Metabolites from *Induratia* spp. Modulating Key Enzymes in Human Hemostasis

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Abstract

Fungi *Induratia* have been poorly evaluated for their non-volatile secondary metabolites. In the present work, we evaluated the effects of non-volatile secondary metabolites released into the culture medium by *Induratia* spp. upon toxic alterations induced by *Bothrops* spp. venoms. *B. atrox* venom phospholipase was inhibited by *Induratia* sp. and *I. yucatanensis* around 12 and 16%. The extracts of two strains inhibited 12 to 25% of the hemolysis induced by *B.moojeni* venom. Thrombolysis was inhibited by 30 to 60% by the compounds present in two extracts. The coagulation induced by *B. moojeni* venom was prolonged by 26 to 41 s by the action of the extracts of *I. yucatanensis* and *I. coffeana*. The fungal extracts did not exerted any cytotoxic effect, nor did they induce any alteration in the other evaluated substrates show the potential use of non-volatile metabolites produced by the fungi evaluated as enzyme modulators, especially for proteases with a fundamental role in human hemostasis.

Introduction

Endophytic fungi belonging to the genus *Muscodor* are known for the production of volatile organic compounds (VOCs) with antimicrobial activity (Strobel 2011; Monteiro et al. 2017; Pena et al. 2017). The first species was isolated from *Cinnamomum zeylanicum* and identified as *Muscodor albus* (Sears et al. 2001). The genus has 25 described species so far (Zhang et al. 2010; Suwannarach et al. 2013; Saxena et al. 2015; Chen 2019; Samarakoon et al. 2020), including *Muscodor coffeanun* which is isolated from organic coffee plants from Brazil (Hongsanan et al. 2015). Recently, the genus *Muscodor* was transferred to *Induratia*, after phylogenetic studies carried out by Samarakoon et al. (2020). Two new species, *Induratia thailandica* and *Induratia ziziphi*, were described by these same authors.

The various metabolites, volatile or not, produced by the fungi of the genus *Induratia* (e.g., alcohols, amines, acids, esters, ketones, and some hydrocarbons) can perform hormonal, antibiotic, antitumor, antidepressant, antifungal, cytotoxic, antiviral, immunosuppressive, antiparasitic, and antioxidant activities (Kusari and Spiteller 2011; Strobel 2014; Demain 2014; Meshram et al. 2014; Saxena et al. 2015; Li et al. 2018; Kaddes et al. 2019). VOCs produced by *Induratia yucatanensis* (as *Muscodor yucatanensis*) showed lethal effects against different phytopathogenic fungi as *Guignardia mangiferae*, *Colletotrichum* sp., *Phomopsis* sp., *Alternaria solani*, *Rhizoctonia* sp., *Phytophthora capsici*, *P. parasitica*, *Botrytis cenerea, Rhizoctonia solani*, and *Cercospora coffeicola* (Macías-Rubalcava et al. 2010; Monteiro et al. 2017). Despite the small number of scientific studies on the excretion of metabolites into the culture medium by fungi belonging to the genus *Induratia*, the synthesis of Brefeldin A (a polyketide with antibacterial, antiviral, antinematoid, and antifungal activities) by *I. yucatanensis* has already been reported (Qadri et al. 2017). *I. darjeelingensis* (as *Muscodor*) showed inhibitory activity on xanthine oxidase, the main enzyme responsible for the production of uric acid (Kapoor and Saxena 2016). Therefore, some species of this genus showed promising antimicrobial, anti-obesity, and antioxidant activities and were reported as possible sources for the development of new pharmaceutical products (Kusari and Spiteller 2011; Demain 2014; Gupta and Meshram 2018; Hyde et al. 2019).

Considering the wide variety of toxic-pharmacological activities carried out by their different enzymes, researchers have used snake toxins as tools in the laboratory (*in vivo*, *in vitro*, and *in silico*) when searching for new medicines based on natural products. These studies make it possible to prospect the effects of natural inhibitors on human physiopathological processes since many of these snake toxins have structural and functional similarity with human enzymes (Andrade et al. 2018; Marques et al. 2018; Cardoso et al. 2019; Cesar et al. 2019). Phospholipases A_2 (PLA_2s) and proteases related to the process of inflammation, blood clotting, and fibrinolysis are the main targets when prospecting for modulating molecules (Kini 2011; McCleary and Kini 2013).

Our research group has worked with *I. coffeana*, *I. yucatanensis*, and *Induratia* sp. isolated from *Coffea arabica*. We previously reported, for these species of fungi, the synthesis of enzymes that degrade different carbon sources and the production of volatile compounds with antifungal activity (Alves et al. 2016; Monteiro et al. 2020). Bastos et al. (2020) investigated for the first time the presence of enzymatic modulators in ethyl acetate extracts obtained from four endophytic fungi belonging to the species *I. coffeana* and *I. yucatanensis* (Bastos et al. 2020). The authors suggest the presence of specific interactions between molecules present in the extracts of *Induratia* spp. and venom proteases.

In the present study, we evaluated the effects of non-volatile secondary metabolites released into the culture medium by others *Induratia* spp. on activities related to human hemostasis induced by proteases and phospholipases A_2 present in snake venoms.

Material And Methods

Endophytic Fungi

The fungi used in this work were isolated from stems and leaves of organic plants of *Coffea arabica* collected in Viçosa (Monteiro et al. 2020). They were identified using DNA's internal transcribed spacer (ITS) sequences and deposited in the Coleção Micologica de Lavras (CML) at the Departamento of Fitopatologia da Universidade Federal de Lavras, Lavras, Brazil, as *I. coffeana* (CML 4012, CML 4019), *I. yucatanensis* (CML 4014, CML 4017), and *Induratia* sp. (CML 4013).

Obtaining Fungal Extracts

To obtain the extracts, the fungi were cultivated in Malt Extract Agar (MEA) medium for 7 days at 25 ºC. After fungi growth, five small discs were removed and inoculated into 1 L of MEB (Malt Extract Broth) medium, incubated at 125 rpm for 15 days at 25 ºC. The fungi underwent a filtration process to separate the mycelia from the supernatant. Ethyl acetate was added to the supernatant (1:0.5 ratio; supernatant/ethyl acetate) to separate the organic from the inorganic parts (twice). The ethyl acetate and produced metabolites were subjected to a rotary evaporation process to obtain the extracts. The extracts were weighed (10 mg) and solubilized in 1 ml of phosphate-buffered saline (PBS), obtaining a storage solution at the concentration of 10 μg μl^{-1}.

Snake Venoms

The venoms used for the induction of activities were commercially obtained from the serpentarium Bio-Agents.
Considering the determination of the minimum dose described in the literature, 10 µg of *Bothrops atrox* venom was standardized for the phospholipase activity. The thrombolytic, hemolysis, and coagulant activities were evaluated using *Bothrops moojeni* venom at the doses of 40 µg, 40 µg, and 6 µg, respectively (Cintra et al. 2012a; Oliveira et al. 2016).

Human Biological Material

Blood samples (5 ml) were collected from volunteers, after their written consent, by venipuncture in the cubital vein of the arm using vacutainers. The blood collection tubes contained heparin for the erythrocyte cytotoxicity (hemolysis) test, sodium citrate for the coagulation test, and no anticoagulant for the thrombolytic test.

The performed experiments are in accordance with and approved by the Human Research Ethics Committee (COEP) of the Universidade Federal de Lavras (registered under the number CAAE/80767417.0.0000.5148).

Enzyme Inhibition Assays

Previous incubations of the venom with fungal extract at the ratios 1:0.25, 1:0.5, and 1:1 (w:w) were performed for the thrombolytic and hemolysis activities. For the phospholipase activity, the proportions used were 1:0.5, 1:1, and 1:2 (w:w). 1:0.5 and 1:1 (w:w) were the proportions for the coagulation assay. The use of different venoms is due to the best responses during the standardization of the minimum effective dose. The different proportions were selected according to standardized experimental peculiarities that aimed at maintaining the reaction environment, after a comprehensive screening in pilot studies. Negative controls received only phosphate-buffered saline (PBS). The positive controls were venom or fungal extracts. The samples were incubated for 30 min at 37 ºC and subsequently submitted to the different activities. Assays were performed in triplicates and in two independent experiments.

Phospholipase Activity

The phospholipase activity was evaluated according to the method described by Gutiérrez et al. (1988). The gel was made with 0.01 mol l^{-1} $CaCl_2$, PBS (1:3; v/v; pH 7.2), egg yolk as a source of phospholipids (phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine), 1% bacteriological agar, 0.01% sodium azide. The gel was poured into Petri dishes at 45 ºC to 50 ºC. The samples were applied to holes made in the gel. The gel remained in a cell culture chamber at 37 ºC for 12 h. The formation of a translucent halo around the holes indicates phospholipase activity. The halos were measured (mm) to quantify the activity.

Hemolysis Activity on Human Erythrocytes

This activity was evaluated using the methodology described by Gutiérrez et al. (1988), with some adaptations. The hematocrit was optimized in the gel composition to approximately 1%. The amount of venom (µg) considered as the minimum hemolytic dose (MHD) is the one that forms a halo between 15 and 20 mm in diameter.

Thrombolytic Activity

The thrombolytic activity was evaluated on blood clots formed *in vitro* in microtiter plates, according to the methodology described by Cintra et al. (2012). The samples were applied to the clots, and the microplates were, then, kept in a cell culture chamber for 24 h at 30 ºC. The thrombolytic activity was quantified by the volume of fluid released by the thrombi.

The mean of the volumes obtained for the negative control (PBS) was subtracted from the mean of the volumes obtained in the other treatments.

Coagulant Activity

To evaluate the coagulation time, fungal extracts were incubated with *Bothrops moojeni* venom (6 µg), for 5 min at 37 ºC, in the proportions of 1:0.5 and 1:1 (w:w). Tubes containing citrated human plasma (200 µl) were kept in a water bath at 37 ºC. Immediately after the addition of the samples, the time until clot formation was measured, according to the methodology described by Rodrigues et al. (2000). A minimum coagulant dose was defined as the smallest amount of venom capable of inducing coagulation within 65 to 75 s.

Statistical Analysis

The data correspond to averages of the triplicates evaluated in two independent tests, and their standard error. The significance between the means was determined by analysis of variance, followed by Student's t-test when the treatments were compared with the control ($p \leq 0.05$).

Results

Phospholipase Activity

The extract of *I yucatanensis* (CML 4017), evaluated in the proportions of 1:0.5 and 1:1, promoted a significant reduction of 17.3% and 15.14%, respectively, in the phospholipase activity. The other extracts evaluated also had a statistically significant inhibitory action on the activity of these enzymes, but at lower values (≤ 14.92%) (Fig. 1). The extracts evaluated alone did not induce changes in the phospholipid substrate.

Hemolysis Activity on Human Erythrocytes

The extracts from *I. coffeana* (CML 4012) and *Induratia* sp. (CML 4013) in the proportion of 1:1 inhibited approximately 26.67% of the *B. moojeni* venom-induced hemolysis. Significant inhibitions between 13

and 20% were also observed for the *Induratia* sp. extract in the proportions of 1:0.5 and 1:0.25 and *I. yucatanensis* (CML 4014) extract at 1:1, 1:0.5, and 1:0.25 (Fig. 2). When evaluated alone, the extracts did not exert cytotoxicity on human erythrocytes.

Thrombolytic Activity

In a 1:1 ratio, extracts of *I. coffeana* (CML 4019) and *I. yucatanensis* (CML 4017) inhibited by 62% and 59%, respectively, the thrombolysis induced by *B. moojeni* venom. The extracts also performed statistically significant inhibitions in lower proportions 35 to 50% (Fig. 3).

The extract of *I. coffeana* (CML 4012) inhibited thrombolysis in approximately 40% in all evaluated proportions. This result suggests a saturation in the possible interactions between the compounds present in the extract and proteases (main enzymes responsible for the dissolution of thrombi). Although they had the least inhibitory effects, all of the extracts from *Induratia* sp. (CML 4013) and *I. yucatanensis* (CML 4014) exerted statistically significant inhibitions between 15 and 35% (Fig. 3).

Coagulant Activity

The extracts of *I. yucatanensis* (CML 4017, CML 4014) prolonged the venom-induced clotting time in 41 s and 31 s, respectively (1:1; w:w). The extract of *I. coffeana* (CML 4019) also extended the clotting time in the two proportions evaluated 1:1 (26 s) and 1:0.5 (18 s).

On the other hand, the extract of *I. coffeana* (CML 4012) showed a procoagulant effect, reducing the venom-induced clotting time in 16 s (1:1 ratio) and 40 s (1:0.5 ratio) (Fig. 4).

Discussion

Endophytes are microorganisms that live synergistically with their host. They provide a wide array of substances, in exchange for nutrients, that are able to help the plant fight diseases, predators, and develop (Kaul et al. 2012). Endophytic fungi are known to produce the greatest variety of metabolites in comparison to any other endophytic microorganism (Shipunov et al. 2008). Their metabolites possess a broad spectrum of activities (e.g.: immunomodulatory, antioxidant, antibiotic, antiparasitic, and antitumoral), which can be distributed in a wide diversity of classes such as alkaloids, steroids, terpenoids, aliphatic, and phenolic compounds (Alurappa et al. 2018).

Fungi belonging to the genus *Induratia* have been poorly evaluated for their non-volatile secondary metabolites. In this work, we focused on evaluating the interaction of this metabolites from *Induratia* spp. (obtained by ethyl acetate extraction) with proteases (serine proteases and metalloproteinases) and phospholipases A_2 present in *Bothrops* spp. venoms. The use of snake venoms as tools in the laboratory makes it possible to prospect the effects of fungal compounds on the physiopathological processes developed in the human body, since many of these snake toxins have structural and functional similarity with human enzymes. Snakes phospholipases A_2 are a group of enzymes able to induce neurotoxic,

myotoxic, membrane degrading, and inflammatory activities. In comparison, snake venom proteases may act upon several classes of proteins resulting in the degradation of extracellular matrix and hemostatic alterations, mainly related to the activation of the coagulation cascade (breakdown of fibrinogen), fibrinolysis and inhibition of platelet aggregation (Marcussi et al. 2013). Our results contributed to expand the knowledge about the biological activities exerted by these compounds, describing their inhibitory action on proteases and phospholipases A_2. This approach allows, in an initial phase, to comprehend the possible effects upon key classes of enzymes with high structural homology with human enzymes. In addition, microorganisms have beneficial advantages because of their biotechnological applications, such as the production of metabolites on a large scale and low cost.

PLA_2 and proteases rely on metallic ions to exert their catalytic activity upon the substrates. One of the main actions by which natural products can inhibit enzymatic activity is chelating metallic ions from the medium (Lago et al. 2014). Therefore, if chelating was indeed a response from fungi extracts incubated with snake venom, consistent inhibitions and dose-dependent values would be noticed throughout the treatments. We believe that the mild inhibitory actions observed here (less than 17%; $p > 0.05$) is due to molecules present in small quantities in the extract capable of interacting with specific sites in the PLA_2, impairing its proper binding to the substrate or competing with the substrate for the catalytic site.

Hemolysis arises from two main reasons. The first one is the direct interaction of phospholipases with erythrocytes membrane causing rupture and extravasation of cellular content. The second one is the action of proteases (metalloproteinases and serine proteases) upon cell membrane proteins, resulting in the degradation of membrane components and loss of membrane integrity. The inhibitions observed were understood as a cumulative effect in proteases and phospholipases A_2, especially by *I. coffeana* (CML 4012) and *Induratia* sp., which yielded around 26% of enzyme inhibition.

The most noticeable results were observed in the thrombolytic and clotting time activities, performed mainly by proteases. The extracts were tested alone and did not cause the formation or dissolution of thrombi, observed when comparing with the control. The previous incubation with snake venoms resulted in the preservation of the thrombi for all extracts tested. Though we may have observed significant inhibitions towards PLA_2 and proteases alone, they do not explain the results obtained in this assay. We believe this may be caused by interactions with fibrinogenolytic enzymes in snake venoms, which indicate that the action of the extracts is not indiscriminate but more directed to certain types of enzymes. The coagulant activity resulted in both coagulant and procoagulant actions. *I. yucatanensis* (CML 4017, CML 4014) and *I. coffeana* (CML 4019) prolonged the clot formation time, while *I. coffeana* (CML 4012) accelerated clot formation. These controversial results indicate that their different phytochemical constitution could account for different behaviors. For example, the presence of antioxidants may delay plasma clotting and platelet aggregation (Ratnasooriya et al. 2005). In smaller doses, antioxidants are unable to alter clotting time significantly. However, clotting would be delayed by increasing antioxidants content. That being said, the reduction in clotting time could be related to the antioxidant action instead of direct action upon snake venoms or clotting cascade components.

Comparing the results in this present study with the ones obtained by Bastos *et al.*, we can assume that the isolates from the species of *Induratia* produce different secondary metabolites since the extracts did not behave similarly (Bastos et al. 2020).

Excessive blood loss and lethargy in producing strong clots are important aspects to consider in cases of accidents and delicate surgical interventions. The search for hemostatic drugs with low-cost, biosecurity, and effectiveness are of paramount importance. Many natural products have shown to modulate the clotting cascade either by promoting blood clotting or inhibiting coagulation.

Conclusion

In this preliminary work, we found out that the extracts tested had the capacity of protecting blood clots from enzymatic degradation by snake venom proteases and phospholipases, and to accelerate fibrinogen breakdown, resulting in the formation of a fibrin network. More in-depth analysis and phytochemical screening are needed to comprehend the mechanism of action of *Induratia* spp. extracts upon blood components and which classes are involved in these biological activities.

Declarations

Ethics approval and consent to participate

The methods that required the use of human biological material (cells and blood components) were previously approved by UFLA's Human Research Ethics Committee (COEP), under the registration number CAAE/**80767417.0.0000.5148.**

Human and animal rights

No humans were used in this research. All research procedures followed were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2008 (http://www.wma.net/en/20activities/10ethics/10helsinki/)

Consent for publication

An informed consent was taken from all individuals.

Availability of data and materials

Not applicable.

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Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

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Authors contribution statement

M.G.B.C.: conceptualization, methodology, validation, formal analysis, investigation, data curation, and writing. M.V.C.T.: writing, review, and editing. P.H.S.C.: writing, review, editing, and creation of images. S.M.: conceptualization, methodology, resources, writing, review, editing, and project administration. P.G.C.: conceptualization, resources, writing, review, editing, supervision, and funding acquisition. All authors read and approved the manuscript.

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Figures

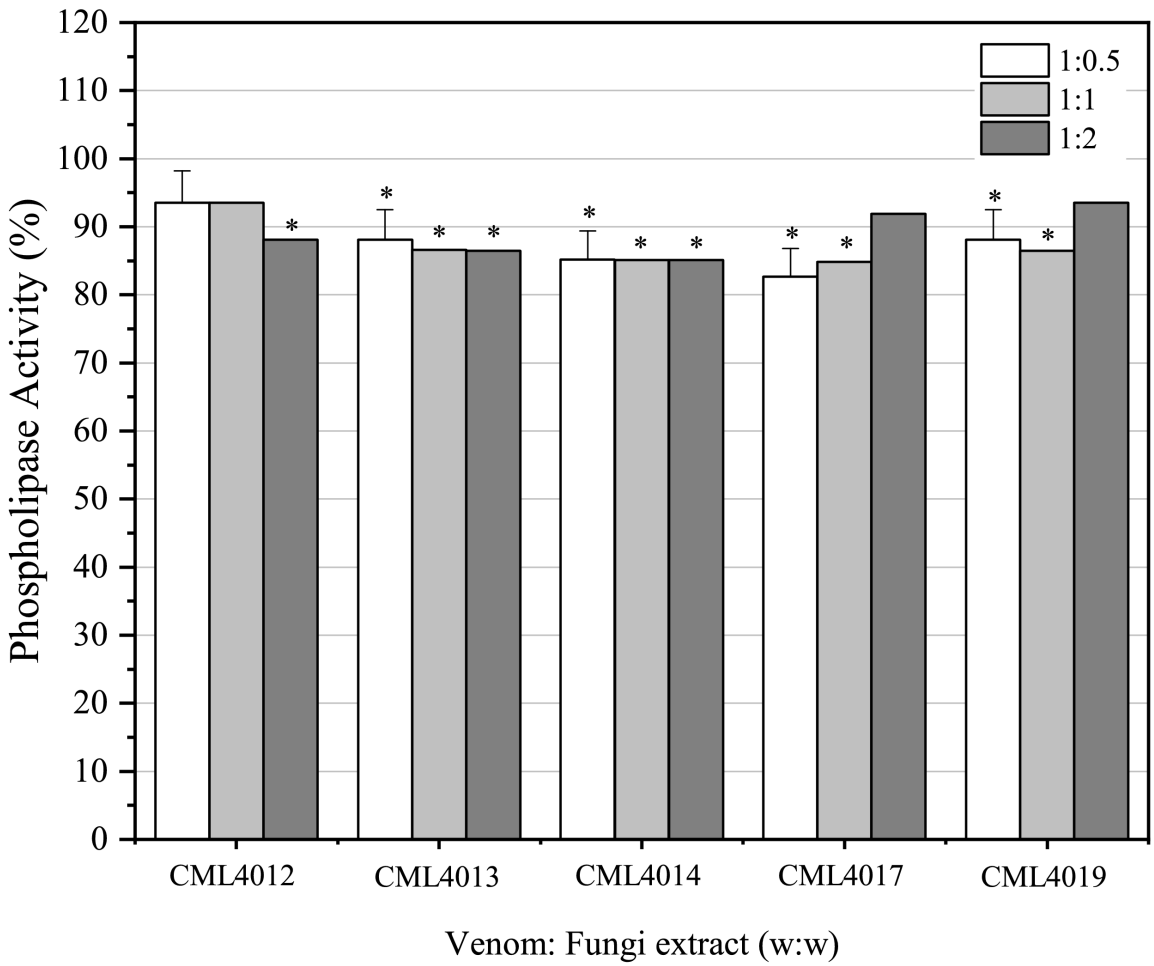

Figure 1

Effect of fungi extracts on the venom-induced (B. atrox) phospholipase activity. The average of the results obtained in the positive control (B. atrox venom at 10 µg) was considered as 100% of activity. The data correspond to the triplicate averages evaluated in two independent tests, and standard error. The venom: fungi extract (w: w) sample were previously incubated for 30 min at 37 ºC. (*) Differ from positive control at a 5% significance level

Figure 2

Effect of fungi extracts on the venom-induced (B. moojeni) hemolysis. The average of the results obtained in the positive control (B. moojeni venom at 40 µg) was considered as 100% of activity. The data correspond to the triplicate averages evaluated in two independent tests, and standard error. The venom: fungi extract (w:w) sample were previously incubated for 30 min at 37 ºC. (*) Differ from positive control at a 5% significance level

Figure 3

Effect of fungi extracts on venom-induced thrombolysis of B. moojeni. The average of the results obtained in the positive control (B. moojeni venom at 40 µg) was considered as 100% of activity. The data correspond to the triplicate averages evaluated in two independent tests, and standard error. The ratios in weight (w), venom: fungi extract, were previously incubated for 30 min at 37 ºC. (*) Differ from positive control at 5% significance level

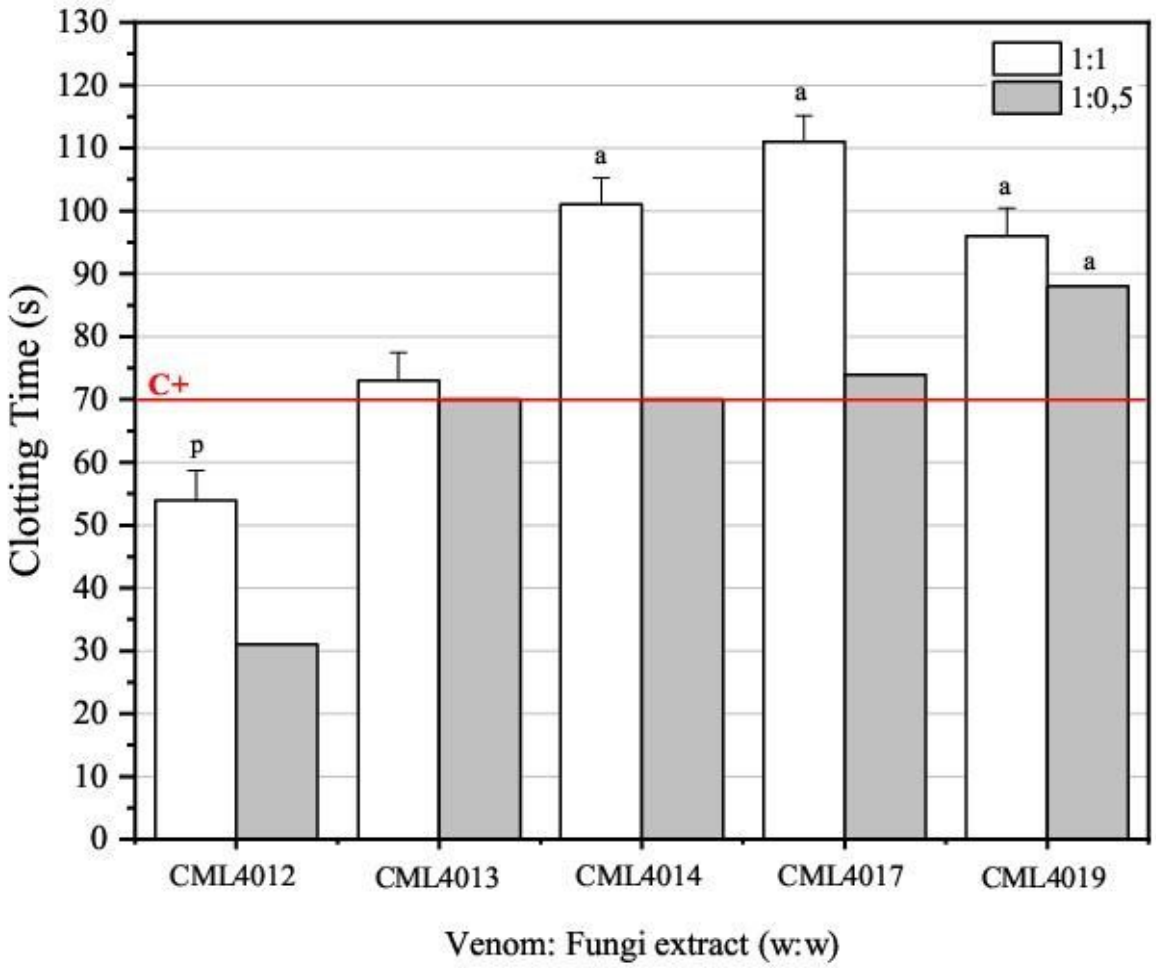

Figure 4

Effect of fungi extracts on venom-induced coagulation of B. moojeni in citrated human plasma. The data correspond to the triplicate averages evaluated in two independent tests, and standard error. The ratios in weight (w), venom: fungi extract, were previously incubated for 5 min at 37 ºC. Positive control: B. moojeni at 6 µg. (a) Differs statistically from the positive control in anticoagulant effect. (p) Differs

statistically from the positive control in procoagulant effect. Differences in values equal to or greater than 10 s were considered significant, considering the prothrombin activation time between 10 and 14 s