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Isoquercitrin and *Baccharis spicata* (Lam.) Baillon (Asteraceae) inflorescence extract as potential biocontrol agents against coleopteran rice weevil

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Abstract

***Sitophilus oryzae* L. causes significant damage during storage of agricultural products. The use of synthetic pesticides renders effective results in the short term, but has also caused pest resistance and environmental pollution. Integrated pest management programs promote the development of new pesticides from natural resources, such as those derived from plants. The objective of this work was to evaluate the insecticide activity of *Baccharis spicata* (Lam.) Baillon extracts and their major phenolic compounds as reduced-risk alternative substances against coleopteran rice weevil. The inflorescence methanol extract was toxic against rice weevil. Three phenolic compounds were identified in this extract by HPLC UV/DAD. Isoquercitrin showed a good toxic activity againt *S. oryzae*. The inflorescence methanol extract and isoquercitrin inhibited the cellulase activity on cellulose agar plate assay method, with these results and additional studies it will be possible to elucidate a mechanism of action. These findings will provide opportunities and challenges to search new botanical insecticides to control pest in stored grains.**

Keywords: *Baccharis spicata*, botanical insecticides, insecticide activity, flavonoids, *Sitophilus oryzae*.

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Introduction

In recent years, the use of plants and their derivatives for pest control has been increasing (Kambou et al., 2008). The real benefits of botanical insecticides can be best seen in countries with small-scale farmers that generally use plants and plant derivatives for protection of stored products (Isman, 2008; Tapondjou et al., 2002). The rice weevil, *Sitophilus oryzae* L. (Coleoptera: Curculionidae), is one of the major pests of cereal grains and their products. This species causes heavy losses and damage to different types of food grains. Thus, several insecticides are used to control it. Although some of them provide a considerable level of residual efficacy, *S. oryzae* is resistant to organophosphates and pyrethroids (Athanassiou et al., 2005). This, combined with the consumers' demand for residue-free food, has motivated researchers to evaluate other reduced-risk alternative substances for use in stored grains. Pesticides derived from plants are organic, non-toxic to non-target organisms, and in addition, do not generate resistance. Most of these compounds are known to be rapidly degraded after application, leaving minimal residues (Kambou et al., 2008). Little is known about phenolic compounds as part of *Baccharis* L. inflorescences. Although they play an important role in attracting pollinating insects, studies on this subject have been focused on volatile compounds of some *Baccharis* species (Ferracini et al., 1996). Among phenolic compounds, flavones and flavonols proved to act as pollinator attractants, oviposition stimulants, feeding attractants, feeding deterrents and insecticides (Iwashina, 2003; Harborne, 2001;Harborne & Williams, 2000). Beninger and Abou-Zaid (1997) reported that rutin and isoquercitrin incorporated in an artificial diet inhibited the growth of gypsy moth larvae at the second instar stage. Some phenylpropanoids and related structures are of widespread occurrence in the plant kingdom and feeding inhibition has been observed in this type of compounds. In addition, phenylpropanoids, such as chlorogenic acid that occurs in leaf trichomes of tomato, reduce growth of early instars of the cotton bollworm, *Helicoverpa zea* (Boddie) (Harborne, 2001). The nature of the phenolic compounds acting as insecticides has not been fully revealed. However, there are reports on inhibition of digestive enzymes by phenolic compounds (Kawaguchi et al., 2007; Bell et al., 1965). Sami and Haider (2007) proposed the inhibition of cellulase activity by an inhibitor isolated from grapes (flavonoid/anthocyanidin). This inhibitor forms a complex with the enzyme due to structural similarity to the substrate (cellulose). The present study investigated the toxic activities of extracts and major phenolic compounds from *Baccharis spicata* (Lam.) Baillon against the rice weevil *S. oryzae*. For pest management strategies to be successful, it is necessary to understand the molecular basis of insect attack. Therefore the present work also tested the cellulase activity of *S. oryzae* and its inhibition by active extracts and compounds of *B. spicata*.

Materials and methods

Plant material and extracts: Aerial parts of *B. spicata* wild plants were collected and identified in Argentina, Santa Fe province, San Lorenzo department, Roldán city, at 32º54'04"S 60º54'26"O, 24/III/2014, by Rodriguez M.V. 1 (UNR) at the flowering period. The material was stored in the UNR

herbarium. The plants were shrubs established in the field and an area of 100 m^2 was delimitated for collection. The fresh aerial parts were air dried in the shade for 10 days at environmental temperature. The dried parts were powdered in a Fristzch Pulverisette 15 mill (Germany). Stems/leaves and inflorescences were separately extracted three times with dichloromethane (DCM) or methanol (MeOH) at room temperature, under stirring for 24 h. The extracts were filtered and concentrated under reduced pressure to give the corresponding dried residues. The yields (%) were: stem/leaf dichloromethane (SLD) and methanol (SLM) extracts, 5.43 and 9.23, respectively; inflorescence dichloromethane (ID) and methanol (IM) extracts, 6.60 and 22.16, respectively.

Chemicals: Chlorogenic acid, isoquercitrin and quercetin were purchased from Extrasynthese (France) and used as reference compounds in this study. All other chemicals and reagents used were of the highest commercially available purity.

Insects: *Sitophilus oryzae* L. insects used in this study were taken from laboratory rearing colonies initiated from infested grains since 2013. The cultures were maintained in the dark in growth chamber set at 27 ± 1 ºC and 70 ± 5% RH and a 12:12 light: dark photoperiod. Once a month insects were removed from the vials and introduced into 1 L glass jars, which was covered with gauze for sufficient aeration, with wheat infestation and pesticide free. The vials with remaining wheat were left in the incubators for an additional period and the emerged *S. oryzae* were used in bioassays. Thus, cohorts of individuals of the same age are obtained to reduce the error introduced by the different base mortality depending on the age of the adult individuals. To ensure insect death due to insecticide (not for age), mixed-sex adults of 14 days old were chosen for toxicity assays.

Toxicity Assays: Grains were treated with three extract concentrations (150.00; 400.00 and 800.00 mg/mL) and five concentrations of phenolic compounds (0.40, 0.80, 1.20, 2.00, 8.00 mg/mL) and were manually shaken to achieve a uniform distribution of the extract. Additional grains were treated with solvent or chlorpyrifos methyl ($0.75.10^{-3}$, $1.50.10^{-3}$, $3.00.10^{-3}$, $6.00.10^{-3}$, $9.00.10^{-3}$ mg/mL) as negative and positive controls, respectively. Fifty grams of samples were taken from treated and control groups and placed separately in glass jars (7cm diameter x 12 cm in height). Thirty adults were added in each vial and were left in a dark place at 30º C and 70% RH. Efficacy was recorded after 24, 48, 72, 96, 120, 144 and 168 h of exposure on treated samples or controls. The whole procedure was repeated 3 times. Abbott (1925) correction formula was applied to assess % mortality. Calculations using this method eliminate errors due to deaths in the control sample which were not caused by the insecticide. Probit analyses were carried out to determine the curves of lethal concentration (LC_{50} and LC_{90}) of the *B. spicata* methanolic inflorescence extract, isoquercitrin and clorpirifos metil. Curves with probability ($P<0.05$) of acceptance of the null hypothesis were accepted by the $\chi 2$ test. These curves of lethal concentrations and

their respective confidence intervals at 95% probability (CI_{95}) were obtained by using StatsDirect3 software.

HPLC UV/DAD: HPLC UV/DAD analyses were carried out on an Agilent Serie 1200 (Agilent Technologies, Germany) liquid chromatograph connected to a model VWD G1314B (DAD) diode array detector and controlled by EZchrome software. The separation of samples was performed using a Phenomenex® C18 column (Luna, 250 mm long×4.6 mm i.d.; 5 μm), preceded by a guard column (2.0 cm long×4.0 mm i.d.; 5 μm), containing the same stationary phase. The samples were automatically injected (20.0 μL). The flow rate was 1.2 mL min^{-1}. A linear gradient of 2% in water (solvent A) and 2% acetic acid in methanol (solvent B) was used. The optimized gradients employed were: 0–30 min, 15%–40% B in A; 30–40 min, 40%–75% B in A; 40–45 min, 75%–85% B in A and 45–50 min, 85%–100% B in A. The chromatogram was monitored at 325 and 254 nm, and UV spectra of individual peaks were recorded in the range of 200–400 nm.

Protein extraction from insects: Adults were frozen at -20ºC until required. Five grams were homogenized in cold 100 mL of 0.5 M Tris-HCl buffer 8.5 and centrifuged for 10 min at 10000g at 4ºC. The supernatant was removed and used as a source of enzymes.

Cellulase activity inhibition studies: Congo red plate assay method was performed according to Sami & Shakoori (2006). 200 μL *B. spicata* IM extract (10; 50; 200 mg/mL); isoquercitrin (1; 4 mg/mL) or solvent as positive control were mixed with 100 μL enzyme solution and were incubated at 50ºC for 15 min. The samples were loaded onto agar plates with CMC substrate (1% agar, 0.5% CMC, 0.5 M Tris-HCl buffer 7.1). The plates were stained with 0.1 % Congo red and washed with 0.1M NaCl. Enzyme activity appeared as a lighter area around the hole, against the red background.

Results and Discussion

Table 1 shows the results for dichloromethane and methanol extracts of aerial parts (stems/leaves and inflorescences) of *B. spicata* against rice weevil *S. oryzae*. SLD extract showed no insecticidal activity during the experimental period. The SLM extract presented low activity with 16.66±6.07 % of mortality at 168 h of treatment at the highest concentration tested. The ID extract exhibited a higher activity with 32.50±7.67 % at 168 h of treatment at the highest concentration tested. The IM extract showed insecticide activity with a concentration required to kill 50% of weevil populations of 280.82 mg/mL. HPLC UV/DAD was developed to analyze the methanol extracts. HPLC chromatograms of IM and SLM extracts were quite different; suggesting a distinct chemical composition, wich explains their different insecticide behavior. Flavonoids and phenylcarboxylic acids were detected in both methanol extracts. The IM extract showed quercetin, isoquercitrin and chlorogenic acid in its composition (Figure 1). Flavonoids, quercetin and isoquercitrin were not present in the SLM extract.

Table 1: LC_{50} and LC_{90} values of *Baccharis spicata* (Lam.) Baillon extracts and their flavonoid components against *Sitophilus oryzae* L.

Extract/component	N insects	LC_{50} (95%CI) (mg/mL)	LC_{90}(95%CI) (mg/mL)	β	χ^2	*P-value*
B. spicata stem/leaf DCM extract (SLD)	100	>1000.00[a]	-	-	-	-
B. spicata inflorescence DCM extract (ID)	100	>1000.00 [a]	-	-	-	-
B. spicata stem/leaf MeOH extract (SLM)	100	>1000.00 [a]	-	-	-	-
B. spicata Inflorescence MeOH extract (IM)	100	280.82 (244.39-316.25)	520.43 (465.91-602.56)	$5.34.10^{-3}$	0.39	0.52
Isoquercitrin	100	1.76 (1.41-2.88)	3.09 (2.29-5.83)	0.96	0.08	0.78
Quercetin	100	>10.00 [a]	-	-	-	-
Chlorpyrifos methyl (positive control)	100	$1.21.10^{-3}$ ($1.09.10^{-3}$- $1.34.10^{-3}$)	$2.05.10^{-3}$ ($1.85.10^{-3}$- $2.37.10^{-3}$)	$1.52.10^{3}$	0.54	0.76

LC= lethal concentration; CI= confidence interval; β= slope

[a]Lethal Concentration curves could not be traced due to the low toxicity of the extract/compound

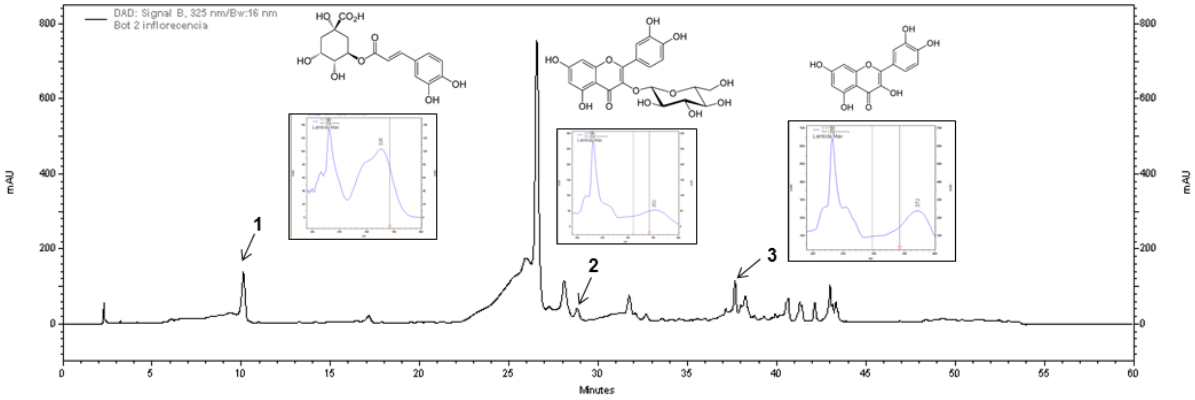

Figure 1: HPLC-UV/DAD chromatogram of *Baccharis spicata* (Lam.) Baillon inflorescence metanol extract. Reference compounds: 1, chlorogenic acid (Rt: 9.96 min); 2, isoquercitrin (Rt: 28.66 min); 3, quercetin (Rt: 37.64 min).

Phytochemical and biological investigations in about 120 *Baccharis* species were carried out (Gonzaga Verdi et al., 2005). Phytochemistry of *Baccharis* species is highlighted by the presence of flavonoids, mainly flavanones and flavones (Abad & Bermejo, 2007). 298 flavonoids were isolated from species of this genus. Among them, 24 were flavanones and 85 were flavones (Gonzaga Verdi et al., 2005). However, only a few chemical and biological investigations have been carried out on the species *B. spicata* (Vieira et al., 2011; Oliveira et al., 2004). Oliveira et al. (2004) reported the presence of phenolic derivatives in ethanol and aqueous extracts of aerial parts of *B. spicata.* Here, a chemical profile analysis of the highest activity extract allowed the detection of two flavonoids and one phenylcarboxylic acid. The insecticide activity of quercetin and isoquercitrin was tested against *S. oryzae* with the aim of finding the components of the extract that display this activity. Toxic activity was only observed for flavonol glycoside isoquercitrin at all concentrations tested with a LC_{50} of 1.76 mg/mL. Nenaah (2013) reported 48.7 % of mortality of *S. oryzae* at 96 h of exposure with flavonoid quercetin-3-O-rutinoside at a concentration of $5mL.cm^{-2}$ using impregnated filter paper bioassay. Nenaah (2013) also reported that the crude flavonoid fraction of *Calotropis procera* (Ait.) (Asclepiadaceae) showed

higher toxic activity than the flavonoids tested individually. The literature has reported that multiple compounds of plant extracts are usually responsible for biological effects (Nenaah 2011; 2013). Chan et al. (1978) reported that in leaves of *Gossypium* spp., quercetin acted as a growth inhibitor of *Pectinophora gossypiella* (Saunders), *Heliothis virescens* (Fabricius) and *H. zea* together with terpene aldehyde, cyclopropenoid fatty acids and condensed tannins. Although isoquercitrin may have potential for *S. oryzae* control, further investigation is needed in order to find other compounds which may act synergistically in inflorescence MeOH extract of *B. spicata*. Although plant phenolics have been regarded as one of the most important defenses against insects in various studies (Barbehenn et al., 1996; Barbehenn & Martin, 1994), their specific mode of action is not yet clearly known. Some authors suggest that the negative effects of phenol compounds are post-ingestive mechanisms wich occur in the midgut of insects (Simmonds, 2003; Barbehenn, 2002). Insects from order Coleoptera may produce their own cellulases in their midguts (Oppert et al., 2010; Watanabe & Tokuda, 2010). It has been reported that phenolic compounds inhibit digestive enzyme activities. Thus, in order to determine the presence of cellulase activity and its inhibition by IM extract and isoquercitrin, a Congo red plate assay was performed. The whole body extract of *S. oryzae* showed cellulase activity with a hydrolized lighter area against the red background (Figure 2a). The cellulase activity was completely inhibited by isoquercitrin at both concentrations tested (1.00 and 4.00 mg/mL) and by IM extract at concentrations of 50.00 and 200.00 mg/mL (Figure 2c-f). A minimum hydrolyzed area was observed when IM extract was used at 10mg/mL (Figure 2b).

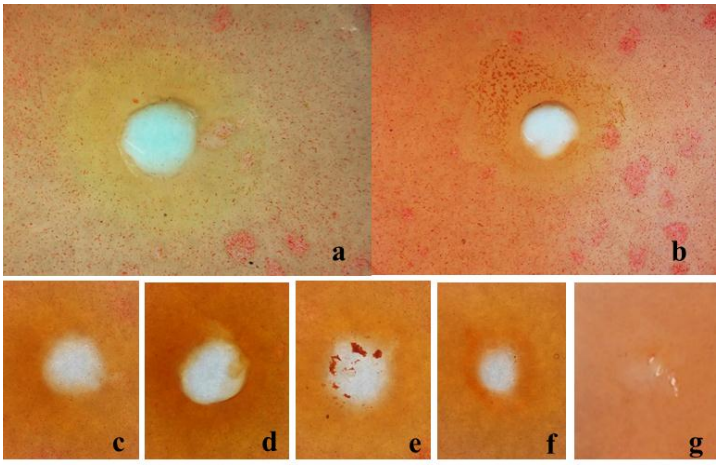

Figure 2: Congo red plate assay method. A-F, Enzymatic extract of *Sitophilus oryzae* L. with: a, solvent (positive control); b, 10 mg/mL of inflorescence methanol (IM) extract ; c, 50 mg/mL of IM extract; d, 200 mg/mL of IM extract; e, 1mg/mL of isoquercitrin; f, 4 mg/mL of isoquercitrin; g, negative control.

This study constitutes, to our knowledge, the first report showing the presence of cellulase activity in *S. oryzae.* A polygalacturonase (pectinase) has already been purified from *S. oryzae* and it seems likely that it is used as a

digestive enzyme due to the fact that cereal seeds contain pectin and cellulose (Shen et al., 1996). Sami and Shakoori (2008) reported the inhibition of a cellulase enzyme (endo-1,4-beta-D-glucanase) of *Aulacophora foveicollis* (Lucas) by a flavonoid compound. In the current study, the toxic activity of isoquercitrin present in *B. spicata* IM extract against *S. oryzae* was demonstrated, as well as the inhibition of cellulase activity of *S. oryzae* by the same flavonoid. The possibility of using digestive enzymes as targets of inhibitors will benefit from knowledge about insect digestion biochemistry. Post-ingestive toxic effects of isoquercitrin on *S. oryzae* could be attributed to their interference with digestive mechanisms; however, more research is needed in order to establish a possible mode of action as botanical insecticide. The results presented here introduce a new perspective on the use of *B. spicata* extract for future research in pest control of stored products. Although the toxic activity is lower when compared to synthetic insecticides, such as chlorpyrifos methyl (Table 1), this extract and its components pose no risk to human beings and other mammals, which is evidenced by the fact that the aerial parts are commonly used as a natural medicine for digestive problems in South Brazil, Paraguay, Uruguay and Argentina (Vieira et al., 2011; Oliveira et al., 2004).

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