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RESEARCH ARTICLE

EVALUATION OF PHYTOTOXIC ACTIVITY OF *Blechum pyramidatum*, A WEED OF PANAMANIAN COFFEE CROPS

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ABSTRACT

The coffee production is a high-value activity for the economy of the Chiriqui province highlands, Republic of Panama, being of particular importance for the development of the rural communities, as well as for indigenous groups. Frequently, this activity is affected by several factors, including weeds, pests, climate change, among others. Weeds provoke great economic losses for coffee producers by interference with the growth and development of the coffee in the cultivated area. Because of *Blechum pyramidatum* significantly affects coffee plantations in Santa Clara, Chiriquí, this plant was selected to evaluate its *in vitro* phytotoxic effect. The results revealed that a *B. pyramidatum* organic extract did not possess significant phytotoxic effect, this finding allows us to propose that negative effects against coffee provoked by *B. pyramidatum* might be by competition for water and nutrients available in cultivation. Finally, we evaluated the effect on the modulation of alpha glucosidase function of primary fractions from this plant.

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INTRODUCTION

Coffee production in the highlands of Chiriqui, province located in the western part of the Republic of Panama, began in the end of 19th century, thanks to on that site exist favorable environmental conditions (altitude, precipitation, temperature, relative humidity, etc.) for this activity. Today, the production of highest quality coffee is one of the most important agricultural practices in the region. In fact, coffee production is a high-value activity for the economy from this province, and in general for all the country, being of particular importance for the development of the surrounding communities, as well as for indigenous groups (Cherigo *et al*., 2012). Unfortunately, every year coffee production is widely affected by several factors including pests, weeds, climate change, among others. Weeds provoke great economic losses for coffee producers by interference with the growth and development of the coffee plants in the fields. In addition, weeds compete with coffee trees for soil nutrients in the plantations. Weeds can also limit coffee yields by producing allelopathic chemicals, by competition for space in plantations and/or by containing pests and pathogens (De Graaff, 1986; Radosevic *et al*., 1997, Cherigo *et al*., 2012). Weed competition against commercial interest plants could be minimized by eliminating or reducing weed presence. However, not all weeds are totally harmful even there are some weeds that provide the positive aspects for plant community and they can often be used as cover crops.

Therefore, farmers need information to detect weeds with negative effects on crops (Altieri, 1995; Cherigo *et al*., 2012). Based upon this premise, our group conducted an evaluation of the phytotoxic potential of more abundant weeds in the coffee plantations from Santa Clara, Chiriqui, Panama. We postulate the hypothesis that major adverse effects caused by a weed on commercially important plants are produced by two main ways: 1) Physical competition for nutrients and water available in the soil, and 2) Chemical competition by producing phytotoxic compounds. In a preliminary assessment, we detected that *Blechum pyramidatum* is one of the most abundant weed in coffee plantations in the area of Santa Clara, Chiriqui (Republic of Panama). *B. pyramidatum* is a perennial herb from Mexico to the northern of South America.

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It is a common weed of roadsides, fields and disturbed areas that generally grow less than 50 cm tall and has small white or purple flowers (Csurhes, 2010). Here, we report the information generated from the evaluation of the phytotoxic activity from organic extract and primary fractions of *B. pyramidatum* and the chemical studies of the active fraction. This information could allow us to identify by which of the two ways proposed in our hypothesis *B. pyramidatum* interfere with the coffee plants in cultivation fields. Finally, in order to identify a beneficial application of this weed we also evaluated the effect of the primary fractions against the modulation of alpha glucosidase function.

MATERIAL AND METHODS

Plant material and extract preparation

B. pyramidatum (Acanthaceae) was collected by August 2012 in a coffee-growing field of Santa Clara, Chiriqui, Panama. This plant was identified by Jorge Lezcano and was deposited at the University of Panama Herbarium. The plant aerial parts were dried at room temperature for a week and then were ground for further study. This material (102.5 g) was extracted by maceration at room temperature with a mixture of $CHCl_3$-MeOH (1:1).

Inhibition of radical elongation of *Amaranthus hypochondriacus*

The growth inhibitory activity of the extract on seedlings of *A. hypochondriacus* was evaluated using the Petri dish radicle elongation and germination bioassay at 28 °C (Mata *et al*., 1998, Cherigo *et al*., 2012). The results were analyzed by ANOVA ($P<0.05$), and IC_{50} values were calculated by Probit analysis based on percent of radicle growth or germination inhibition. The extract was evaluated at three concentrations (10, 100 and 1000 µg/mL) and 2, 4-D was used as the positive control.

General Chemical Experimental Procedures

NMR spectra were acquired on Jeol Eclipse 400 MHz spectrometer and referenced to residual solvent ^1H and ^{13}C signals (δ_H 7.26, δ_C 77.0 for $CDCl_3$). APCIHR-MS were acquired on a JEOL LC-mate mass spectrometer. The purification of the compounds was carried out on Agilent 1100 HPLC system equipped with a quaternary pump, a diode array detector, a normal phase silica gel column (Phenomenex Luna, 4.6 mm × 100 mm, 5 µm) at a flow rate of 1 mL/min. Column chromatography used silica gel 60 (70-230 mesh, Merck). TLC (analytical) was performed on precoated silica gel 60 F254 plates (Merck). All solvents were HPLC grade and used without further purification.

Chemical studies

The resulting crude extract was subjected to bioassay guided fractionation by resuspend the extract in a mixture water-MeOH 70:30 and sequential partition with hexane (5×400 mL) and ethyl acetate (5×400 mL). Each obtained fraction, including the final hydromethanol fraction, was evaporated to dryness and subjected to phytotoxic assays. Ethyl acetate fractions (moderately active, 6.53 g) was fractionated by column chromatography on silica gel (70 g). The column was eluted with hexane, followed by a gradient of hexane: EtOAc (1:0→0:1) and finally with a gradient of EtOAc: MeOH (1:0→1:1). Altogether, 133 fractions (50 mL each) were collected and combined according to their TLC profiles to yield twelve primary fractions (FA to FF), which were re-evaluated against *A. hyponchondriacus* and the activity was presented in fraction FB [eluted with 75 % hexanes: 25 % EtOAc].

Fraction FB (2.01 g) was further subjected to silica gel column chromatography and eluted with a gradient of hexane: EtOAc (1:0→0:1). This process led to nineteen fractions (FB-1 to FB-19). At this stage due to the low yield and high complexity of fractions, we worked only with major fractions. Fraction FB-2 eluted with Hexane: EtOAc (9:1), was purified by normal phase HPLC (Luna silica 250 x 10 mm column, isocratic elution of 90 % hexanes: 10 % EtOAc, UV detector at 254 nm, flow of 1 mL/min) to afford 4.1 mg of compound **1** and 1.3 mg of compound **2**. From fraction FB-3 eluted with Hexane: EtOAc (8:2), purified by normal phase HPLC (Luna silica 250 x 10 mm column, isocratic elution of 85% hexanes: 15% EtOAc, UV detector at 254 nm, flow of 1 mL/min) was obtained 2.8 mg of compound **3**. Fraction FB-6 (1.78 g) was further subjected to silica gel column chromatography and eluted with a gradient of hexane: EtOAc (1:0→0:1) and EtOAc: MeOH (1:0→1:1). This process led to six fractions (FB-6-A to FB-6-F). Fraction FB-6-B eluted with Hexane: EtOAc (4:6), was purified by normal phase HPLC (Luna silica 250 x 10 mm column, isocratic elution of 40 % hexanes: 60 % EtOAc, UV detector at 254 nm, flow of 1 mL/min) to afford 1.2 mg of compound **4**. Finally, Fraction FB-6-E eluted with 100 % EtOAc, was purified by normal phase HPLC (Luna silica 250 x 10 mm column, isocratic elution of 20 % hexanes: 80 % EtOAc, UV detector at 254 nm, flow of 1 mL/min) to afford 2.3 mg of compound **5**.

Spectral data for isolated compounds

Triacontanol (1). Colourless powder, M.P. 82-84 °C, ^1H-NMR(400 MHz) ($CDCl_3$): δ_H 3.86 (2H, brs, H-1), 1.52 (2H, brs, H-2), 1.20 (brs, H-3 – H-29),0.82 (3H, t, *J*=7.1 Hz, CH_3-30); 13C-NMR (100 MHz) ($CDCl_3$): δ_C 63.1 (CH_2), 32.8(CH_2), 31.9 (CH_2), 29.7-29.3 (CH_2 x 24), 25.7 (CH_2), 22.7 (CH_2), 14.1 (CH_3). APCI-HR-MS *m/z* 439.4884 $[M+H]^+$ (calcd for $C_{30}H_{63}O$, 439.4879).

Palmitic acid (**2**). White crystalline scales, M.P. 63-64 °C, ^1H-NMR (400 MHz, $CDCl_3$): δ_H 0.84-0.93 (m, CH_3), 1.25-1.33 (m, 13×CH_2), 2.17-2.30 (s, CH_2, C-2). ^{13}C-NMR ($CDCl_3$): δ_C 179.0, 33.9 (CH_2), 32.0 (CH_2), 29.8-29.1 (CH_2 x 10), 24.8 (CH_2), 22.8 (CH_2), 14.2 (CH_3). APCI-HR-MS *m/z* 257.2486 $[M+H]^+$ (calcd for $C_{16}H_{33}O_2$, 257.2481).

Stigmasterol (**3**). Colorless crystalline solid, M.P. 170-172 °C. ^1H-NMR ($CDCl_3$, 400 MHz): δ_H 5.33 (m, H-6), 5.15 (dd, *J* = 15.3, 8.0 Hz, H-22), 5.02 (dd, *J* = 15.3, 8.0 Hz, H-23), 3.28 (m, H-3), 0.90 (d, *J* = 6.5 Hz, CH_3-21), 0.83 (d, *J* = 6.6 Hz, CH_3-26), 0.84 (t, *J* = 7.0 Hz, CH_3-29), 0.81 (d, *J* = 6.5 Hz, CH_3-27), 0.80 (s, CH_3-19), 0.65 (s, CH_3-18). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ_C 140.9 (C-5), 138.4 (C-22), 129.4 (C-23), 121.7 (C-6), 71.9 (C-3), 57.0 (C-14), 56.0 (C-17), 51.3 (C-24), 50.3 (C-9),

42.5 (C-13), 42.2 (C-4), 40.5 (C-20), 39.7 (C-12), 37.5 (C-1), 36.6 (C-10), 32.2 (C-8), 32.0 (C-25), 31.9 (C-7), 31.8 (C-2), 28.9 (C-16), 25.4 (C-28), 24.4 (C-15), 21.2 (C-27), 21.1 (C-21), 21.0 (C-11), 19.4 (C-19), 19.0 (C-26), 12.4 (C-18), 12.0 (C-29). APCI-HR-MS *m/z* 413.3787 $[M+H]^+$ (calcd for $C_{29}H_{49}O$, 413.3783).

Ligulariaphytin A (4). ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 9.76 (s, H-10), 9.54 (s, H-5), 8.72 (s, H-20), 8.03 (dd, *J* = 12.0, 18.0 Hz, H-3'), 6.32 (brd, *J* = 18.4 Hz, H-3^{2}a), 6.17 (brd, *J* = 11.2 Hz, H-3^{2}b), 4.47 (q, *J* = 7.6 Hz, H-18), 4.09 (d, *J* = 9.2 Hz, H-17), 3.97 (m, CH_2-17^4), 3.90 (s, CH_3-12^1), 3.77 (s, CH_3-13^4), 3.74 (m, CH_2-8^1), 3.45 (s, CH_3-2^1), 3.27 (s, CH_3-7^1), 2.55 (m,H-17^{2}a), 2.45 (m,H-17^{1}a), 2.18 (m,H-17^{2}b), 1.87 (m,H-17^{1}b), 1.72 (t, *J*=7.6, CH_3-8^2), 1.62 (d, J = 7.6, CH_3-18^1), 1.07 (t, *J* = 7.2 Hz, CH_3-17^5). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ_C 173.5 (C-17^3), 171.3 (C-19), 171.1 (C-13^3), 166.4 (C-16), 161.2 (C-5), 155.9 (C-6), 150.2 (C-7), 145.7 (C-8), 141.6 (C-1), 138.9 (C-11), 136.7 (C-7), 136.2 (C3, C4), 134.6 (C-15), 131.7 (C-15), 131.6 (C-2), 129.1 (C-3^1), 122.9 (C-3^2), 111.3 (C-13), 104.3 (C-10), 102.1 (C-13^1), 100.6 (C-13^2), 99.8 (C-5), 94 (C-20), 60.6 (C-25), 54.4 (C-13^4), 53.8 (C-17), 50.3 (C-18), 32.3 (C-17^2), 31.4 (C-17^1), 22.4 (C-18^1), 19.7 (C-8^1), 17.8 (C-8^2), 14.2 (C-17^5), 12.3 (C-2^1, C-12^1), 11.5 (C-7^1). APCI-HR-MS *m/z* 653.2980 $[M+H]^+$ (calcd for $C_{37}H_{41}N_4O_7$, 653.2975).

Stigmasterol 3-O-D-glucoside (5). Colorless needles. M.P. 289-290 °C. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ_H 5.23 (brd *J* = 5.4 Hz, H-6), 5.14 (dd, *J* = 15.2, 8.0 Hz, H-22), 5.02 (dd, *J* = 15.3, 8.0 Hz, H-23), 4.78 (d, *J* = 7.5 Hz, H-1), 3.83 (m, H-3), 3.84-4.44 (m, Glc-H), 1.01 (s, CH_3-19), 0.90 (d, *J*= 6.2 Hz, CH_3-21), 0.83 (d, *J* = 6.6 Hz, CH_3-26), 0.82 (t, *J* = 7.0 Hz, CH_3-29), 0.80 (d, *J* = 6.5 Hz, CH_3-27), 0.66 (s, CH_3-18). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ_C 141.5 (C-5), 138.9 (C-22), 129.1 (C-23), 121.1 (C-6), 102.8 (C-1') 79.8 (C-3'), 76.7 (C-5'), 74.2 (C-2'), 70.6 (C-4'), 62.2 (C-6'), 57.0 (C-14), 56.1 (C-17), 52.1 (C-24), 50.8 (C-9), 43.9 (C-4), 43.1 (C-13), 40.5 (C-20), 39.9 (C-12), 37.8 (C-1), 36.9 (C-10), 32.9 (C-25), 32.8 (C-2), 31.9 (C-7), 31.7 (C-8), 28.9 (C-16), 25.6 (C-28), 24.5 (C-15), 21.9 (C-21, C-24), 21.7 (C-27), 21.5 (C-11), 19.5 (C-19), 19.1 (C-26), 12.6 (C-18), 12.1 (C-29). APCI-HR-MS *m/z* 575.4317 $[M+H]^+$ (calcd for $C_{35}H_{59}O_6$, 575.4312).

Alpha glucosidase inhibitory assay

The alpha glucosidase inhibitory assay was performed according to Chan and collaborators (2010), with modifications (Lopez *et al*., 2015). α-Glucosidase from baker's yeast purchased from Sigma Chemical Co. The inhibition was measured spectrophotometrically at pH 7.0 and 37 °C employing 2 mM p-nitrophenyl α-D-glucopyranoside (PNP-G) as a substrate and 32 mU/mL of enzyme, in 100 mM potassium phosphate buffer (enzyme stock). Acarbose was dissolved in phosphate buffer, and serial dilutions (in order to obtain the IC_{50}) were prepared and employed as positive control. The absorbance (A) of 4-nitrophenol released by the hydrolysis of PNP-G was measured at 400 nm by Synergy HT Bio Tek microplate spectrophotometer. A 20 µL of acarbose or plant extract solution was incubated for 7 min with 150 µL of enzyme stock at 37 °C. After incubating, 150 µL of substrate was added and further incubated for 20 min at 37 °C.

All assays are performed in 96-well microplates (Greiner bio-one 655101) in duplicate. The activity of samples was calculated as a percentage in comparison to a control (DMSO or MeOH instead of sample solution) according with the following equation:

$$\%Inhibition = \left({}^{(\Delta A_{control} - \Delta A_{sample})}/_{\Delta A_{control}} \right) \times 100\%$$

The concentration required to inhibit activity of the enzyme by 50 % (IC_{50}) was calculated by regression analysis (Lopez *et al*., 2015).

RESULTS AND DISCUSSION

The crude $CHCl_3$-MeOH (1:1) extract from *B. pyramidatum* was prepared and evaluated against germination and initial radical elongation of *A. hypochondriacus*, a specie frequently utilized in biological testing for phytotoxicity. Radical elongation of *A. hypochondriacus* seemed to be affected only for a high concentration of *B. pyramidatum* organic extract because at 1000 ppm exhibited 55.1 % of inhibition whereas the other two serial lower concentrations (100 ppm and 10 ppm) did not showed significant effect (Fig. 1). Subsequently, we carry out the primary fractionation and found that activity was found in the ethyl acetate fraction (60 % of inhibition at 1000 ppm). This result led the chemical study to the fraction of ethyl acetate in order to find the main phytotoxic compounds from this plant.

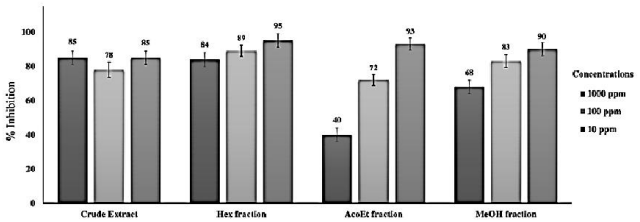

Figure 1. Phytotoxic activities of crude extract and primary fractions from *B. pyramidatum*

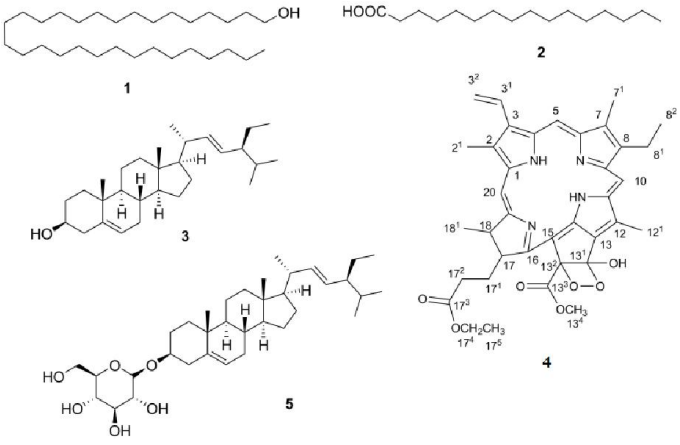

Figure 2. Structures of compounds isolated from ethyl acetate fraction

Extensive chromatography of the active fraction led to the isolation of five known compounds, identified as 1-triacontanol (**1**), palmitic acid (**2**), stigmasterol (**3**), ligulariaphytin A (**4**), and stigmasterol-D-glycoside (**5**). All compounds (Fig. 2) were

identified by analysis of the spectroscopic data including HRESITOF-MS, NMR, and the structures were confirmed by comparing with reported data (Kang and Cho, 1989; Allerhand and Maple, 1988; Kojima *et al*., 1990; Li *et al*., 2012). The spectroscopic characteristics of the isolated compounds were the following. The ^{1}H NMR spectrum of compound 1 showed signals for a methylene adjacent to an OH group [3.64 (2H, t)], a methylene [1.56 (2H, m)], a multiplets for 25 methyl's (~1.30) and a terminal methyl group [0.88 (3H, t)]. The mass spectral data of compound **1** gave a molecular formula $C_{30}H_{62}O$ (pseudo molecular ion at 439.4872 $[M + H]^{+}$), which was supported by the ^{13}C NMR spectral data.

Analysis of all NMR data together with mass spectrometry information allowed us to detect that this compound belongs to saturated fat family containing a terminal hydroxyl group, and specifically it was 1-triacontanol (Kang and Cho, 1989). 1-triacontanol (1-TRA) has been reported as one of the natural components of epicuticular waxes of several plants, where it has showed a positive role in enhancing growth, increasing plant dry weight, protein and chlorophyll contents, photosynthesis, nitrogen fixation, enzymatic activities, and levels of free amino acids, and reducing sugars (Ries *et al*., 1977; Uchiyama and Ogasawara, 1981; Naeem *et al*., 2011).

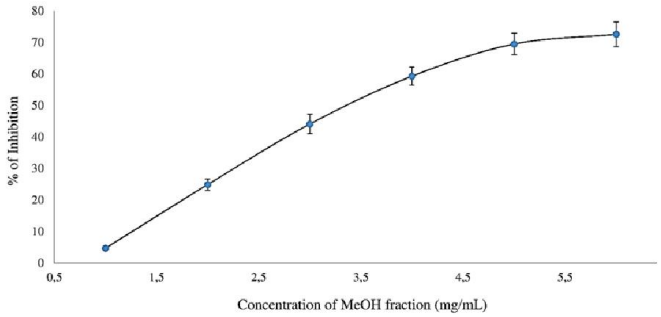

Figure 3. Percent of inhibition of alpha glucosidase at different concentrations MeOH fraction

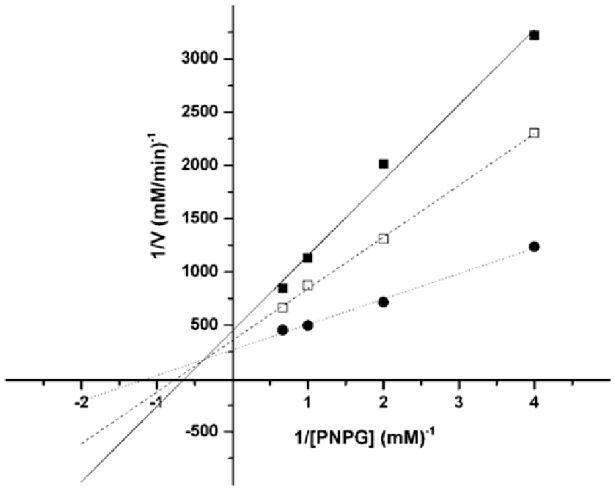

Figure 4. Lineweaver–Burk plots of alpha glucosidase inhibition at different concentrations of substrate and MeOH fraction

^{1}H NMR spectrum for compound **2** showed a signal at δ_H 0.84 which indicates a terminal methyl group, broad multiplets at δ_H 1.2-1.4 attributable to a several methylene groups and a signal at δ_H 2.17 belong to methylene protons adjacent to carboxylic group. All NMR evidences coupled with the mass obtained by APCIHR-MS were consistent with reported data for palmitic acid (Allerhand and Maple, 1988). For compound **3**, ^{1}H NMR spectra showed the presence of two methyl singlets at δ_H 0.71, and 1.03; three methyl doublets at δ_H 0.80, 0.82, and 0.91; and a methyl triplet at δ_H 0.83. Moreover, the presence of three proton signals at δ_H 4.98, 5.14, and 5.31 suggested the presence of a trisubstituted and a disubstituted olefinic bonds. This data supported the presence of sterol skeleton having a hydroxyl group at C-3 position with two double bonds and six methyl groups which was supported by the COSY and HMBC correlations. Thus, the structure of **3** was assigned as stigmasterol (Kojima *et al*., 1990).

For compound **4**, ^{1}H NMR spectrum displayed characteristic signals for a porphyrin core, as denoted the signal of two methyl groups directly attached to a conjugated ring system [δ_H 3.39 (s, H_3-2^1) and 3.68 (s, H_3-12^1), a methyl group [δ_H 1.82 (d, J= 7.2 Hz, H_3-18^1)], a vinyl group [δ_H 7.95 (dd, J= 18.0, 12.0 Hz, H-3^1), 6.25 (d, J=18.0 Hz, H-3^2) and 6.15 (d, J= 12.0 Hz, H-3^2)] and three olefinic singlets at δ 9.47 (H-10), 9.31 (H-5) and 8.56 (H-20). It also can be appreciated signals for one methyl ester group [δ_H 3.90 (s, H-13^4)], one ethyl group [δ_H 3.57 (q, J=7.2 Hz, 8^1) and 1.67 (t, J= 7.2 Hz, 8^2)], one methyl propionate group [3.59 (s, H-17^4)], and two amino groups [δ_H =1.65]. The ^{13}C- and DEPT (135 and 90) NMR revealed the presence of seven methyl, four methylene, seven methine and eighteen quaternary carbons. Every proton was assigned to its corresponding carbon by using of HSQC experiment, and protonated carbons and quaternary carbons is confirmed by an HMBC data. All spectral properties of compound **4** were comparable with those reported for ligulariaphytin A (Li *et al*., 2012).

Finally, compound **5** exhibited great similarities with spectroscopic data of compound **3**, only it presents additional signals in the region of δ_H 2.03-3.31 which was assigned as the five protons of sugar moiety. On the basis of all spectral data compound **5** was identified and established as stigmasterol-D-glycoside (Kojima *et al*., 1990).

As noted in the experimental section, the obtained amounts of relatively pure compounds were less than those required for biological evaluation (10 mg). For this reason, we could not perform this evaluation. In a survey of the literature, we found that none of compounds have shown phytotoxic activity. Most likely, the phytotoxic effect of ethyl acetate fraction may be related to the concentration of compounds present in this fraction. Moreover, triacontanol has also been found in some plants that have proved phytotoxic activity (Ahmed *et al*., 2004; Chung *et al*., 2005; Silva *et al*., 2015). In some of those studies the active components could not be identifying, and so researchers hypothesized that triacontanol, at high concentrations, has similar phytotoxic effect to those observed with auxins (Sitinjak and Pandiangan, 2014, Silva *et al*., 2015). Finally, we proceeded to evaluate the hypoglycemic activity of primary fractions by using an enzymatic assay (alpha glucosidase) previously implemented in our laboratory. Methanol fraction inhibited alpha glucosidase enzyme in a

concentration-dependent manner (Fig. 3) with IC_{50} value of 3.47 mg/mL. In order to obtain further evidence of the nature of the interaction of methanol fraction with alpha glucosidase kinetic analyses were carried out. Lineweaver–Burk plots (Lopez *et al*., 2015) were constructed using different concentrations of substrate and methanol fraction. The results in figure 4 indicated that methanol fraction showed typical reversible competitive plots, with series of lines having the same y-intercept as the enzyme without inhibitors. These results suggested that methanol fraction bind to alpha glucosidase or to the substrate-enzyme complex. Acarbose also behaved as competitive inhibitor (Lopez *et al*., 2015). This result show that methanol fraction is a competitive inhibitor of the alpha glucosidase enzyme.

Conclusions

The phytochemical study of the ethyl acetate fraction from *B. pyramidatum* leaves led to the isolation of five compounds. Even thought metabolites isolated have been reported from other plants; to our knowledge, there are no reports of their presence in *B. pyramidatum*. The phytotoxic activity AcOEt fraction may be related to the presence of triacontanol. Triacontanol stimulates plant growth at very low concentrations, but it can have an inhibitory effect at higher concentrations, such as those reported for auxin. In general terms, phytotoxic activity only was detected at high concentrations of the extract and fractions tested, so it is possible to predict that *B. pyramidatum* mainly affects other commercial plants (as coffee) due to its ability to capture water and nutrients available in the area culture. Finally, we also evaluated the effect of the primary fractions of the enzyme alpha glucosidase, with the methanol fraction showing a good inhibitory effect. Therefore this plant might also be an interesting alternative for reducing levels of blood sugar of people affected by Diabetes Mellitus.

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