Current Microbiology (2020) 77:3603–3611
https://doi.org/10.1007/s00284-020-02170-5

Enzymatic Modulators from *Induratia* spp.

Andréa Patrícia da Silva Pomposo Bastos[1] · Patrícia Gomes Cardoso[2] · Ítalo Augusto Férrer Melo Santos[2] · Marcus Vinicius Cardoso Trento[3] · Laura Cristina Jardim Porto[1] · Silvana Marcussi[3]

Received: 6 March 2020 / Accepted: 19 August 2020 / Published online: 31 August 2020
© Springer Science+Business Media, LLC, part of Springer Nature 2020

Abstract

In the present work, ethyl acetate extracts, consisting of non-volatile compounds, from the culture of endophytic fungi isolated from coffee plants, *Induratia coffeana* and *Induratia yucatanensis*, were prospected in enzyme modulation tests that act in human hemostasis. Dry extracts of the fungi were diluted in dimethyl sulfoxide p.a. 99.9% (DMSO), and then tested. *Bothrops atrox* venom was used as an enzyme source and tool to induce the activities. Prior to the evaluation of the activities, incubations of the extracts with the venom were performed in the proportions 1: 0.01, 1: 0.25, 1: 0.5, and 1: 1 (venom: extract; mass: mass). The extracts of all fungi promoted a significant increase in the clotting time induced by the venom, which was even longer when the extracts were previously incubated with the citrated plasma. The activity of phospholipases A_2 did not significantly change when evaluated in the presence of fungal extracts. However, the evaluated extracts inhibited proteases by 73% and 30% in the thrombolytic and caseinolytic tests, respectively. In addition, the extracts did not induce cytotoxicity on human erythrocytes when evaluated in the absence of the venom. Thus, it is possible to suggest the presence of specific interactions between molecules present in extracts of *Induratia* spp. and venom proteases, highlighting non-volatile metabolites as promising sources of compounds of medical and scientific interest.

Introduction

Microorganisms are a potential source of new bioactive metabolites that can be used in human health. The endophytic fungi isolated from different plants, mainly medicinal plants with the capacity to produce a range of biologically active metabolites, shifted the focus of new drug sources from plants to fungi [1]. One advantage in producing functional fungal metabolites is the possibility of large-scale and short time cultivation, usually using substrates of low cost. Steroids, alkaloids, peptides, terpenoids, tannins, polyketones, flavonoids, and phenolics are some examples of compounds produced by fungi. Several secondary metabolites produced by endophytic fungi had their biological activities (e.g., antimicrobial, antiviral, cytotoxic, immunosuppressive, antiparasitic, antioxidant) and mode of action already reported [2–8]. Endophytic fungi from the order Xylariales constitute one of the sources of secondary metabolites with huge chemical diversity, which makes it possible to suggest a partnership between mycologists and chemists [9]. Some natural products from fungi have been used to develop commercial drugs, as cited by Helaly et al. [9] and Hyde et al. [10]. Besides producing bioactive compounds, the

✉ Patrícia Gomes Cardoso
patricia@ufla.br

Andréa Patrícia da Silva Pomposo Bastos
andrea_p.bastos@hotmail.com

Ítalo Augusto Férrer Melo Santos
italo.ferrer@hotmail.com

Marcus Vinicius Cardoso Trento
marcusvinicius.ct@gmail.com

Laura Cristina Jardim Porto
laurap@ufla.br

Silvana Marcussi
marcussi@ufla.br

[1] Nutrition Department (Departamento de Nutrição), Universidade Federal de Lavras (UFLA), Lavras, MG 37200-900, Brazil

[2] Biology Department (Departamento de Biologia), BIOGEN Laboratory (Laboratório BIOGEN), Universidade Federal de Lavras (UFLA), Lavras, MG 37200-900, Brazil

[3] Chemistry Department (Departamento de Química), Biochemistry Laboratory (Laboratório de Bioquímica), Universidade Federal de Lavras (UFLA), Lavras, MG 37200-900, Brazil

endophytes present beneficial functions to the host plant by modulating responses in biotic and abiotic stresses [11].

Recently, molecular phylogenetic studies using ITS, LSU, *rpb2*, and *tub2* DNA sequence shows that *Muscodor* species have affinities to the genera *Emarcea* and *Induratia*. Thus, all *Muscodor* species were transferred to *Induratia* [12]. Endophytic strains of the genus *Induratia* are important mycofumigation agents. They produce a mixture of volatile organic compounds (VOCs) composed of organic acids, alcohols, esters, ketones, and aromatic hydrocarbons [12–14].

VOCs produced by *Induratia* spp. completely suppressed the growth of phytopathogenic fungi *R. solani*, *C. coffeicola*, and *Phoma* sp. and showed fungicidal effect against *A. ochraceus* on coffee beans and *F. verticillioides* in corn seeds [15]. The extrolites of *Induratia* species have also shown promising biological activity with possible applications in the agriculture and pharmaceutical industries, and many patents have been granted to various companies [16].

Induratia species were reported as enzymes producer, which includes extracellular amylase, cellulase, lipase, pectinase, phytase, protease, endo β-1,4 glucanase, and exo β-1,4 glucanase [17, 18]. Kapoor and Saxena [19] described a potent antioxidant activity in the extract obtained from *Induratia indica* (as *Muscodor indica*) and inhibitory action of xanthine oxidase in the extract of *Induratia darjeelingensis* (as *Muscodor darjeelingensis*). These actions help in the development of alternatives for the control of hyperuricemia and diseases related to oxidative stress.

The effect of VOCs of *Induratia yucatanensis* (as *Muscodor yucatanensis*) were lethal to the endophytes *Colletotrichum* sp., *Phomopsis* sp., and *Guignardia mangiferae*, and to the phytopathogens *Phythophthora capsici*, *Phythophthora parasitica*, *Rhizoctonia* sp., and *Alternaria solani*. It also inhibited root elongation in amaranth, tomato, and barnyard grass, particularly those produced during the first 15 days of fungal growth [20]. The same authors evaluated organic extracts from the culture medium and mycelium of *I. yucatanensis* on the same endophytes, phytopathogens, and plants. They observed that the mycelial extract inhibited plants more than endophytes or phytopathogens, and that both extracts share 12 allelochemicals including benzene derivatives, phenolic compounds, cyclopentadienes, esters, lactones, alkanes, aldehydes, and carboxylic acids.

Qadri et al. [21] characterized the soluble compounds produced by *I. yucatanensis* Ni30 (wild type) isolated from *Elleanthus* sp. and found brefeldin A as the major compound in the culture broth, whereas its mutant type EV1 presented ergosterol and xylaguaianol C. *Induratia cinnamomi* (as *Muscodor cinnamomi*) was reported to control *Rhizoctonia solani* AG-2 root rot in tomato plants, produce the phytohormone indole-3-acetic acid that increases seed germination and root elongation, solubilize metal minerals, and tolerate herbicides and insecticides [22].

Protease inhibitors can be found as one of the molecules present in fungal extracts. Proteases, especially serine proteases and metalloproteases, help in hemostasis by acting during the activation and at the end (dissolving thrombi, clots, and fibrin networks) of blood's coagulation cascade [23]. Due to its regulatory activity in the formation and dissolution of clots and thrombi, substances that modulate proteases are often used as antithrombotic and anticoagulant drugs [24].

Phospholipase A_2 inhibitors, which also may be present in fungal extracts, are molecules of great importance in hemostatic modulation. This is because phospholipases A_2 are capable of promoting an intense toxic activity [25], resulting mainly from their catalytic activity on membrane phospholipids, which leads to structural breakdown, alteration in the flow of ions behind the membranes, activation of endogenous phospholipases of cells, or even cell death. In addition, the breakdown of phospholipids can result in the release of arachidonic acid, which is a precursor to eicosanoids via cyclooxygenase and lipoxygenase pathways and, therefore, causing changes in hemostasis and inflammatory responses [26].

Several natural inhibitors of proteases and phospholipases A_2 have already been isolated from plants and animals to be used in the control of various pathological processes, such as thrombosis, hemorrhage, and cancer [27]. The extract produced by a fungus isolated from caves in Brazil, *Lecanicillium aphanocladii*, promoted an increase in plasma clotting time, induced by snake venom [28]. Most of the natural enzymatic modulators described in literature act reversibly through transient interactions, such as hydrogen and hydrophobic interactions [26]. Other inhibitors act as metal chelators, sequestering or forming complexes with ions, which are essential cofactors for the catalytic activity of enzymes. They may also interfere with other biological activities that depend on catalytic activity [27–29].

There is still little information about the soluble molecules produced by *Induratia* species. Thus, we investigated the presence of enzymatic modulators in ethyl acetate extracts (consisting of non-volatile compounds) obtained from four endophytic fungi belonging to the species *I. coffeana* and *I. yucatanensis* on parameters that assist in the maintenance of human hemostasis. Snake venom enzymes, mainly phospholipases A_2 and proteases, have high structural and functional homology with human enzymes. Therefore, they were used to induce the necessary effects in this study, which allowed the evaluation of the bioactivity of compounds of the fungi on processes that occur in the human body.

Materials and Methods

Human Biological Material

Human blood was used for the in vitro evaluation of the action of the fungal extracts on the cytotoxicity on erythrocytes, plasma coagulation, and thrombolytic activities. Blood samples (10 mL) from 2 volunteers (aged over 18 and healthy)—for each test—were collected by venipuncture in tubes for vacuum collection in the median cubital vein. The tests were carried out after approval by the human research ethics committee of the Federal University of Lavras (COEP—UFLA), with a project registered under the number CAAE/80767417.0.0000.5148.

Obtaining the Fungal Extracts

Fungi of the *Induratia* genus were isolated from fresh and healthy leaves of organic coffee plantations (*Coffea arabica*) from Zona da Mata region, Viçosa municipality, Minas Gerais, Brazil. They were identified using DNA's internal transcribed spacer (ITS) sequences. Single spore cultures were cryopreserved and deposited in the Mycological Collection of Lavras (CML) at the Department of Phytopathology at the Federal University of Lavras, Brazil, under the culture accession numbers (GenBank accessions number): *I. coffeana* CML4009 (MN658674), *I. coffeana* CML4010 (MN658675), *I. coffeana* CML4020 (MN658680) and *I. yucatanensis* CML4016 (MN658684). The fungi were grown in plates containing the medium PDA (Potato Dextrose Agar) for seven days at 25 ℃. After growth, six disks of approximately 5 mm diameter from the fungal colonies were transferred to 1 L of ME broth (Malt Extract), incubated at 25 ℃ in the dark on a shaker at 125 rpm for 12 days. The supernatants were separated from the mycelia by vacuum filtration. The extraction was carried out with the addition of ethyl acetate to the supernatants, in the proportion of 1: 0.5 (supernatant/ ethyl acetate), followed by removal of the solvent by rotary evaporation process. The extracts were then dissolved in Dimethyl sulfoxide p.a. 99.9% (DMSO) at a concentration of 10 mg mL^{-1}.

Snake Venom

The *Bothrops atrox* venom, used as a tool to induce activities, was bought from the Bio-Agents serpentarium (Batatais, São Paulo, Brazil). The venom was weighed and dissolved in phosphate-buffered saline (PBS, pH 7.4) at a concentration of 10 mg mL^{-1}, and stored at − 20 ºC. Screening tests were carried out to define the minimum inducing doses for each activity [27]. The use of venom in our research was registered at the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen, Brazil) under the number ADF95EA.

Evaluation of Fungal Extracts Effects on the Coagulation of Citrated Human Plasma Induced by Enzymes Present in the Snake Venom (Previous Incubation of the Extracts with the Venom)

The extracts were previously incubated with the *Bothrops atrox* venom (6 µg) in the proportions of 1: 0.01, 1: 0.25, 1: 0.5, and 1: 1 (venom: extract; weight: weight) for 10 min at 37 °C. The samples were then added to tubes containing citrated plasma (200 µL) and then timed until a hard clot was formed. Controls containing only the venom were also performed. The minimum coagulant dose used in the tests was previously defined, which was the minimum amount of venom capable of inducing plasma coagulation (6 µg) in an interval between 50 and 70 s.

Evaluation of the Effects of Fungal Extracts on the Coagulation of Citrated Human Plasma Induced by Enzymes Present in the Venom (Previous Incubation of the Extracts with Plasma)

The effects exerted by the extracts of endophytic fungi on plasma coagulation were evaluated as in the previous test. However, the extracts were incubated with citrated plasma (200 µL) for 10 min at 37 °C, and then the *B. atrox* venom (6 µg) was added at the same proportions 1:0.01, 1:0.25, 1:0.5, and 1:1 (venom: extract; w: w). Controls containing only the venom (6 µg) or containing only the fungal extracts were also performed.

Phospholipase A_2 Activity

The phospholipase activity was evaluated on agar gel according to the method described by Gutiérrez et al. [30]. The gel was made with 0.01 M $CaCl_2$, egg yolk as a source of phospholipids (phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine), 1: 3 v/ v of PBS pH 7.2, 1% bacteriological agar, and 0.005% sodium azide. The medium was then poured into Petri dishes at 45 to 50 ºC. The samples were previously incubated for 30 min at 37 °C in the proportions of 1: 0.01, 1: 0.25, 1: 0.5, and 1: 1 (venom: extract; w: w), and then applied to holes made in the gel. After that, they were placed into a cell culture chamber for 12 h at 37 ºC. The formation of a translucent halo around the hole is indicative of phospholipase activity. The halos were measured in millimeters by a digital caliper to quantify the activity and its inhibition. The values were then converted

into a percentage, considering the positive control (*B. atrox* venom at 10 µg) as 100% activity.

Cytotoxicity Test on Human Erythrocytes

This activity was evaluated using the methodology described by Gutiérrez et al. [30], with adaptations referring to the replacement of the phospholipid substrate by human erythrocytes. A human blood sample (5 mL) was collected and diluted in PBS (25 mL). The blood was then washed twice, using centrifugation at 1200 RPM for 10 min each and the supernatant removed and replaced with PBS at each centrifugation step. The red blood cell concentrate obtained was adjusted to a hematocrit of approximately 1% in the gel composition. The amount of venom (20 µg) considered as the minimum hemolytic dose was determined in a pilot test. It corresponded to the dose responsible for the formation of a halo in the gel with a diameter between 15 and 18 mm. The tests were performed with venom, fungal extracts, and both previously incubated in the proportions of 1: 0.01, 1: 0.25, 1: 0.5, and 1: 1 (venom: extract; w: w) for 30 min at 37 °C, and then applied to the holes in the gels. The gels remained in a cell culture chamber at 37 ºC for 24 h. The cytotoxic activity and its inhibition were quantified by measuring the translucent halos formed by the samples. The values obtained were converted into a percentage, considering the values of the venom alone as 100% activity.

Thrombolytic Test

The thrombolytic activity was evaluated on blood clots formed in vitro, in 96-well plates, according to the methodology described by Cintra et al. [31]. Samples of fungal extracts were evaluated separately. Its modulating action on enzymes present in venoms was also evaluated, after previous incubations of the extract with venom in different proportions (1: 0.01, 1: 0.25, 1: 0.5, and 1: 1—venom: extract; w: w) for 30 min at 37 °C. Controls containing only PBS or venom were also performed. The samples were applied, in triplicate, to the clots and the plates remained in a cell culture chamber for 48 h at 37 °C. The thrombolytic activity was quantified by the volume of liquid released by the thrombi, being subtracted from all treatments the average of the sample volumes (controls with PBS) and considered as 100% of lysis the average of the volumes released by the thrombi in the positive controls (*B. atrox* venom at 20 µg).

Proteolytic Test Using Casein as Substrate

This activity was evaluated using the methodology described by Gutiérrez et al. [30], with adaptations referring to the replacement of the phospholipid substrate by casein. Casein was used at a concentration previously described by Wang et al. [32] in a liquid caseinolytic test. A casein solution of 5 mg mL^{-1} in 50 mM Tris–HCl buffer (pH 8.0) was used for the preparation of the gel.

The *B. atrox* venom (20 µg) and the extracts were previously incubated in the proportions of 1: 0.01, 1: 0.25, 1: 0.5, and 1: 1 (venom: extract; w: w), for 30 min at 37 °C. Subsequently, they were applied to the holes made in the gel and incubated for 48 h at 37 ºC in a cell culture chamber. Controls containing only venom or extracts were also evaluated.

The gel was stained with 1% black starch solution and destained in 10% acetic acid solution. The quantification of the activity was performed by measuring the diameters of the translucent halos, formed around the holes. The results were expressed in percentage, in which the controls containing only venom corresponded to 100% of proteolytic activity.

Statistical Analysis

The results were presented as the mean of the triplicates ± standard deviation. The significance between the means was determined by analysis of variance, followed by the Tukey's test when the treatments were compared with the control ($P < 0.05$).

Results

Evaluation of Fungal Extracts Effects on the Coagulation of Citrated Human Plasma Induced by Enzymes Present in the Snake Venom (Previous Incubation of the Extracts with the Venom)

Non-volatile metabolites present in the extracts from the culture filtrate of the fungi *Induratia coffeana* (CML4009, CML4010, CML4020) and *I. yucatanensis* (CML4016) increased the clotting time of the citrated plasma when previously incubated with the venom, in most proportions evaluated (Table 1). However, the extract of *I. coffeana* (CML4020), in the proportion 1: 0.1, showed a pro-coagulant activity by reducing the observed clotting time by half when compared to the positive control (6 µg of snake venom). This same extract, however, was also the one that demonstrated the greatest inhibition effect of venom-induced clotting, prolonging the clotting time by 105 s when evaluated in the proportion of 1: 0.5.

Evaluation of the effects of Fungal Extracts on the Coagulation of Citrated Human Plasma Induced by Enzymes Present in the Venom (Previous Incubation of the Extracts with Plasma)

The previous incubation of the extracts with the plasma resulted in an increase in the clotting time, induced by the

Table 1 Coagulation activity of *Induratia* spp. extracts when first incubated with *Bothrops atrox* venom, or previously incubated with the citrated plasma

Samples	Clotting time (s)				
	Without extract	Venom: fungal extract (w: w) ratio			
		(1:1)	(1:0.5)	(1:0.25)	(1:0.1)
B. atrox venom (control)	55 ± 1	–	–	–	–
I. coffeana CML4009	–	88 ± 1*	148 ± 7*	88 ± 2*	88 ± 3*
I. coffeana CML4010	–	95 ± 1*	91 ± 2*	77 ± 1*	76 ± 3*
I. yucatanensis CML4016	–	111 ± 1*	100 ± 1*	84 ± 2*	86 ± 2*
I. coffeana CML4020	–	111 ± 5*	160 ± 7*	115 ± 11*	35 ± 1
Samples	Without extract	venom: fungal extract with citrated plasma (w: w) ratio			
		(1:1)	(1:0.5)	(1:0.25)	(1:0.1)
B. atrox venom (control)	51 ± 4	–	–	–	–
I. coffeana CML4009	–	219 ± 9*	152 ± 6*	131 ± 3*	86 ± 0*
I. coffeana CML4010	–	600 ± 0*	385 ± 2*	279 ± 4*	146 ± 5*
I. yucatanensis CML4016	–	342 ± 23*	173 ± 1*	166 ± 26*	104 ± 8*
I. coffeana CML4020	–	482 ± 1*	296 ± 1*	165 ± 2*	150 ± 6*

Values are means ($n = 3$) ± standard deviation. The tests were performed with samples containing different proportions of venom: fungal extract (w:w)

*Different from positive control by increasing clotting time. Because of the activation time of some factors of the coagulation cascade (e.g., the prothrombin activation time is between 10 and 14 s), values equal to or greater than 10 s in relation to the control were considered statistically significant. Control: 6 µg of *Bothrops atrox* venom

venom, in comparison to the data obtained after incubation of the extracts with the venom (Table 1).

Phospholipase A_2 Activity

The highest percentage of inhibition observed was 7.2% for the extract of *I. coffeana* (CML4020) evaluated in the proportion 1: 0.5 (venom: extract; m: m). All the other fungal extracts, under the conditions evaluated, did not have a statistically significant inhibitory effect on phospholipases A_2 activity (Fig. 1b).

Cytotoxicity Test on Human Erythrocytes

The fungal extracts did not induce cytotoxic action on human erythrocytes in the absence of the venom, under the conditions evaluated in this study (data not shown). After interaction venom-fungal extract, statistically significant inhibition on the cytotoxic activity exerted by the venom was observed in the extract of *I. coffeana* (CML4020) in the proportion of 1: 0.1, *I coffeana* (CML4010) in the proportion of 1: 1, and *I. coffeana* (CML4009) in the proportions 1: 0.5, 1: 0.25, and 1: 0.1. In contrast, potentiation of the venom-induced hemolytic activity was also observed, mainly, for the extracts of *I. yucatanensis* (CML4016) in the proportion of 1: 0.5 (62.6%) and *I. coffeana* (CML4009) in 1: 1 (57.6%) (Fig. 1c).

Thrombolytic Test

The extracts in all proportions showed a statistically significant reduction in the venom-induced thrombolytic activity, except the extract of *I. coffeana* (CML4020) at the 1: 0.25 ratio (Fig. 1d). The extract of *I. coffeana* (CML4009) presented the most promising result with a 73.6% inhibition in the proportion 1: 0.5. This same extract induced 25.4% of thrombolytic activity (data not shown) when evaluated in the absence of venom. Once again, since it was already observed in the coagulate test, it is demonstrated the interactions between compounds present in the extract and blood components, which results in the destabilization of thrombi.

Proteolytic Test Using Casein as Substrate

All extracts showed inhibitory activity on the breakdown of casein induced by proteases present in the venom, except for *I. coffeana* (CML4010, CML4009) in the ratio 1: 1, *I. coffeana* (CML4009) and *I. yucatanensis* (CML4016) and in the ratio 1: 0.1 (Fig. 1a).

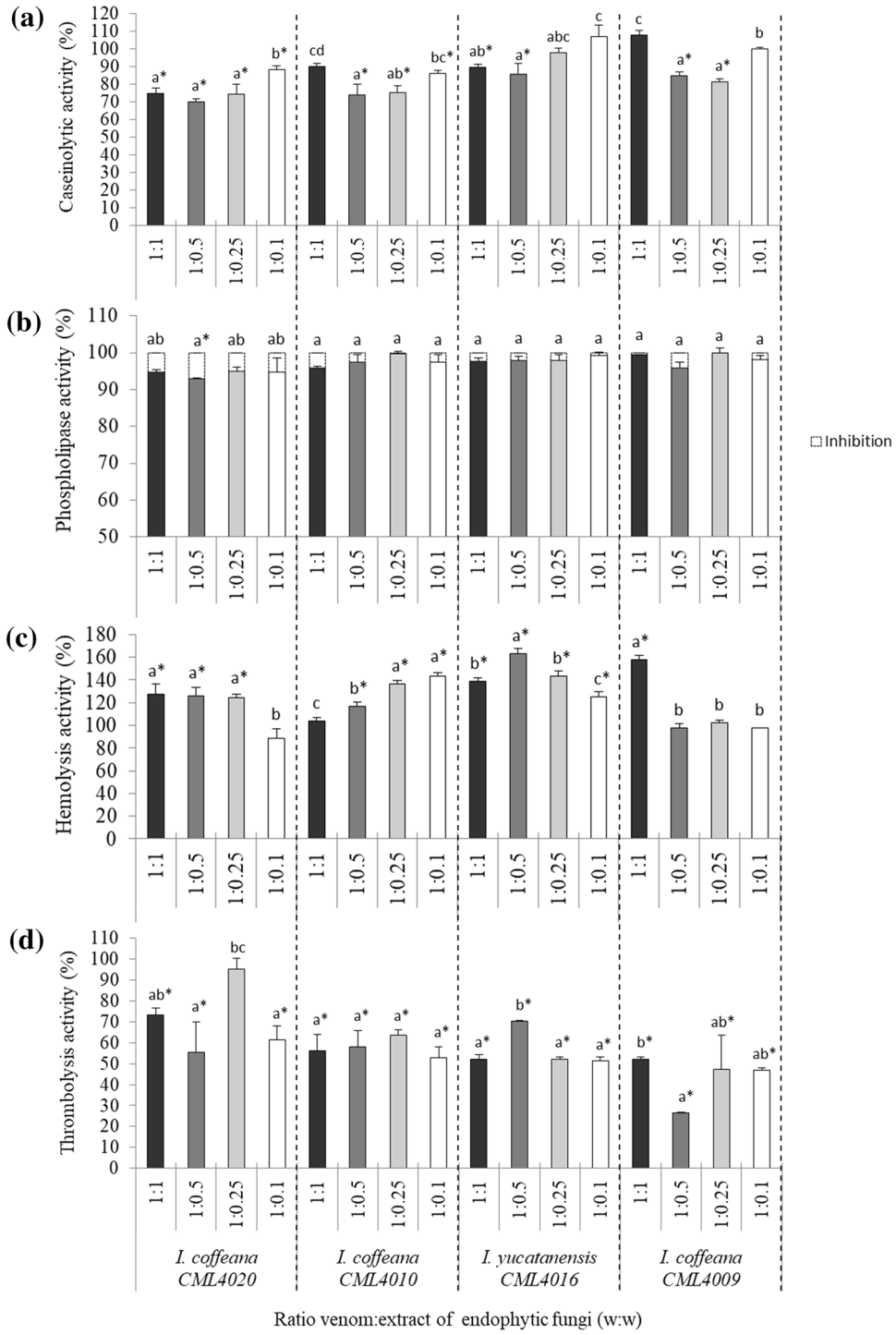

Fig. 1 Caseinolytic activity (**a**), Phospholipase activity (**b**), Hemolysis activity (**c**), and Thrombolysis activity (**d**) induced by *Bothrops atrox* venom, after the incubation with extracts from the endophytic fungi of the genus *Induratia*. Controls containing only venom (20 µg) were considered as 100% activity. The results correspond to the average of the data obtained for each proportion (venom: extract, w: w) in triplicate, and the upper vertical bars represent the standard deviation of the samples. *The means statistically differ from the respective control. Different letters indicate that the means of the different proportions differ from each other by the Tukey test ($P < 0.05$)

Discussion

Considering the presence of several bioactive components in fungal extracts [1–23], the data obtained in our work suggest that the increase in plasma coagulation time, as well as the pro-coagulant action, may be resulting of weak and reversible interactions of compounds present in the extracts with the coagulant proteases present in the venom. In addition, at some point, they may have enhanced the action of hemorrhagic proteases. As these are mixtures containing varied molecular structures and in unknown quantities, the molar ratios that can interact and change the evaluated activity may be random between the doses evaluated. This is well observed in the effect variation (inhibition or potentiation) resulting from the evaluation of the *I. coffeana* (CML4020) extract.

Natural enzyme inhibitors have been widely described with inhibition mechanisms based on weak (hydrogen and/or hydrophobic interactions) and transients interactions in different regions of the enzyme structures (e.g., active site and cofactor binding site) [26]. Considering that the fungi of the genus *Induratia* have also been reported as producers of compounds with bioactive action [12, 14], it is likely that metabolites produced by these fungi have mechanisms of action similar to those already described for some natural compounds. The extract produced by a fungus isolated from Brazilian caves, *Lecanicillium aphanocladii*, also promoted an increase in plasma clotting time, induced by snake venom [21]. Based on the literature, the authors of this work suggest that the action of the molecules present in the fungus extract is through the hydrophobic interactions with the aromatic rings that make up the structure of amino acid residues present in the active site of proteases, or other regions. Links in different regions of the active site can result in conformational changes in enzymes, with a consequent reduction in catalytic activity [33, 34].

The previous incubation of the extracts with the plasma resulted in an even greater increase in the clotting time. This result may have occurred because the bioactive molecules of the evaluated fungi have a greater number of interactions with plasma proteins than with proteases present in the venom, making it difficult for proteolytic enzymes to access the cascade factors and, thus, delaying their activation.

In addition to the possible interactions of fungal bioactive compounds with the components of the coagulation cascade, which are made up of enzymes and proteins, it is also necessary to consider interactions of fungal compounds with anticoagulation regulators present in the plasma, such as capture and/or formation of complexes with ions that are cofactors of the coagulation cascade and venom enzymes.

It should be noted that the possible effects of fungal extracts on the coagulation cascade depend not only on the proportion of chemical compounds in relation to the enzymes present in the venom, but also on the type and quantity of molecules present in the different extracts, on the composition of the plasma, the incubation time of the samples, and other reaction conditions, such as pH and temperature.

Thrombin inhibitors correspond to a class of drugs that are prominent in the treatment of bleeding, in which they act on the coagulation cascade. Such inhibitors may inhibit some metalloproteases, preventing the transformation of prothrombin (Factor II) into thrombin. Thus, they reduce the action of this enzyme in fibrinogen proteolysis for the formation of fibrin, and, consequently, decrease the formation of clots and thrombi [35]. To be an ideal thrombin inhibitor, it must be effective, selectively, and have an extended half-life [36].

Our results suggest modulation of enzymes exerted by the evaluated extracts and that the compounds present in the extracts act selectively on the different classes of enzymes present in the *B. atrox* venom. Although the venom-induced effects are attributed to several enzymes that act by different mechanisms, a class of enzyme can be highlighted in each test carried out. The coagulation of citrated plasma is mainly attributed to serine proteases, which have an action similar to thrombin [26, 27]. Thus, the inhibitory action of fungal extracts on these enzymes and the activation of prothrombin present in the plasma can be suggested, which results in the observed increases in clotting time.

I. coffeana (CML4020) extract evaluated in the proportion of 1: 0.5 showed a promising inhibitory effect on phospholipases A_2 activity. Phospholipases A_2 comprise one of the most common classes of toxins in the composition of snake venoms, along with the various classes of proteases. They are enzymes capable of promoting an intense catalytic activity on phospholipids, affecting the transport across biological membranes, blood clotting, inflammatory response, and hemostasis [25]. In addition, they may have genotoxic and mutagenic activities [26]. Thus, phospholipase A_2 inhibitors are also promising molecules for the development of therapeutic agents, with possible use in the prevention, control, and treatment of pathologies associated with exacerbated inflammation. The present work highlights a potential future application for the extract of the fungus *I. coffeana* (CML4020).

Regarding cytotoxicity on human erythrocytes, the fungal extracts did not induce cytotoxic action in the absence of the venom and, after the incubation venom-fungal extract, some extracts reduced the cytotoxicity of the venom, while others increased the cytotoxicity. In the cytotoxicity test, there is a more complex reaction context. Cells can undergo the action of peptides that bind to membrane-carrying proteins and thus, changing the influx of ions and the membrane potential. It can also go through the action of proteases that degrade protein-membrane components, activating endogenous proteases, and phospholipase A_2 proteins that degrade membrane phospholipids. In addition, other molecules present in lesser amounts in the venom composition may also exert activities, such as biogenic amines [37].

In this context, the potentiation of the cytotoxic activity can be attributed to a variety of molecular interactions whose mechanisms are still unknown. The importance of the results obtained is in the fact that the extracts have had modulating effects on the activities induced by the venom, and have not induced activity on the molecules and cells when evaluated in the absence of the venom.

Fungal extracts showed a reduction in the venom-induced thrombolytic and proteolytic activity. Thrombi dissolution mechanisms are related to fibrin degradation by the fibrinolytic system [23]. Thus, considering the results obtained,

we can suggest the presence of plasminogen-activating molecules, for their conversion into plasmin, in some extracts. Fungal extracts also increased the dissolution of venom-induced thrombi. Therefore, they characterize the action of molecules that exert positive modulation on metalloproteases, especially fibinogenolytic, fibrinolytic, hemorrhagic, and hemolytic, in which contains disinterring domain in its structure, according to Gutiérrez et al. [38]. This demonstrates that the selected microorganisms have a wide range of bioactive compounds, capable of acting both in the coagulation cascade (probably as protease inhibitors or through interactions with cofactors) and in the clot and thrombus dissolution system (degrading the final product of the cascade).

Casein represents a random protein substrate since it is not related to the context of hemostasis. Thus, it was possible to observe that the fungal extracts are also able to inhibit proteases that act in the extracellular context, for example, in the digestive and defense processes of the organism.

It can be concluded by the results presented in this work that the extracts of the endophytic fungi studied of the genus *Induratia* have molecules that exert modulating activity on the blood coagulation process. In addition, there is a possible mechanism of interaction with the components of the coagulation cascade and with coagulant/fibrinogenolytic proteases present in the *Bothrops atrox* venom. Although the inhibitory effect of fungal extracts was less on the enzymatic activity of phospholipases A_2, the data indicate the presence of inhibitory molecules, mainly in the extract of *I. coffeana* (CML4020). This is the first report on the modulation activity of the non-volatile metabolites from *Induratia* spp. extracts on the blood coagulation process.

It is possible to suggest that the protease modulators present in the fungal extracts can be widely explored for future pharmaceutical applications, once they were tested using snake venoms as controls and activity-inducing substances. The various classes of proteases in the *B. atrox* venom act in processes closely related to a variety of human pathologies. As for example, they induce or inhibit blood clotting and platelet aggregation, degrade intra and extracellular components (apoptosis, cell replacement, debridement, and tissue regeneration), and activate the inflammatory response [38, 39].

The extracts of the endophytic fungi *I. coffeana* (CML4009, CML4010, CML4020) and *I. yucatanensis* (CML4016) evaluated in this study demonstrated modulating activity on the blood coagulation process, acting as inhibitors or potentiators of the catalytic activity exerted by different classes of proteases (hemorrhagic or fibrinogenolytic serine proteases and metalloproteases—mainly from classes P-II and P-III).

The intrinsic variations in the production of metabolites, existing in each microorganism, are responsible for the various applications attributed to them [31]. However, the fungi whose extracts were evaluated in the present study correspond to species little explored in the pharmaceutical and clinical area. Thus, future studies are necessary to enable a greater understanding of the classes of molecules produced by them, as well as their identification and wide characterization (pharmacological and toxicological).

Acknowledgements This research was supported by Coordenação de Aperfeiçoamento de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Universidade Federal de Lavras (UFLA).

Author Contributions Conceptualization: APDSPB, PGC, LCJP, SM. Data acquisition: Bastos APDSPB, MVCT. Data analysis: APDSPB, ÍAFMS, SM. Design of methodology: APDSPB, PGC, MVCT, LCJP, SM. Writing and editing: APDSPB, PGC, ÍAFMS, MVCT, LCJP, SM.

Compliance with Ethical Standards

Conflict of interest We declare that we have no conflict of interest in this work.

References

1. Nisa H, Kamili AN, Nawchoo IA, Shafi S, Shameem N, Bandh SA (2015) Fungal endophytes as prolific source of phytochemicals and other bioactive natural products: a review. Microb Pathog 82:50–59
2. Ali L, Khan AL, Hussain J, Al-Harrasi A, Waqas M, Kang SM, Al-Rawahi A, Lee IJ, Ali L (2016) Sorokiniol: a new enzymes inhibitory metabolite from fungal endophyte *Bipolaris sorokiniana* LK12. BMC Microbiol 16:103
3. Patil RH, Patil MP, Maheshwari VL (2016) Bioactive secondary metabolites from endophytic fungi: a review of biotechnological production and their potential applications. In: Rahman AU (ed) Studies in natural products chemistry. Elsevier, Netherlands, pp 189–205
4. Yan L, Zhao H, Zhao X et al (2018) Production of bioproducts by endophytic fungi: chemical ecology, biotechnological applications, bottlenecks, and solutions. Appl Microbiol Biotechnol 102:6279–6298
5. Tanapichatsakul C, Khruengsai S, Pripdeevech P (2019) Production of eugenol from fungal endophytes *Neopestalotiopsis*. sp and *Diaporthe*. sp isolated from *Cinnamomum loureiroi* leaves. PeerJ 7:e6427
6. Ancheeva E, Daletos G, Proksch P (2020) Bioactive secondary metabolites from endophytic fungi. Curr Med Chem 27:1836–1854
7. Suwannarach N, Kumla J, Sujarit K, Pattananandecha T, Saenjum C, Lumyong S (2020) Natural bioactive compounds from fungi as potential candidates for protease inhibitors and immunomodulators to apply for Coronaviruses. Molecules 25:1800
8. Kaaniche F, Hamed A, Abdel-Razek AS et al (2019) Bioactive secondary metabolites from new endophytic fungus *Curvularia* sp. isolated from *Rauwolfia macrophylla*. PLoS ONE 14:e0217627
9. Helaly SE, Thongbai B, Stadler M (2018) Diversity of biologically active secondary metabolites from endophytic and saprotrophic fungi of the ascomycete order Xylariales. Nat Product Rep 35:992–1014

10. Hyde KD, Xu J, Rapior S et al (2019) The amazing potential of fungi: 50 ways we can exploit fungi industrially. Fungal Divers 97:1–136
11. Kuźniar A, Włodarczyk K, Wolińska A (2019) Agricultural and other biotechnological applications resulting from trophic plant-endophyte interactions. Agronomy 9:779
12. Samarakoon MC, Thongbai B, Hyde KD, Brönstrup M, Beutling U, Lambert C, Miller AN, Liu J-KJ, Promputtha I, Stadler M (2020) Elucidation of the cycle of the endophytic genus *Muscodor* and its transfer to *Induratia* in Induratiaceae fam. nov., based on a polyphasic taxonomic approach. Fungal Divers 101:177–210
13. Strobel GA, Dirkse E, Sears J, Markworth C (2001) Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. Microbiology 147:2943–2950
14. Strobel G (2011) *Muscodor* species—endophytes with biological promise. Phytochemistry 10:65–172
15. Monteiro MCP, Alves NM, Queiroz MV, Pinho DB, Pereira OL, Souza SMC, Cardoso PG (2017) Antimicrobial activity of endophytic fungi from coffee plants. Biosci J 33:381–389
16. Gupta M, Meshram V (2018) The biological promises of endophytic Muscodor species. In: Gehlot P, Singh J (eds) Fungi and their role in sustainable development: current perspectives. Springer, Singapore, pp 51–74
17. Alves NM, Guimarães LHS, Piccoli RH, Cardoso PG (2016) Production and partial characterization of an extracellular phytase produced by *Muscodor* sp. under submerged fermentation. Adv Microbiol 6:23–32
18. Monteiro MCP, Tavares DG, Nery EM, Queiroz MV, Pereira OL, Cardoso PG (2020) Enzyme production by *Induratia* spp. isolated from coffee plants in Brazil. Braz Arch Biol Technol 63: e20180673. https://doi.org/10.1590/1678-4324-2020180673
19. Kapoor N, Saxena S (2016) Xanthine oxidase inhibitory and antioxidant potential of Indian *Muscodor* species. 3 Biotech 6:248
20. Macías-Rubalcava ML, Hernández-Bautista BE, Oropeza F, Duarte G, González MC, Glenn AE, Hanlin RT, Anaya AL (2010) Allelochemical effects of volatile compounds and organic extracts from *Muscodor yucatanensis*, a tropical endophytic fungus from *Bursera simaruba*. J Chem Ecol 10:1122–1131
21. Qadri M, Nalli Y, Jain SK, Chaubey A, Ali A, Strobel GA, Vishwakarma RA, Riyaz-Ul-Hassan S (2017) An insight into the secondary metabolism of *Muscodor yucatanensis*: Small-molecule epigenetic modifiers induce expression of secondary metabolism-related genes and production of new metabolites in the endophyte. Microb Ecol 73:954–965
22. Suwannarach N, Kumla J, Matsui K, Lumyong S (2015) Characterization and efficacy of *Muscodor cinnamomi* in promoting plant growth and controlling *Rhizoctonia* root rot in tomatoes. Biol Control 90:25–33
23. Franco RF (2001) Fisiologia da coagulação, anticoagulação e fibrinólise. Medicina (Ribeirão Preto. Online) 34:229–237
24. Choi BS, Sapkota K, Choi JH, Shin C, Kim S, Kim SJ (2013) Herinase: a novel bi-functional fibrinolytic protease from the monkey head mushroom, *Hericium erinaceum*. Appl Biochem Biotechnol 170:609–622
25. Dennis EA, Cao J, Hsu YH, Magrioti V, Kokotos G (2011) Phospholipase A2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. Chem Rev 111:6130–6185
26. Marcussi S, Santos PRS, Menaldo DL, Silveira LB, Santos-Filho NA, Mazzi MV, Da Silva SL, Stábeli RG, Antunes LMG, Soares AM (2011) Evaluation of the genotoxicity of *Crotalus durissus terrificus* snake venom and its isolated toxins on human lymphocytes. Mut Res Genet Toxicol Environ Mutagenes 724:59–63
27. Marques TR, Braga MA, Cesar PHS, Marcussi S, Corrêa AD (2019) Jabuticaba (*Plinia jaboticaba*) skin extracts as inhibitors of phospholipases A2 and proteases. Anais da Acad Bras de Ciências 91:16
28. Cardoso MGB, Trento MVC, Reis CH, Marcussi S, Cardoso PG (2019) *Lecanicillium aphanocladii*: snake venom phospholipases A2 and proteases as tools to prospect enzymatic inhibitors. Lett Appl Microbiol 69:88–95
29. Cesar PHS, Trento MVC, Sales TA, Marques TR, Braga MA, Ramalho TC, Marcussi S (2019) Molecular interactions between *p*-coumaric acid and snake venom toxins. J Cell Biochem 120:14594–14603
30. Gutiérrez JM, Chaves F, Gené JA, Lomonte B, Camacho Z, Schosinsky K (1989) Myonecrosis induced in mice by a basic myotoxin isolated from the venom of the snake *Bothrops nummifer* (jumping viper) from Costa Rica. Toxicon 27:735–745
31. Cintra ACO, De Toni LGB, Sartim MA, Franco JJ, Caetano RC, Murakami MT, Sampaio SV (2012) Batroxase, a new metalloproteinase from *Bothrops atrox* snake venom with strong fibrinolytic activity. Toxicon 60:70–82
32. Wang WJ, Shih CH, Huang TF (2004) A novel P-I class metalloproteinase with broad substrate-cleaving activity, agkislysin, from *Agkistrodon acutus* venom. Biochem Biophys Res Commun 324:224–230
33. Dunaevsky YE, Popova VV, Semenova TA, Beliakova GA, Belozersky MA (2014) Fungal inhibitors of proteolytic enzymes: classification, properties, possible biological roles, and perspectives for practical use. Biochimie 101:10–20
34. Saavedra SL, Avila L, Giudicessi SL, Albericio F, Camperi SA, Cascone O, Martinez-Ceron MC (2018) Natural snake venom inhibitors and their pharmaceutical uses: challenges and possibilities. Curr Pharm Des 24:1737–1747
35. Mehta AY, Jin Y, Desai UR (2014) An update on recent patents on thrombin inhibitors (2010–2013). Expert Opin Ther Pat 24:47–67
36. Sixma JJ, De Groot PG (1992) The ideal anti-thrombotic drug. Thromb Res 67:305–311
37. Costa JO, Fonseca KC, Mamede CCN, Beletti ME, Santos-filho NA, Soares AM, Arantes EC, Hirayama SNS, Selistre-Dearaújo HS, Fonseca F, Henrique-Silva F, Penha-Silva N, Oliveira F (2010) Bhalternin: functional and structural characterization of a new thrombin-like enzyme from *Bothrops alternatus* snake venom. Toxicon 55:1365–1377
38. Gutiérrez JM, Rucavado A, Escalante T, Lomonte B, Ângulo Y (2010) Tissue pathology induced by snake venoms: how to understand a complex pattern of alterations from a systems biology perpective? Toxicon 55:166–170
39. Kini RM, Kho CY (2016) Metalloproteases affecting blood coagulation, fibrinolysis and platelet aggregation from snake venoms: Definition and nomenclature of interaction sites. Toxins 8:284

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.