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Evaluation of phytotoxic, cytotoxic and antiparasitic *in vitro* activities of *Borreria verticillata*, a weed of Panamanian coffee crops.

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In recent years, there have been significant changes in weed populations in different agricultural production systems. Coffee production is economically important in the Republic of Panama, and the specie *Borreria verticillata* affects a significant portion of this crop. Weeds may directly affect the yields of economically important plants through two main ways: by producing allelochemicals which inhibit plant growth or by competition for nutrients and water availability in the soil. *Borreria verticillata* was selected to evaluate its phytotoxic activity by which this weed affects the coffee crops. In addition, we carried out antiparasitic evaluations for determining the activity of *Borreria verticillata* extract against three human parasites: *Leishmania donovani*, *Trypanosoma cruzi* and *Plasmodium falciparum*. The experimental results revealed that the extract prepared using the aerial parts of *Borreria verticillata* did not show significant phytotoxic and cytotoxic effects. On the other hand, the antiparasitic evaluations showed that the extract possessed only moderate activities against *Plasmodium falciparum.* Finally, we proceeded to identify the major chemical components of this extract and we obtained three known compounds: scualene (**1**), epoxy-scualene (**2**) and borrecapine (**3**).

Key words: *B. verticillata*, coffee weed, Phytotoxicity, antiparasitic activity, metabolites.

Coffee (*Coffea arabica* L.) production is one of the major activities from the province of Chiriquí, in western Panama. Each year, the coffee crops are broadly affected by various factors, one of them is weeds. Weeds cause great economic losses for producers of coffee, because they interfere with the growth and development of the coffee plants, which influences the yield of the fruit. In coffee plantations, weeds also compete with the coffee plants for soil macronutrients. Weeds can also limit coffee yield directly by producing allelopathic chemicals, by competition for space in the area of cultivation and/or, indirectly, by containing pests and pathogens (De Graaff, 1986, Radosevic et al. 1997).

Weed competition could be minimized by eliminating or reducing their presence, which ultimately could increase the coffee yields. However, not all weeds compete directly with coffee plants. For that reason, it is important that farmers can distinguish between benign and malign weeds (Altieri, 1995).

In recent years, there have been significant changes in weed populations in different agricultural production systems (Ngouajio and McGiffen, 2002). *Borreria verticillata*, one of the most problematic and aggressive weed, is considered a major problem because it is resistant to burning land.

Borreria verticillata is a plant, which grows in wild form mostly in the tropical and subtropical areas of America, Africa, Asia and Australia during the whole year and it has been used medicinally by different ethnic groups in these areas. In Brazil, *B. verticillata* is widely used in traditional folk medicine as antipyretic, analgesic, emetic, antidiarrheal, and to treat erysipelas and hemorrhoids. Previous studies with *B. verticillata* have shown the presence of terpenoids, indole alkaloids and iridoid type compounds (Conservaand Ferreira, 2012; Vieira et al. 1999; Moreira et al. 2010; Lorenzi and Matos, 2002).

The purpose of this research was to prepare an organic extract using the aerial parts of this plant to evaluate their phytotoxic activity and to find out if this weed affects the coffee crops by producing allelochemicals or by competition for the nutrients and the water availability in the ground. Finally, we described the isolation of the major components of this organic extract and some of its biological properties.

MATERIALS AND METHODS

General Experimental Procedures.

NMR spectra were acquired on Jeol Eclipse 400 MHz spectrometer and referenced to residual solvent ^{1}H 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, and a normal phase silica gel column (PhenomenexSphereclone, 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.

Plant material

B. verticillata (Rubiaceae) was collected by December 2010 in a coffee-growing field of Santa Clara, Chiriqui, Panama. The plant was identified for comparison with a *B. verticillata* previously deposited in the herbarium of the University of Panama.

Extraction and isolation of borrecapine, scualeno and 2,3-epoxyscualene from *B. verticillata* extract.

Air-dried aerial plant material (37 g) was extracted by maceration at room temperature with a mixture of MeOH:$CHCl_3$ (1:1). The resulting extract was evaporated and the residue (7.03 g) was fractionated by column chromatography on silica gel (50 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, 179 fractions (50 mL each) were collected and combined according to their TLC profiles to yield eight primary fractions (FI to FVIII). Because of the low yield and high complexity of fractions we proceeded to work only with major fractions. Fraction FI (2.77 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 eigth secondary fractions (FI-A to FI-G). Fraction FI-A eluted with 100 % Hexane, was purified by normal phase HPLC (Sphereclone 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 1.8 mg of scualene (**1**). From fraction FI-B eluted with Hexane: EtOAc (8:2), purified by normal phase HPLC (Sphereclone 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 0.9 mg of epoxy-scualene (**2**). Fraction F6 (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 secondary fractions (F6-A to F6-F). Fraction FI-C eluted with 100 % EtOAc, was purified by normal phase HPLC (Sphereclone silica 250 x 10 mm column, isocratic elution of 30% hexanes: 70% EtOAc, UV detector at 254 nm, flow of 1 mL/min) to afford 1.0 mg of borrecapine (**3**).

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). 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 four

concentrations (1, 10, 100 and 1000 µg/ml) and 2,4-D was used as the positive control.

Brine shrimp lethality assay

In vitro lethality assay of *Artemia salina* was used for detecting cell toxicity (Meyer et al. 1982). Brine shrimp eggs were placed in sea water (3.8% w/v sea salt in distilled water) and incubated at 28 ^{o}C. Eggs were hatched within 48h providing large number of larvae (nauplii). Ten nauplii were placed in each vial containing 5 ml of sea water and increasing concentrations of *B. verticillata* extract (10, 50, 100, 500 and 1000 ppm). Each concentration was assessed by triplicate. The percentage lethality was determined by comparing the mean surviving larvae of the test and control tubes. LC values were obtained from the best-fit line plotted concentration verses percentage lethality. Potassium dichromate was used as a positive control in the bioassay while the negative control was vials that contain only the solvent used for the preparation of the test samples.

Antiparasitic bioassays

All bioassays were performed in duplicate, testing at 10, 2, 0.4, 0.08, and 0.016 µg/mL. The antiplasmodial activity was evaluated using a fluorometric method based on the detection of parasite DNA with the fluorochrome PicoGreen using a chloroquine-resistant strain (Indochina W2) of *P. falciparum* (Corbett et al. 2004; Martinez-Luis et al. 2011).The parasites were maintained *in vitro* by a modification of the method of Trager and Jensen (1976). We used chloroquine as a positive control (IC_{50} = 80-100 nM). Chagas bioassays were performed following the protocol of Buckner et al. (1996) and using nifurtimox as a positive control (IC_{50}= 3-5 µg/mL). Leishmaniasis bioassays were performed using a method based on parasite DNA fluorescence (Calderon et al. 2004; Martinez-Luis et al. 2011). In this latter assay, amphotericin-B was used as the positive control and had an IC value of 80 ng/mL.

RESULTS AND DISCUSSION

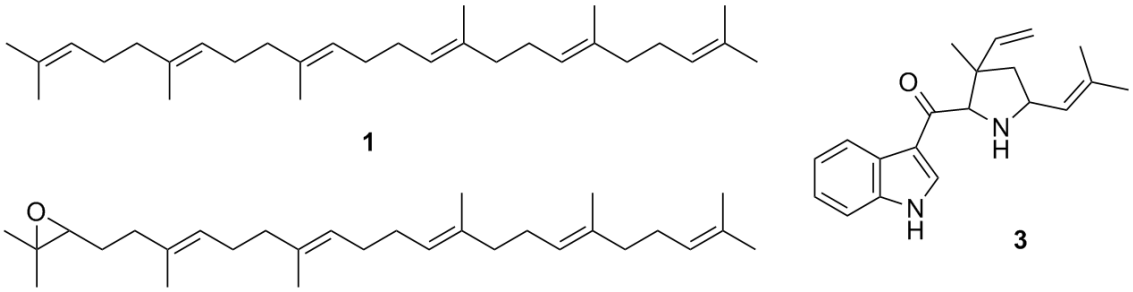
A crude $CHCl_3$-MeOH (1:1) extract was prepared using aerial parts of *B. verticillata* and we evaluated its effect on radicle growth of *Amaranthus hypochondriacus*. We observed that this crude extract possessed a weak phytotoxic effect, because at the highest concentration tested (1000 ppm) presented only inhibited the 32.5 percent of *A. hypochondriacus* growth a well know weed for detecting phytotoxic activity. Next, we evaluated the toxicity of the extract against the crustaceous *Artemia salina* to detect indirectly the cytotoxicity of the plant and we detected that extract had a weak toxicity (IC_{50} = 672 ppm). The level of mortality was found to be directly proportional to the concentration of the extract added. Then we proceeded to test the activity of the extract of *B. verticillata* against *in vitro* promastigotes form of *Leishmania donovani*, epimastigotes of *Tripanosoma cruzi* and *Plasmodium falciparum* trophozoites, which are routine assays in our institute, with the aim to detect if *B. verticillata* extract possessed antiparasitic activity. The results showed that the extract had only activity against *P. falciparum* presenting 65 % growth inhibition at maximum concentration tested (10 µg/mL), and was completely inactive against *T. cruzi* and *L. donovani*.

Finally, we proceeded to identify the major chemical components of *B. verticillata*. Extensive chromatography of the extract resulted in the isolation of the three known compounds (Figure 1), scualene (1), epoxy-scualene (2) and borrecapine (3). The chemical properties of these compounds, including HRESITOF-MS, NMR and optical rotations data, were identical to those previously reported in the literature (Pogliani et al.1994; Willett et al. 1967; Jossang et al. 1977). We also evaluated the antiparasitic activity of these compounds and the results showed that only compound 3 exhibited antiplasmodial activity against a chloroquine resistant strain of *P. falciparum* with IC value of 11.4 µM. It is not rare for indolic alkaloid borrecapine has shown activity against malaria, as there are several reports in literature of certain compounds with similar core that have shown activity against *P. falciparum* (Frederich et al. 2008).

CONCLUSIONS

Biological assessments performed with *B. verticillata* organic extract allowed us to detect that this plant posse a weak phytotoxic and cytotoxic activities. These results suggest that the presence of *B. verticillata* in the coffee field decreases the yields of this commercial plant by probable competition for nutrients and water available in the soil and not for an

Figure 1. The chemical structures of metabolites isolated from *B. verticillata.*



allelopathic effect induced by weed. On the other hand, we evaluate the antiparasitic properties of *B. verticillata* extract and its isolated compounds and we found that this plant possessed antiplasmodial activity and the alkaloid borrecapine is one of the active component. To our knowledge, this is the first report of the antimalarial activity of *B. verticillata* and this constitutes the first report of a biological activity of the known natural alkaloid borrecapine.

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