLOGICAL ACTIVITIES

3.2.1 ANTIMICROBIAL EVALUATION

The reason for selecting the tested strains is due to their importance in human infections and their presence in wounds, which delays the healing process. Unfortunately, it was impossible to evaluate the compound in the agar diffusion test as it did not diffuse into the solid medium. However, for the liquid medium incubation technique, followed by plating after serial dilution and colony counting, the results were statistically significant, showing the antimicrobial action of the compound for both *S. aureus* and *P. aeruginosa*, as shown in Tables 1 and 2.

Table 1 - Antimicrobial activity of the molecule 4-(4-hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydro--quinoline-3-carboxylic-acid ethyl ester and positive control on *Staphylococcus aureus* ATCC 25923.

Time (h)	Antimicrobial activity (Colony Forming Units, CFU)			
	Control	Compound (250 mg)	CV (%)	P-value
* 0	5.09	5.00	1.94	0.308
* 24	10.28	4.04	47.83	0.033
* 48	15.32	2.22	83.32	0.033

CV (%): Coefficient of Variation. P-value less than 0.05 is statistically significant and is highlighted in red. * Data have a normal distribution.
Source: Research data

Table 2 - Antimicrobial activity of the molecule 4-(4-hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydro--quinoline-3-carboxylic-acid ethyl ester and positive control on Pseudomonas aeruginosa ATCC 27853.

Time (h)	Antimicrobial activity (Colony Forming Units, CFU)			
	Control	Compound (250 mg)	CV (%)	P-value
* 0	4.52	4.52	0.76	0.920
* 24	12.36	0.00	109.55	0.011
* 48	18.44	0.00	109.69	0.011

CV (%): Coefficient of Variation. P-value less than 0.05 is statistically significant and is highlighted in red. * Data have a normal distribution.
Source: Research data

There is no significant difference between the compound and the control for both bacteria at time zero, which was expected since this time elapses immediately after dilution of the bacterial inoculum. However, when the plates containing TSA are incubated for 24h and 48h, a significant result is observed for both *S. aureus* and *P. aeruginosa*. For *S. aureus*, the number of viable cells decreased over 24h and 48h (Table 1), while for *P. aeruginosa*, the 24h treatment time was sufficient to eliminate viable cells. Thus, the compound presented a robust bactericidal action against Gram-negative bacteria, which can be more difficult to be eliminated from host organisms, as stated by Jubeh *et al*. (2020).

The antibiotic Chloramphenicol was used as a positive control (30 *μg*), according to prior standardization in the microbiology laboratory. However, it was not possible to establish a comparison profile between the drug and the compound, as no bacterial growth was observed at any of the time points evaluated for chloramphenicol.

Jamale *et al*. (2019) also studied polyhydroquinoline derivatives. Said derivatives were potentially significant as antimicrobial and antituberculosis agents, with some of the compounds proving effective against *Mycobacterium tuberculosis*, considered an extremely difficult bacterium in terms of elimination.

Recently, Mane *et al*. (2021) carried out antimicrobial tests of various polyhydroquinoline derivatives against Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacterial strains and against filamentous fungi (*Aspergillus niger*) and yeast (*Saccharomyces cerevisiae*), and determined their minimum inhibitory concentrations (MIC).

3.2.2 EFFECTS ON THE COAGULATION OF CITRATED HUMAN PLASMA

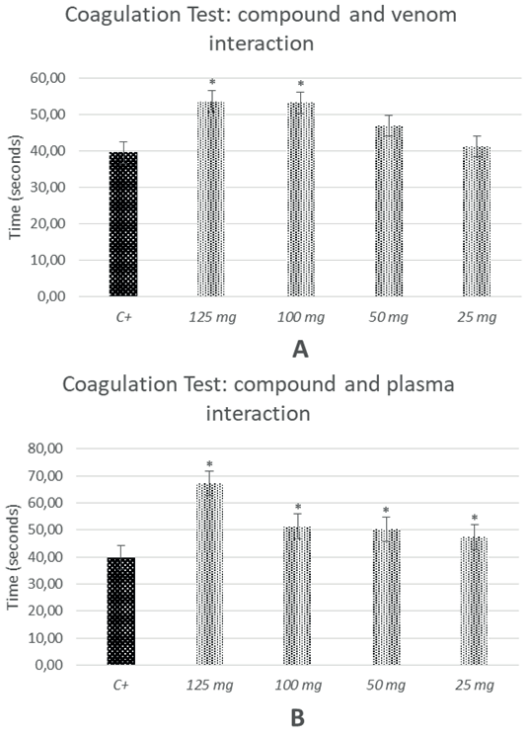
When previously incubated with *Bothrops moojeni* venom (40μg), the compound at doses of 125 and 100 mg was able to reduce the coagulant activity induced by proteases and prolong the plasma clotting time by 14 and 13.66 seconds ($p < 0.05$), respectively (Figure 2A).

However, when the compound is previously incubated with citrated human plasma, followed by the addition of the venom, clotting time increases at all doses tested ($p<0.05$). Doses of 125, 100, 50,

and 25 mg induced increased clotting times of 27.66, 11.66, 10.66, and 7.66 seconds, respectively, compared to the time obtained in the positive control - *B. moojeni* venom (Figure 2B).

Compound 4-(4-hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester did not induce coagulation when added directly to plasma (data not shown). However, it was able to interact with factors of the coagulation cascade, inhibiting the action of coagulating enzymes present in the venom. Furthermore, interactions of the molecule with the coagulation-inducing proteases present in the venom can also be suggested when we consider the results of the assay carried out with their previous incubation and subsequent addition of plasma.

Figure 2 - (A) Effect on the coagulation of citrated human plasma exerted by the synthesized molecule 4-(4-Hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester previously incubated with *Bothrops moojeni* venom. **(B)** Effect on the coagulation of citrated human plasma exerted by the synthesized molecule 4-(4-Hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8--hexahydroquinoline-3-carboxylic acid ethyl ester, previously added to the plasma and only them inducing coagulation with *Bothrops moojeni* venom.



Positive control (C+): *Bothrops moojeni* venom (40 µg). Samples: compound in the doses of 125, 100, 50, and 25 mg. Data represent the means and standard deviation of triplicates. *Statistically significant – differs from the positive control ($p < 0.05$).
Source: Research data

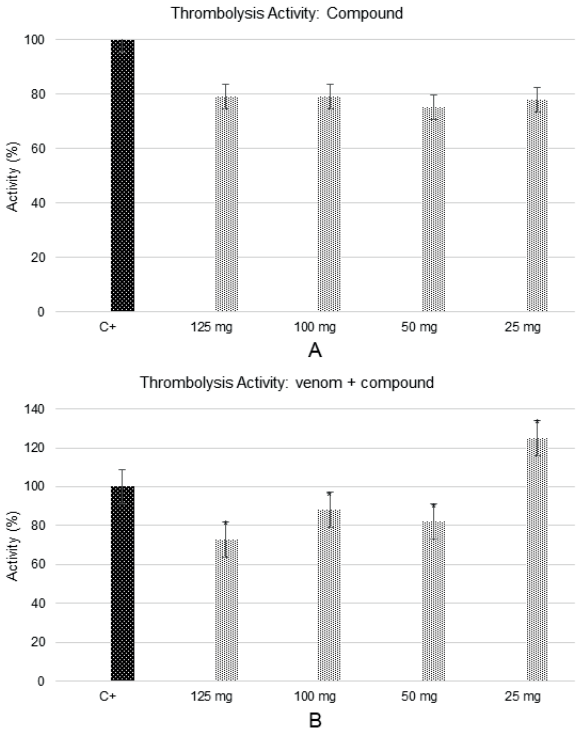
Thrombosis is one of the primary causes of heart attacks and cardiovascular diseases (LICHOTA *et al.*, 2020). In the prevention and/or treatment of this disease and others related to it, the most used drugs are anticoagulants, antiplatelet agents that inhibit the formation of blood clots, and fibrinolytic drugs that dissolve the clot (RINGLEB, 2006). Thus, the interaction potential with cascade factors and thrombin-like proteases can be widely investigated in the search for formulations of new drugs. Future studies that make it possible to understand the mechanisms of action of this compound are highly relevant to enable its clinical use.

3.2.3 EFFECTS ON HUMAN BLOOD THROMBI AND VENOM-INDUCED THROMBOLYTIC ACTIVITY

4-(4-hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester did not differ statistically from the positive control (*Bothrops moojeni* - 20µg) at the tested doses. Thus, the synthesized compound exerts thrombolysis effects similar to the venom (Figure 3A).

When incubated with the synthesized compound at doses of 125, 100, and 50 mg, the thrombolytic activity exerted by the venom led to reductions of 73, 88, and 82%, respectively ($p<0.05$). These results characterize the inhibitory effect of 4-(4-hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8--hexahydroquinoline-3-carboxylic acid ethyl ester on protease-inducing thrombolysis. However, the compound boosts this activity at its lowest concentration (25 mg) (Figure 3B). This result is consistent with the effect shown in the coagulation test since the compound was able to interact with plasma/blood proteins and inhibit the action of coagulation proteases.

Figure 3 - **(A)** Effect on human blood thrombi exerted by the synthesized molecule 4-(4-Hydroxy-phenyl)-2--methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester. **(B)** Evaluation of the thrombolytic activity induced by *Bothrops moojeni* venom when incubated with the synthesized molecule 4-(4-Hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester.

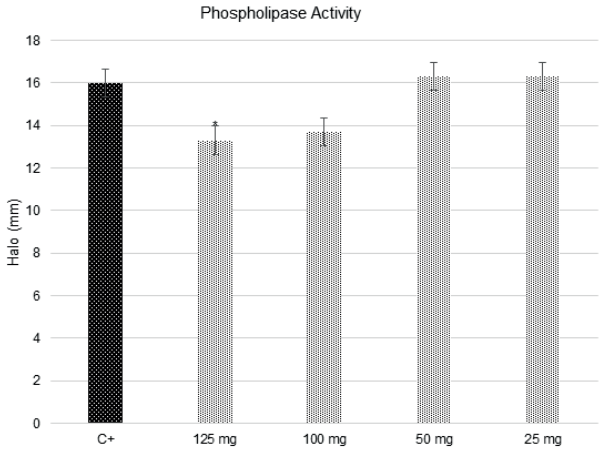


Positive control (C+): *Bothrops moojeni* venom (20 µg). Samples: compound in the doses 125, 100, 50, and 25 mg. Data represent the means and standard deviation of triplicates. *Statistically significant – differs from the positive control ($p < 0.05$).
Source: Research data

3.2.4 EFFECTS ON THE ACTIVITY OF PHOSPHOLIPASES A_2

The synthesized compound did not induce phospholipase activity under the conditions and doses tested (data not shown). However, when evaluated as to the phospholipase activity induced by *Bothrops moojeni* venom (20µg), compound 4-(4-Hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8- hexahydroquinoline-3-carboxylic acid ethyl ester at a dose of 125 mg was able to inhibit phospholipases A_2, causing a reduction of 2.7 mm ($p<0.05$) of the halo formed in the positive control (16 mm). A trend towards inhibition of phospholipase activity was observed at a dose of 100 mg, which resulted in a halo of 13.7 mm ($p=0.06$) (Figure 4).

Figure 4 - Effect of the compound 4-(4-Hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester on the phospholipase A_2 activity induced by *Bothrops moojeni* venom.



Positive control (C+): *Bothrops moojeni* venom (20 µg). Samples: compound in the doses 125, 100, 50, and 25 mg, previously incubated with venom. Data represent the means and standard deviation of triplicates. *Statistically significant – differs from the positive control ($p < 0.05$).

The breakdown of membrane phospholipids can trigger inflammatory processes in the human body by producing metabolites such as arachidonic acid and lysophospholipids (MEYER *et al.*, 2005). Thus, finding natural or synthetic compounds capable of inhibiting phospholipases' action is of paramount importance in the pharmaceutical sector. The significant inhibitions of PLA_2 activity observed at the highest doses of the compound highlight an anti-inflammatory potential that can be better investigated in future studies.

Using the same method employed in the present study, Abreu (2020) observed inhibitions of phospholipase A_2 activity of 10%, 12.5%, and 20% for Betamethasone valerate at concentrations of 0.1, 1 and 10 µg, respectively. The compound evaluated in the present study induced inhibitions greater than 12.5% only when assessed at doses of 100 and 125 mg.

Considering that commercially available steroid drugs and polyhydroquinolines, act as anti-inflammatory agents through different mechanisms, future studies with a broader scope to determine comparisons between efficacy and safety would be highly valuable in determining the pharmaceutical application potential of the synthesized molecule.

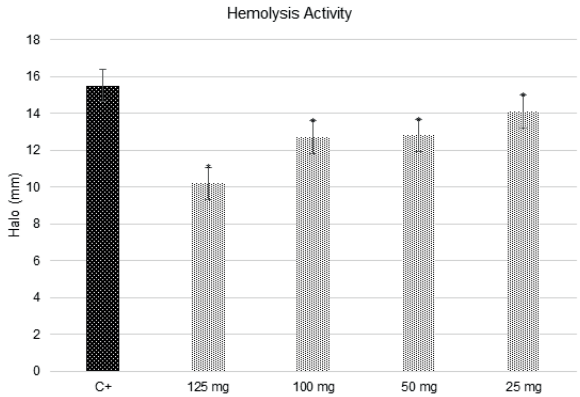
The use of snake venom as a tool for investigating the action of phospholipases A_2 and their possible inhibitors was evaluated in a manuscript published by Sales *et al.* (2017). The work demonstrates the similarity between human and venom phospholipases and uses computational and experimental models to assess the anti-inflammatory activity of vanillic acid. Thus, phospholipase inhibitors, such as the compound evaluated here, can be considered potential anti-inflammatory agents.

3.2.5 CYTOTOXIC ACTIVITY ON HUMAN ERYTHROCYTES

The synthesized compound was not cytotoxic on human erythrocytes when studied *in vitro*, under the evaluated conditions and doses (data not shown). However, a protective effect exerted by the compound on erythrocyte membranes was observed, reducing cytotoxicity induced by enzymes present in the venom (when previously incubated). The positive control (*Bothrops moojeni* - 20µg) induced 15.5 mm halos and reduced activity exerted by the compound by 5.3, 2.8, 2.7, and 1.4 mm at doses of 125, 100, 50, and 25 mg, respectively ($p<0.05$) (Figure 5).

The cytotoxic activity of polyhydroquinoline derivatives was evaluated in tests with several cell lines (FAN *et al*., 2018), and significant inhibition was observed on tumor cells (osteosarcoma) for 3 synthesized derivatives (Methyl 2,7,7-trimethyl- 4-(2-cyanophenyl)-5-oxo1,4,5,6,7,8-hexahydroquinoline-3-carboxylate, Methyl 2,7,7-trimethyl-4-(2-methoxyphenyl)-5-oxo-1 ,4,5,6,7,8-hexahydroquinoline-3-carboxylate and Methyl 2,7,7-trimethyl-4-(3,4-dimethylphenyl)-5-oxo-1,4,5,6,7, 8-hexahydroquinoline-3-carboxylate) with IC_{50} ranging between 25 and 45 µM.

Figure 5 - Effect of the compound 4-(4-Hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester on the cytotoxic activity induced by *Bothrops moojeni* venom in human erythrocytes.



Positive control (C+): *Bothrops moojeni* venom (20 µg). Samples: compound in the doses 125, 100, 50, and 25 mg, previously incubated with the venom. Data represent the means and standard deviation of triplicates. *Statistically significant – differs from the positive control ($p <0.05$).

Cabrera *et al*. (2016) reported the antiproliferative effect of several PHQ derivatives on glial cells (glioma). The authors observed a dose-dependent, decreasing effect (concentrations ranged from 5 to 50 µM) of PHQs on cell viability. They also described that the activity exerted by the analyzed derivatives was more effective in compounds that contained a hydroxyl group bonded to the aromatic ring. In this paper, the PHQ derivative 4-(4-hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester also contains such a group.

4 CONCLUSION

This paper demonstrated the multicomponent synthesis without using solvents and catalysts of 4-(4-Hydroxy-phenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester, according to the principles of green chemistry. The molecule exhibited after the biological screening on the antimicrobial and anti-cytotoxic activities and a modulating potential on enzymes that act in hemostatic and inflammatory processes (phospholipases A_2, and serine and metalloproteases). Further studies should be performed in order to establish the best concentrations and formulations for a possible future applications.

AKNOWLEDGEMENTS

The authors would like to thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Universidade Federal de Lavras (UFLA) for their support. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (CAPES) – Finance Code 001.

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Recebido em: 16 de Novembro de 2024
Avaliado em: 4 de Fevereiro de 2025
Aceito em: 18 de Maio de 2025

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