Journal of Entomology

ISSN 1812-5670

Academic Journals Inc.

www.academicjournals.com

Journal of Entomology 9 (2): 115-122, 2012
ISSN 1812-5670 / DOI: 10.3923/je.2012.115.122
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Effects of Organic Extracts of *Bursera copallifera* and *B. lancifolia* Leaves in the Development of *Spodoptera frugiperda*

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ABSTRACT

The genus *Bursera* has worldwide presence, some of its representative species are distributed in México, where are locally used as insecticides. Phytochemical studies of *Bursera* species have mentioned the presence of terpenoids, flavonoids and lignans. The plants extracts have been shown to possess a range of biological activities. Within the search of natural products with activity on insects, organic extracts (hexane, ethyl acetate and methanol) of *Bursera copallifera* and *B. lancifolia* leaves were tested for its effect on *Spodoptera frugiperda* development and acetylcholinesterase inhibitory activity. The extracts were analyzed by planar chromatography using reagents (anisaldehyde and natural products reagent) to detect specific groups of compounds. Neonate larvae of *S. frugiperda* were fed with insect diet mixed with extracts in a concentration of 1000 ppm. After seven days of the initial dosing, surviving larvae were weighed and its length measured. Administration of the hexane, ethyl acetate and methanol extracts from both species were correspondent with reduced larval weight and size. Insects treated with *B. copallifera* extracts were the most affected (73±0.005%, $p<0.05$ and 35±0.04% for weight and size reductions in ethyl acetate treatment). Acetylcholinesterase of *S. frugiperda* was inhibited i*n vitro* to similar extent by methanol (IC_{50} 367 μg mL^{-1}) and ethyl acetate extracts (IC_{50} 397 μg mL^{-1}) from *B. copallifera* and *B. lancifolia*, respectively. Presence of terpenoids and flavonoids were confirmed after planar chromatography analysis. Results indicate that the extracts reduced larval growth and suggest that detected constituents may be partially responsible for the biological activity observed. Further studies on the physiological response when administered to insects and chemical characterization of the extracts are recommended.

Key words: Acetylcholinesterase inhibition, *Bursera*, *Spodoptera frugiperda*, copal, flavonoids, terpenoids

INTRODUCTION

The plant family Burseraceae consists of approximately twenty genera and six hundred species that are widely distributed at low elevations in subtropical regions (Becerra and Venable, 1999).

In México, one of the most representative genus is *Bursera*, locally known as cuajiote or copal with over a hundred species present from the south of the United States of America to Peru (Becerra and Venable, 1999).

Phytochemical studies of the genus *Bursera* have reported the presence of lignans, bilignans, flavonoids, flavonoid glycosides, steroids, short chain aliphatic alkanes, acetates, alcohols, ketones and terpenoids, the latter mostly monoterpenes while diterpenes and triterpenes occur at lesser extent (Alonso-Castro *et al*., 2011; Evans *et al*., 2000; Evans and Becerra, 2006; Hernández-Hernández *et al*., 2005; Peraza-Sánchez *et al*., 1995; Souza *et al*., 1989; Zuñiga *et al*., 2005). Presence of resins and essential oils is characteristic of the genus and many studies have focused on the chemical characterization of bark or volatiles, however, little is known about the compounds present in leaves and its biological activity.

Bursera extracts and compounds have been investigated for a wide range of biological activities for example antioxidant, cytotoxic, nematicidal, bactericidal and acaricidal (Ara *et al*., 2009; López-Aroche *et al*., 2008; Rosado-Aguilar *et al*., 2010; Salinas Sánchez *et al*., 2009). The species *B. copallifera*, as well as others present in México, is traditionally used to kill insects (Aldana Llanos *et al*., 2010; Evans and Becerra, 2006; Maldini *et al*., 2009).

Plant extracts can exhibit different modes of action when administered to insects, for example amino acid arrest, ecdysis disruption, inhibition of gut proteases and inhibition of acetylcholinesterase (AChE). These effects are attributed to the secondary metabolites synthesized by the plants. Recent studies have shown the AChE inhibitory activity of flavonoids such as quercetin, rutin, macluraxanthone, quercitrine, tiliroside and kaempferol (Jung and Park, 2007; Khan *et al*., 2009). Other references have shown that terpenic compounds, especially monoterpenes have also AChE inhibitory capacity (López and Pascual-Villalobos, 2010).

Within the search for natural products that have effects on insects, extracts from leaves of *B. copallifera* and *B. lancifolia* were investigated to assess its effect in the development of *Spodoptera frugiperda*. Larval weight and size were the evaluated variables. Planar chromatographic analysis of the extracts was performed to evaluate its complexity and the nature of its constituents. Given the previous reports of the found chemical group's activity, the extracts were also tested for inhibitory activity of AChE.

MATERIALS AND METHODS

Reagents and solvents: Analytical grade solvents obtained from J.T. Baker, México, were used in plant extractions. Acetylcholine iodide (AchI), 5,5'-dithio-bis (2-nitrobenzoic acid) (DNTB), p-anisaldehyde, 2-aminoethyl diphenylborinate (NPR) polyethyleneglycol 4000 (PEG 4000), quercetin, kaempferol and rutin were purchased from Sigma-Aldrich Inc, USA.

Plant material: Samples of *B. copallifera* and *B. lancifolia* were collected in the state of Guerrero, México in September 2009. Voucher specimens were deposited at the Herbarium of the Benemérita Universidad Autónoma de Puebla: *B. copallifera* HUAP11770 and *B. lancifolia* HUAP11771.

Preparation of plant extracts: The air-dried and powdered leaves of *B. copallifera* and *B. lancifolia* were subsequently extracted three times with hexane, ethyl acetate and methanol; maceration procedure was carried out at room temperature (22°C). Solvent was separated from the plant material by gravity filtration and then evaporated under reduced pressure using a rotary evaporator.

Insect rearing: Wild *S. frugiperda* larvae were collected in Yautepec, Morelos, México. There after a colony was reared continuously, fed with artificial diet (Mihm, 1989) and kept in a constant climate chamber (KBW 240, Binder GmbH, Germany) with a photoperiod of 16:8 h (L:D), 27°C±2°C and 60% relative humidity. Adults were feed with a sugar solution (Mihm, 1989).

Bioassay: The bioassays were carried out in plastic glasses (Bio-Serv N°9051), the experimental unit was one glass, each treatment and control (solvent alone) consisted of 50 repetitions. A know quantity of the crude extract of *B. copallifera* or *B. lancifolia* were incorporated into the diet to give a final concentration of 1000 ppm. The glasses were filled with 8 mL of freshly prepared diet mixed with the extract or solvent alone and then allowed to congeal at room temperature. Once the diet was cool and the solvent evaporated, two neonate larvae were placed in each well. On the third day only one larva remained. The glasses were kept for 7 days in a growth chamber (KBW 240, Binder GmbH, Germany) at constant temperature and relative humidity (27°C±2°C and 60%, respectively) with a 16:8 h (L:D) photoperiod. During this lapse the glasses were monitored, size and weight of each organism were determined at the seventh day.

Acetylcholinesterase assay: AchE crude extracts were prepared the same day of enzyme assay in a Dounce homogenizer using head, thorax and abdomen from 3 non-treated adults of *S. frugiperda* in 4 mL of 0.1 M phosphate buffer (pH 8). The homogenate was filtered through a fine nylon mesh and kept cold on ice. AchE assay was carried out following a modified Ellman *et al.* (1961) method. The assay media contained 0.1 M phosphate buffer, 0.05% Triton X 100, 0.5 mM AChI, 0.3 mM DNTB and AChE crude extract in a final volume of 250 µL. The reaction was initiated by the addition of AChI 75 mM and then monitored after 5 min at 412 nm with a Spectramax 190 microplate reader (Molecular Devices, USA). The volume of crude extract in the assay was adjusted to have an activity of 0.05 absorbance units per minute. The tested leaf extracts were dissolved in ethanol, diluted with phosphate buffer pH 8 with Triton X100 and added to the assay media to give final concentrations between 2 and 1000 µg mL^{-1}. IC_{50}'s were calculated by the graphic method.

Planar chromatographic analysis: Chromatography was performed on 10×5 cm glass plates precoated with 0.25 mM silica gel (60 F_{254}, Merck, Germany), oven activated at 110°C for 1 h. A 10 µL aliquot of each extract (5 µg mL^{-1}) were applied by means of an automatic TLC sampler 4 (CAMAG, Switzerland). Rutin, quercetin and kaempferol were used as standards for methanol and ethyl acetate extracts. Plates were developed for 9 cm in glass chambers (CAMAG, Switzerland). For hexane extracts the mobile phase used was hexane-ethyl acetate, 75:25 (v/v) and for ethyl acetate extracts the mobile phase was hexane-ethyl acetate-methanol, 12.5:75:12.5 (v/v), visualization was achieved by spraying with anisaldehyde-sulfuric acid reagent and subsequent heating (115°C) (Wagner and Bladt, 1996). The mobile phase used for plates that included flavonoid standards was toluene-acetone-formic acid 6:2:2 (v/v). Visualization was achieved with NP-PEG reagent (Wagner and Bladt, 1996). Images of the chromatograms under VIS and UV_{365} light were obtained with a digital camera.

Statistical analysis: Size and weight data were analyzed by one-way analysis of variance (ANOVA) and a post hoc Tukey test using SIGMA STAT® 3.5 (Systat Software Inc, Chicago, USA). Values of $p<0.05$ were considered significant. Results are expressed as Mean±SEM.

RESULTS AND DISCUSSION

Size and weight

***Bursera copallifera*:** Results of size and weight are shown in Fig. 1. All extracts tested (hexane, ethyl acetate and methanol) caused size and weight reduction ($p<0.05$). Size values ranged from 19% for methanol to 35% for ethyl acetate treatments. Weight was the most affected in all treatments with reductions values ranged 55% from methanol to 73% for ethyl acetate treatments.

***Bursera lancifolia*:** Statistically significant decrease in weight was observed after administration of the extracts. Weight reduction percentages were 22% for hexane extract, 39% for ethyl acetate and 32% for methanol. Significant size reduction (15%) was observed only in ethyl acetate and methanol treatments (Fig. 2).

Inhibition of acetylcholinesterase: Methanol and ethyl acetate extracts of both *Bursera* species showed inhibitory activity of AchE (Table 1). The IC_{50}'s were within the range of 350 to

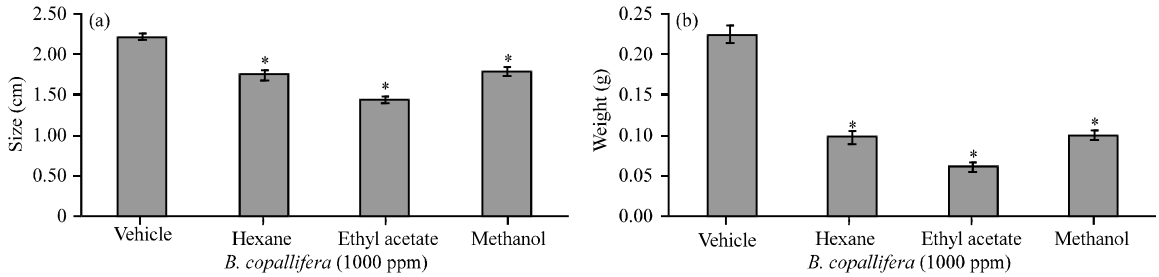

Fig. 1: Effect of hexane, ethyl acetate and methanol extracts from leaves of *B. copallifera* on the larval (a) size and (b) weight of *S. frugiperda*. Each column represents the mean, error bars indicate ±SEM initial n = 50, *$p<0.05$

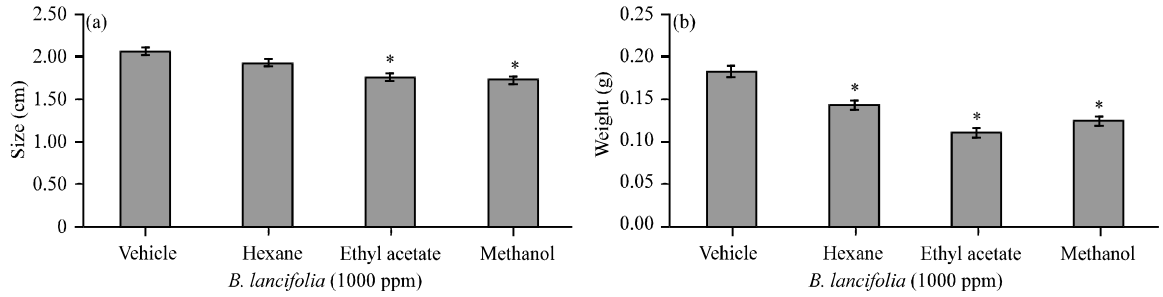

Fig. 2: Effect of hexane, ethyl acetate and methanol extracts from leaves of *B. lancifolia* on the larval (a) size and (b) weight of *S. frugiperda*. Each column represents the mean, error bars indicate ±S.E.M., initial n = 50, *$p<0.05$

Table 1: Acetylcholinesterase inhibiting activity of leaf extracts from *B. lancifolia* and *B. copallifera*

	Extracts IC_{50} (μg mL^{-1})		
Species	Hexane	Ethyl acetate	Methanol
B. lancifolia	----	397	707
B. copallifera	----	553	367

IC_{50} values for AChE for each treatment

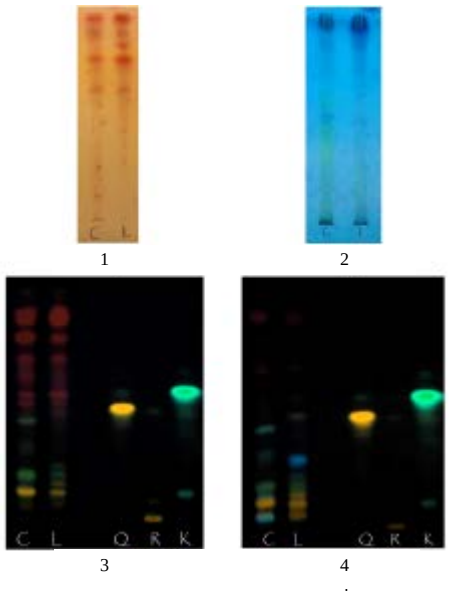

Fig. 3: Chromatograms obtained from the extracts of *B. copallifera* (C) and *B. lancifolia* (L). 1: Hexane extracts after derivatization with anisaldehyde, VIS. 2: Ethyl acetate extracts after derivatization with anisaldehyde, VIS. 3: Ethyl acetate extracts and flavonoid standards after derivatization with NP-PEG reagent, UV_{365}. 4: Methanol extracts and flavonoid standards after derivatization with NP-PEG reagent, UV_{365}. Quercetin (Q), rutin (R) and kaempferol (K)

710 μg mL^{-1}. Methanolic extract from *B. lancifolia* had the highest IC_{50} value while the same solvent extract from *B. copallifera* had the lowest. Hexanic extracts were tested up to 1000 μg mL^{-1} but did not showed activity.

Chromatographic analysis: The technique exposed the presence of terpenes in the hexane and ethyl acetate leaf extracts from both species. Profiles were similar in terms of spot number but there were variations in spot intensity which suggests a difference in the components concentration of said extracts (Fig. 3).

Flavonoids were detected in ethyl acetate and methanol extracts. Profiles of the ethyl acetate extracts from both species were similar. The methanol extracts had different flavonoid pattern according to the observed color. Quercetin and kaempferol presence was observed in methanol extracts, whereas in ethyl acetate extracts only quercetin was observed.

The results showed a significant size and weight reduction of *S. frugiperda* fed on diet containing the leaf extracts of both studied species, values of the evaluated growth parameters for ethyl acetate extract treatments were lower than that of the control larvae. Similar results have been reported for other plant genera and *Spodoptera* species (Sujatha *et al.*, 2010; Vera Curzio *et al.*, 2009) and are attributed to the secondary metabolites contained in the plant extracts. In the present work flavonoid and terpenoid compounds that were detected by TLC could be partly involved in the insect response observed. Previous reports have suggested that flavonoids and its effects play an important role in the feeding behavior of insects (Negi *et al.*, 2011). For

example, when rutin is ingested, the glycosidic bond is hidrolized, liberating the aglycone quercetin which can inhibit mithocondrial ATPase. Furthermore, rutin and quercetin have been related with reduced growth and low survival rate of *Ostrinia nubilalis* (Simmonds, 2001). Regarding the effects of terpenoid compounds, studies with diverse species from different lepidopteran families show feeding inhibition, as well as growth and developmental reduction (Isman, 1994, 2002). In the performed experiment these compounds could be modulating the feeding behaviour of the larvae which, by means of its sensory receptors can respond to a range of fagostimulants and deterrents (Simmonds, 2001).

The AChE inhibitory activity found is consistent with previous reports of the effect of flavonoids like quercetin, rutin and kaempferol (Khan *et al*., 2009; Bangou *et al*., 2011; Benamar *et al*., 2010; Ahmad *et al*., 2003).

It is advisable to follow this study with a systematic investigation focused on the fractionation of extracts and the characterization of the previously detected compounds, as well as tests for evaluation of other activities involved in digestion such as gut enzymes inhibition.

CONCLUSION

Planar chromatography evaluation indicated presence of terpenoids in the hexane and ethyl acetate extracts and flavonoids in the ethyl acetate and methanol extracts. These compounds may be responsible to some extent of the AChE inhibitory activity. The insect test results suggests that *Bursera* extracts contains secondary metabolites acting on the insect development causing growth and size reduction. Present findings could partially support the traditional use of some *Bursera* species as insecticides.

ACKNOWLEDGMENTS

We thank José Luis Contreras for the plant identification and Laura Lina for the assistance in the insect rearing.

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