CHEMICAL CONSTITUENTS AND ANTIFUNGAL POTENTIAL OF THE *Richardia brasiliensis* (Gomes) ETHANOLIC EXTRACT[†]

[COMPONENTES QUÍMICOS Y POTENCIAL ANTIFUNGAL DE LA EXTRACCIÓN ETANÓLICA DE *Richardia brasiliensis* (Gomes)]

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SUMMARY

The objective of this work was to evaluate the fungicide potential of the *Richardia brasiliensis* plant extract or its fractions on *Lasiodiplodia theobromae* and *Colletotrichum gloeosporioides*, as well as the isolation and identification of substances present in these fractions. For both the crude and ethanolic extracts, the plants' aerial part was fractionated and the hexane fractions and ethyl acetate were separately subjected to columns of silica-gel leading to isolation of steroids and triterpene acids respectively. One- and two-dimensional ^{1}H-NMR and ^{13}C-NMR spectral analyses were used for structural elucidation. Concentrations of 10; 20; 40; 80; 160 µg 100mL^{-1} ethanolic extract, hexane fractions and a pure isolated substance (oleanolic acid) with 5 µL of DMSO were utilized for antifungal activity evaluation. Spectral analysis allowed identification and isolation of *β*-sitosterol and a mixture of *β*-sitosterol and stigmasterol. For fungicide action, the hexane and acetate or pure fractions provided reduction of the mycelial growth of *C. gloesporioides*, at the 160 µg100 mL^{-1} concentration. For *L. theobromae*, the acetate or pure fraction and the ethanolic extract were highlighted in the concentrations of 80 and 160 µg100 mL^{-1}. From the results of the evaluation of fungicide potential of the R. *brasiliensis* extract and its fraction against the fungi *C. gloeosporioides* and *L. Theobromae*, as well as the chemical insulation of the substances involved, it can be noted that this weed contains potential fungicidal substances and especially the isolated triterpenes are promising for use in formulations in the control of these pathogens.

Key-words: Rubiaceae; natural fungicide; triterpenes and steroids; *Colletotrichum gloeosporioides; Lasiodiplodia theobromae*.

RESUMEN

El objetivo de este trabajo fue evaluar el potencial fungicida del extracto vegetal y las fracciones de *Richardia brasiliensis* en *Lasiodiplodia theobromae* y *Colletotrichum gloeosporioides*, el aislamiento y la identificación de sustancias presentes en estas fracciones. El extracto crudo como el extracto etanólico de la parte aérea de las plantas de *R. brasiliensis* se fraccionaron y las fracciones de hexano y acetato de etilo, por separado, se sometieron a una columna de gel de sílice que llevó al aislamiento de los ácidos esteroides y triterpenos respectivamente, la elucidación estructural ocurrió a través del análisis de espectros uni y bidimensional de RMN ^{1}H y ^{13}C. Para la evaluación de la actividad antifúngica se utilizaron concentraciones de 10; 20; 40; 80; 160 µg de 100 mL^{-1} de extracto etanólico, fracción

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de hexano y sustancia aislada pura (ácido oleanólico) con 5 µl de DMSO. El análisis espectral permitió la identificación y el aislamiento del *β*-sitosterol y una mezcla de *β*-sitosterol y estigmasterol. Para la acción fungicida, el hexano y el acetato o las fracciones puras proporcionaron una reducción del crecimiento micelial de *C. gloesporioides*, a la concentración de 160 µg100 mL^{-1}. Para *L. theobromae*, el acetato o fracción pura y el extracto etanólico se destacaron en las concentraciones de 80 y 160 µg100 mL^{-1}. A partir de los resultados de la evaluación del potencial fungicida de lo extracto *R. brasiliensis* y su fracción contra los hongos *C. gloeosporioides* y *L. Theobromae*, así como el aislamiento químico de las sustancias involucradas, se puede observar que esta maleza contiene sustancias fungicidas potenciales y especialmente los triterpenos aislados son prometedores para uso en formulaciones para el control de estos patógenos.
Palabras clave: Rubiaceae; fungicida natural; triterpenos y esteroides; *Colletotrichum gloeosporioides; Lasiodiplodia theobromae.*

INTRODUCTION

The mango tree (*Mangifera indica* L.) can be cultivated in tropical and subtropical climates due to its adaptive capacity. Under the subtropical conditions the crop management becomes relatively easy due to the low temperatures facilitating the induction of flowering, although the growth and fruit quality can be negatively affected by these temperatures.

However, the incidence of fungal diseases is one of the causes of production losses. The anthracnose caused by the *Colletotrichum gloeosporioides* (Penz.) and *Lasiodiplodia theobromae* (Patouillard) Griffon & Maublanc fungus are among the main diseases that affect different cultures and can attack all stages of plant development, possibly even causing post-harvest losses affecting commercialization.

In the field, the two diseases are controlled chemically; however, the two fungi present resistance to the commonly used fungicides due to the fact that they are treated preventively with phytosanitary products. Needless to say, the inappropriate use of these products has caused major environmental impact, bringing about economic and social damage (Sales *et al.* 2004).

It has become a requirement for importing markets to acquire export-certified fruit, whose entire production chain is permanently monitored and controlled through programs and specific legislation, aiming for food safety and environmental protection through extinction and/or replacement of phytosanitary products by phytoregulators or biological products (Gouveia, 2007).

Several methods have been tested in order to control post-harvest pathogens, such as biocontrol, controlled and modified atmosphere, refrigerated storage, induction of resistance and use of plant extracts (Mazaro, 2007). The last one reveals a wide diversity of secondary metabolites that act on a wide range of micro-organisms.

The *Rubiaceae*is family has been exploited, either via species of high economic value, such as the *Psychotria ipecacuanha* (brot.) Stockes (medicinal) and the *Gardenia jasminoides* Ellis (ornamental), or via toxic species, such as the *Palicourea marcgravii* St. Hill, or weeds, such as the *Richardia brasiliensis* Gomes (Kissmann and Groth, 2000).

R. brasiliensis, whose studies found in the literature are focused on the area of agribusiness due to its invasive characteristic of other cultures, soybeans and corn, its easiness to adapt to different planting systems (conventional and no-tillage) causing serious problems competing with the crops and its difficulty to be controlled (Pedrinho-Junior et al. 2004). Several phytosociological studies of weed species in Brazil have shown a high incidence of *R. brasiliensis* in the Cerrado region (Adegas *et al*. 2010; Guglieri-Caporal *et al*. 2011; Monquero and Christoffoleti, 2003). Due to its invasive nature, both in annual crops and pasture degradation, this plant has been systematically eliminated by means of herbicides (Monquero *et al*. 2005; Gomes and Christoffoleti, 2008; Akjhisa *et al*., 1987), which can lead to the extinction of the species that has been studied so little phytochemically.

Thus, the objective of this study was to evaluate the fungicide potential of *Richardia brasiliensis* and its fractions against the fungi *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae* and chemical isolation of substances involved.

MATERIALS AND METHODS

Preparation of the plant material, extracts and fractions: The plant *Richardia Brasiliensis* was collected (leaves and stems) in the experimental area of Medicinal and Aromatic Plants at Anhanguera – Uniderp University, Campo Grande - MS, (20º26'47"S; 54º32'7'O), and an exsiccate of the specimen is deposited at the Herbarium of this institution under the registration number 05052.

The botanical material was cleaned, dried in an oven with forced air ventilation at 45 °C (MARCON®, MA35) for 3 days, weighed, sprayed in an electrical mill (MARCONI®, MA048) and sieved (sieve n° 60). From the processed material, 760.5 g were subjected to extraction with ethanol (99.5 %). The extraction occurred in ultrasound bath for 60 minutes (UNIQUE®, 1450) followed by maceration, at room temperature. This procedure was repeated until the medication was depleted, which occurred in 15 days. The solvent was evaporated under vacuum on a rotary evaporator (Tecnal®, MA120), obtaining 19.8 g of crude ethanolic extract (ExEtOH).

Chemical fractioning: The ExEtOH extract (15.5 g) was suspended in methanol/water (1:1, 340 mL) and successively partitioned with hexane (550 mL) and ethyl acetate (750 mL). After solvent removal, the fractions were dried to yield hexane fractions (FHex = 4.1 g), ethyl acetate (FAcoet = 3.2 g) and hydromethanolic (FH/MeOH = 7.1 g).

Phytochemical analysis of the extracts and fractions: The extracts and fractions were subjected to phytochemical tests for plant secondary metabolites: phenolic compounds, tannins, flavonoids, free coumarins, anthocyanins, anthraquinone, steroids, triterpenes, alkaloids, saponins, cardiotonic heterosides, cyanogenic heterosides were tested in accordance with Trease and Evans (1989) and Harborne (1998) with little modification. Alterations in color and/or precipitation when compared with control were observed for results of the assays following the method of Fontoura *et al*. (2015), being strongly positive (+++), moderately positive (++), weakly positive (+) and partially positive (±). However, when demonstrating only hazed and/or partially changed color, as well as the absence of color and / or precipitation, the result was considered as negative (-). The intensity of color and/or precipitation indicates the increased concentration of such class of secondary metabolite.

Isolation of active components: Part of the FAcoet (2.2 g) was fractionated into silica gel with mixtures of hexane (Hex), ethyl acetate (AcOEt) and methanol (MeOH) in increasing gradient of polarity. Purification was performed by recrystallization with acetone in the sub-fraction eluted in Hex:AcOEt at 20%, which provided a mixture of two substances encoded as F_1 and F_2 (7.8 mg).

F_{Acoet} (2.0 g) was subjected to a column of silica gel, using as eluent Hex, AcOEt and MeOH in increasing gradient of polarity. The sub-fractions eluted in Hex:AcOEt (75:25) provided after purification in acetone: MeOH a pure substance (22.0 mg), codified as F_3, and a mixture (61.2 mg) containing the substance F_3 and a second substance codified as F_4. One- and two-dimensional ^{1}H-NMR and ^{13}C-NMR spectral analyses and comparison with data from literature (Mahato and Kundu, 1994; Falcão *et al*., 2003; Hung and Yen, 2001; Takeda *et al*. 2004) were used for structural elucidation.

Antifungal activity: Pure colonies of fungi *Lasiodiplodia theobromae* and *Colletotrichum gloeosporioides*, recorded in the collection of cultures of the University Anhanguera - Uniderp, were previously transferred to Petri dishes containing PDA culture medium (potato-dextrose-agar) and maintained in incubation chamber for 10 days.

The ethanolic extract, the hexane fraction and the pure fraction (F3= oleanolic acid) obtained from fractionation of ethyl-acetate were used for a stock solution (0.05 g 100 mL^{-1}) containing 5 µL of dimethyl sulfoxide (DMSO) and completing the volume to 100 mL with alcohol at 20%.

Four repetitions of each concentration (10; 20; 40; 80; 160 µg 100 mL^{-1}) one only with PDA medium (without extracts or *R. brasiliensis* fractions) called control, were poured into Petri dishes of 90 mm in a volume corresponding to 10 mL per dish. After solidification, a mycelium disc with 5 mm diameter previously transplanted one week before the procedure was peaked in the center of each dish. The dishes were subsequently covered and sealed with plastic film and kept in growth chamber at 25 °C, with a 12 hours photoperiod.
The mycelial growth evaluation was realized through daily measurements of the colony diameter, obtained by the average of two diametrically opposed measurements, realized until the control group reached the edge of the Petri dish.

Statistic analysis

F test was applied for analysis of variance ($\alpha \leq 5\%$) and, when significant, non-linear and linear regression analysis ($\alpha \leq 5\%$) was applied for doses with the ethanolic extract, the hexane fraction and the pure fraction.

RESULTS AND DISCUSSION

The results of the phytochemical analysis of the ethanolic extract and the fractions of leaves and stems of *R. Brasiliensis* are presented in Table 1. Analyses showed that the semi-purification with solvents of different polarity had the capacity to separate the components present in the plant.

The ExtEtOH extract presented the highest diversity of secondary metabolites in relation to fractions. The fraction that stands out in relation to the number of classes is FAcoet fraction, with seven classes (phenolic compounds, flavonoids, coumarins, steroids, triterpenes and alkaloids, followed by the hydromethanolic fraction (five classes).

Table 1. Phytochemical analyses of crude ethanolic extract (ExEtOH) and fractions (Hexane = FHex; Ethyl acetate = FAcoet; Hydromethanol = FH/MeOH) *of Richardia Brasiliensis* leaves and stems, Campo Grande, Mato Grosso do Sul, Brazil.

Secondary metabolites	Extracts and Fractions			
	ExEtOH	FHex	FAcoet	FH/MeOH
Phenolic Compounds	+++	-	+++	+++
Tannins	++	-	-	+++
Flavonoids	+++	+	+++	++
Coumarins	++	-	++	++
Steroids	+++	+++	++	-
Triterpenes	+++	++	+++	-
Alkaloids	++	-	++	-
Saponins	+	-	-	++

(+++) greater intensity, (++) average intensity, (+) lower intensity, (±) partial (-) negative result. ExEtOH = Ethanolic extract. FHex = Hexane. FAcoet = Ethyl acetate. FH/MeOH = Hydromethanolic.

The phytochemical analysis enabled the isolation of two steroids, *β*-sitosterol (F1) and a mixture of *β*-sitosterol and stigmasterol (F2) of the FHex fraction (Figure 1).

In the FAcoet fraction, oleanolic acid (F_3) was isolated and a mixture of triterpenes containing the oleanolic acid and Ursolic-acid (F_4) (Figure 2). The structural elucidation occurred by comparing the spectral data with the data described in the literature for steroids (Mahato and Kundu, 1994) and with pentacyclic triterpenes (Falcão *et al*. 2003; Hung and Yen, 2001; Takeda *et al*. 2004; Pinto *et al*., 2008).

Bernardo *et al.* (1998) reported the isolation and identification of six substances from *A. brasiliensis* leaves, collected in Paraíba, Brazil. Among the isolated substances are 3-O-rutinoside and isorhamnetin-3-O-rutinoside (glycated flavonoids), the oleanolic acid (triterpene), coumarin scopoletin (coumarin) and the acids *p*-hydroxybenzoic acid and *m*-methoxy-*p*-hydroxy-benzoic acid (benzoic). These triterpenes present fungicidal activity once they have a basic lipophilic skeleton, which facilitates the rupture of the cell wall and the passage of the constituents present in the extract.

C_7-R=H, *β*–sitosterol (F_1)

C_8-R=H, stigmaterol (Δ^{22}) (F_2)

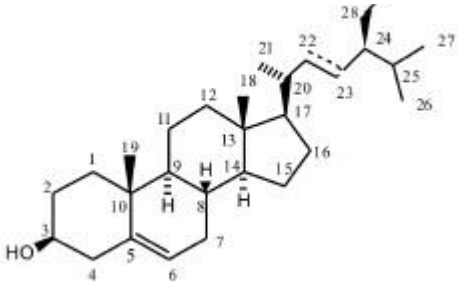
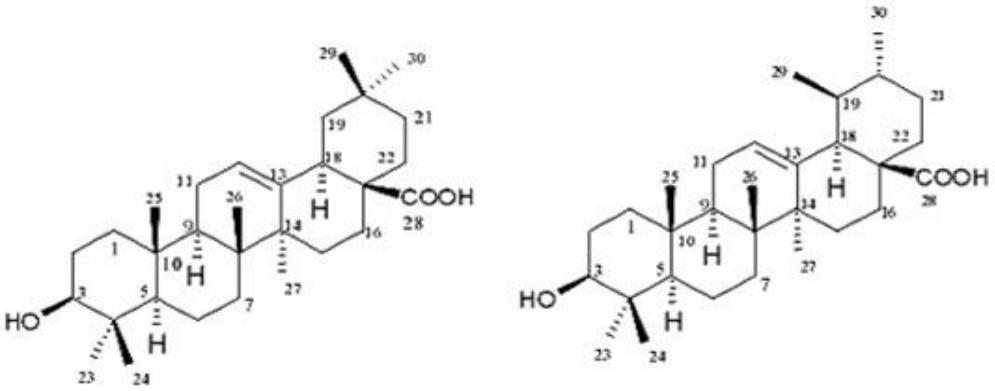


Figure 1. Structure of β-sitosterol (F1) and Stigmasterol (F2) isolated from the hexane fraction of leaves and stems of *R. brasiliensis*, Campo Grande, Mato Grosso do Sul, Brazil.



Oleanoic acid (F_3) Ursolic Acid (F_4)

Figure 2. Structure of the triterpenes oleanolic acid (F_3) and Ursolic acid (F_4) isolated from the Ethyl acetate fraction of leaves and stems of *R. brasiliensis*, Campo Grande, Mato Grosso do Sul, Brazil.

The results, of the antifungal activity, showed spore mycelial *C. gloesporioides* were inhibited by FHex fraction and pure fraction (F3= oleanolic acid), obtained from *R. brasiliense*, treatment and the inhibitory effect was highly correlated with concentration of 160µg mL^{-1} (Figure 3).

For the fungus *L. theobromae* (Figure 4), mycelial growth reduction for oleanolic acid and ExEtOH extract was observed at a concentration of 80 and 160 µg mL^{-1}. The ExEtOH extract and the FHex fraction did not provide significant reductions of the fungi *C. gloesporioides* and *L. theobromae*, respectively.

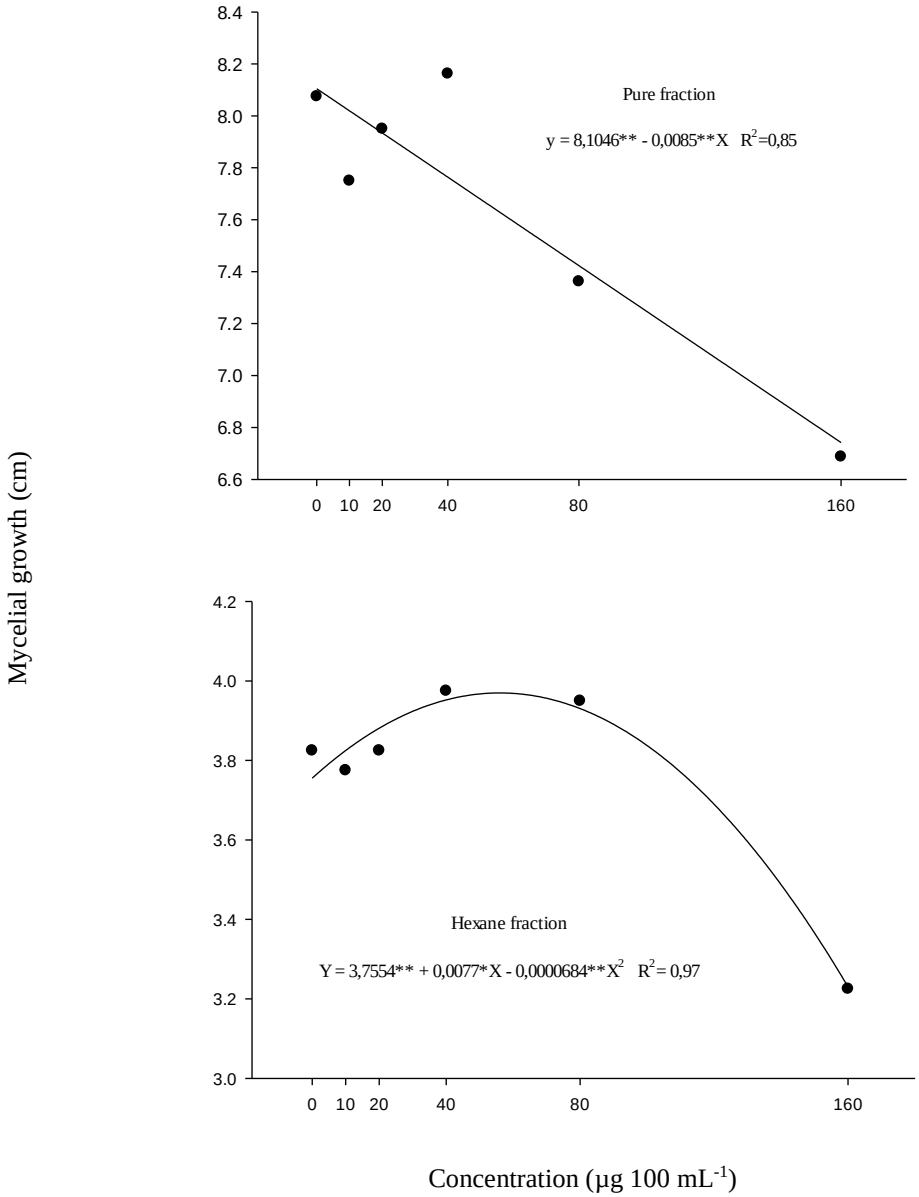


Figure 3. Analysis of regression between the extract/ fractions and dosage used compared to mycelial growth (cm) of *Colletotrichum gloeosporioides*. EtOH = ethanolic extract; Hex= hexane fraction; pure fraction = oleanolic acid. ** significate at P<0.01 and * significate P<0.05.

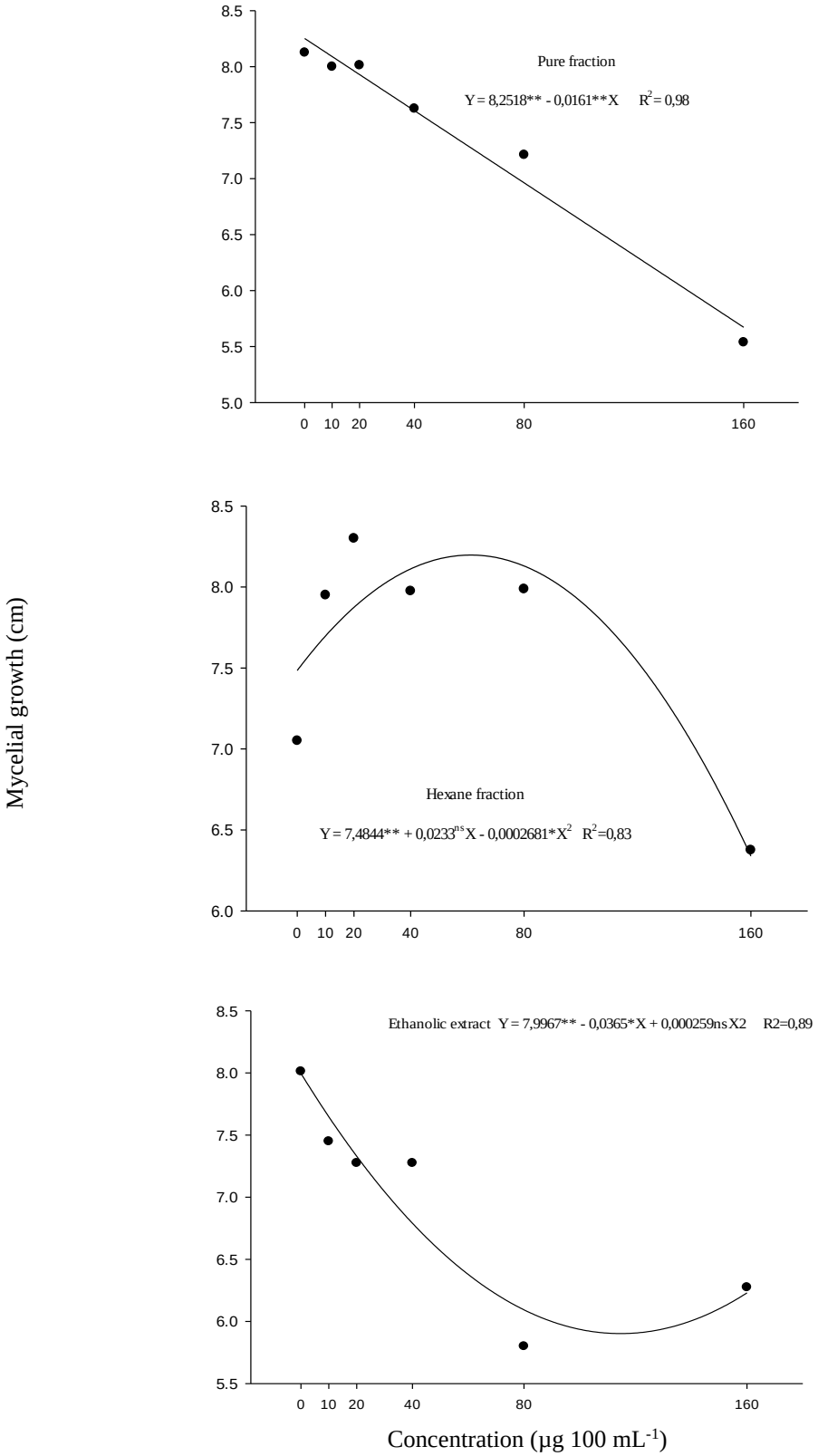


Figure 4. Analysis of regression between the extract/ fractions and dosage used compared to mycelial growth (cm) of *Lasiodiplodia theobromae*. EtOH = ethanolic extract; Hex= hexane fraction; Pure fraction = oleanolic acid. ns= no significate, ** significate at P<0.01 and * significate P<0.05.

According to the Celoto *et al*. (2008), plants are rich in a variety of substances with fungitoxic potential, which, when compared to synthetic fungicides, are practically harmless to the environment, and with respect to fungitoxicity may exceed chemical molecules.

According to Agrios (2005), phenolic compounds, flavonoids and triterpenes are inhibitors of hydrolytic enzymes, responsible for the resistance of young plant tissues to attack by phytopathogens, which may potentiate the control effect when these fractions are sprayed on a host, thus avoiding penetration of phytopathogenic fungi. Similarly, the authors Zuanazzi; Mountain (2004); Cushnie and Lamb (2005) indicate that these compounds can cause metabolic changes in fungal pathogens, acting on the inhibition of nucleic acid synthesis, membrane permeability and energetic metabolism.

Therefore, this fact is justified by the isolated substances in this study, at hexane fraction, the steroids β-sisterol and stigmasterol. The pure fraction (F3) corresponds to oleanolic acid, a pentacyclic triterpene. Based on these data, one may infer that these constituents are probably can be related to the fungitoxic properties of the pathogens investigated.

CONCLUSIONS

From the results of the evaluation of fungicide potential of the *R. brasiliensis* extract and its fraction against the fungi *C. gloeosporioides* and *L. Theobromae*, as well as the chemical insulation of the substances involved, it can be noted that this weed contains potential fungicidal substances and especially the isolated triterpenes are promising for use in formulations in the control of these pathogens.

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