Philipp Agric Scientist
Vol. 103 No. 3, 201-206
September 2020

ISSN 0031-7454

Isolation and Characterization of Bioactive Compounds from Seeds of Ipil-ipil (*Leucaena leucocephala*) and Its Antifeedant Activity Against the Third Instar of Common Cutworm (*Spodoptera litura*)

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Bioactive compounds from ipil-ipil (*Leucaena leucocephala*) seeds were isolated using solvent extraction and silica gel column chromatography. Antifeedant activities of the crude ethanolic extract, chromatographic fractions and crystalline isolate were tested on third instar larvae of the common cutworm (*Spodoptera litura*). Dual choice assay using 10 µg cm^{-2} applied on both adaxial and abaxial parts of castor (*Ricinus communis*) leaves showed that the crystalline isolate had the highest activity, with percentage relative feeding inhibition of 79.93 ± 1.00%. The very high melting point, together with data from chemical and spectroscopic analyses, suggest that the isolate is a saponin-containing complex with molar mass of 662 g mol^{-1} in the major component.

Key Words: antifeedant, biopesticide, common cutworm, Fabaceae, ipil-ipil, *Leucaena leucocephala*, saponin, *Spodoptera litura*

Abbreviations: FT-IR – Fourier-transform infrared, MS – mass spectrum, RFI – relative feeding inhibition, TLC – thin layer chromatography

INTRODUCTION

The common cutworm (*Spodoptera litura*) is an agricultural pest of many plant species including economically important crops such as cabbage, broccoli, cauliflower, radish and castor (Ahmad et al. 2007; Ghumare and Mukherjee 2003). Outbreaks have been reported in Southeast Asia in the past years due to field control failures that resulted in insecticide resistance (Kranthi et al. 2002; Ahmad et al. 2007; Gandhi et al. 2016). *S. litura* showed resistance to the synthetic insecticides cypermethrin, endosulfan and chlorpyriphos (Kranthi et al. 2002). Synthetic insecticides pose a threat to the environment, and have adverse effects on non-target organisms and on human health (Ghosh et al. 2012). As an alternative to synthetic insecticides, phytochemicals from botanical sources such as 3-O-rhamnoside, quercetin and d-onanitol were reported to have antifeedant activity against the third-instar larvae of *S. litura* (Negi et al. 2016).

The search for plant bioactive compounds continues in order to maximize the availability of their sources in different areas of the world where different kinds of plants can thrive, and to make the compounds available for commercial production. In a recent study, dalanghita (*Citrus reticulata* Blanco cv. Ladu) seeds were found to contain a limonoid that can inhibit feeding and growth of the Asian corn borer [*Ostrinia furnacalis* (Guenée)] larvae (Abrera et al. 2015). Ipil-ipil (*Leucaena leucocephala*) is one of the botanical sources that can also be explored. It is a 3–15 m tall shrub or tree that requires warm day temperatures (25-30°C) for optimum growth and has become widely grown in most tropical countries including the Philippines (CABI 2018). Hexane and methanolic extracts of ipil-ipil leaves were recently reported to have antifeedant activity against *S. litura* (Negi et al. 2016). Ipil-ipil seeds are also known to have secondary metabolites such as flavonoids, alkaloids, saponins, and tannins which are also present in other plants with antifeedant activity (Baskar et al. 2011; Paul

and Choudhury 2016). Saponins protect plants from phytopathogenic microorganisms, phytophagous mammals and insects (Chaieb 2010; Milgate and Roberts 1995). A crystalline mixture of saponins extracted from sunflower (*Tithonia diversifolia*) also rendered toxicity against the black armyworm (*Spodoptera exempta*) (Morallo -Rejesus et al. 1999, unpublished). This study conducted bioassay-guided isolation and characterization of a bioactive saponin component of ipil-ipil seeds with significant antifeedant activity against third-instar larvae of the common cutworm.

MATERIALS AND METHODS

Materials, Chemicals and Equipment

Analytical grade petroleum ether, ethyl acetate, methanol, and silica gel were purchased from Scharlau®, Sigma-aldrich® and Kieselgel®. Purified ethanol used for extraction was fractionally distilled from technical grade ethanol and dried using analytical grade anhydrous magnesium sulfate. Silica gel 60 F254 pre-coated thin layer chromatography (TLC) plates (20 cm× 20 cm), 0.20 mm thick, were obtained from Merck®.

Sample Collection

Dried pods of ipil-ipil (*L. leucocephala*) were gathered from trees in Victoria, Laguna in January 2017. The seeds were collected and oven-dried at 40°C (Badmus et al. 2019; Ramsumair et al. 2014) and then pulverized using a household blender. Dried ground seeds were stored in air-tight containers at room temperature for no more than 5 d.

Extraction and Isolation of Bioactive Compounds

The extraction process reported by Gill et al. (2012) was used, with modifications. Pulverized seeds (50 g) were defatted by soaking in 100 mL petroleum ether for 24 h, and shaken using a mechanical shaker. The defatted seed powder was collected through suction filtration and extracted with 100 mL ethanol for 24 h at room temperature, with shaking. The seed extract was collected by suction filtration and then concentrated by rotary evaporation. The concentrated seed extract was stored in a refrigerator until further use. This crude extract was subjected to antifeedant assay and fractionation by column chromatography.

Test for Phytochemicals in the Ethanolic Extract

The test for presence of phytochemical active substances was done using the method of Maryani et al. (2013), with modifications.

Bio-assay Guided Fractionation of Ethanolic Extract

A modified version of the fractionation process done by Simas et al. (2007) was used. The crude extract was fractionated by silica-gel column chromatography using a column with height and radius of 20 cm and 1.1 cm, respectively. Gradient elution was performed using ethyl acetate, 1:1 (v/v) ethyl acetate: methanol and 1:4 (v/v) ethyl acetate: methanol as solvents. Similar eluates were pooled based on their TLC profile, using varying proportions of ethyl acetate and chloroform as developing solvents. Analysis resulted in four distinct fractions (Fractions 1–4).

Antifeedant Activity Assay

The antifeedant activity of the crude extract and fractions were evaluated using the dual choice leaf disc method of Negi et al. (2016), with modifications. Field-collected mature castor (*Ricinus communis*) leaves, taken from leaf positions away from the shoot apex near the base of the tree, were used. Circular discs (area = 79 cm^2) were made in such a way that the parts beyond the leaf sinus were removed and the midrib served as marker between the two equal halves. The crude extract and fractions were diluted with water to achieve a final concentration of 785 ppm. On half of a leaf, 0.5 mL of test solution was applied on the adaxial and abaxial sides using a syringe, and was spread evenly using a spatula. Assuming uniform distribution of the test solution, the final concentration was approximately 10 µg of extract per cm^2 of leaf. The other half of the leaf disc was left untreated. Each leaf disc was air dried at room temperature and placed in a plastic cup. Moist cotton covered with aluminum foil was kept in the leaf stalk to prevent early drying of the leaf. Three laboratory-reared third-instar larvae of *S. litura* (pre-starved for 2 h), obtained from the National Crop Protection Center, were placed in the center of the leaf. Citronella oil diluted with acetone to a concentration equivalent to that of the extracts was used as positive control. Water and acetone served as negative controls for the test solutions and citronella oil, respectively. Two trials, each with three replicates, were done for every treatment. The fed areas in treated and untreated parts of the leaf discs were measured after 24 h using Adobe Photoshop CC 2015 software. Percentage relative feeding inhibition (% RFI) was calculated based on the equation (Kraus et al. 1999, unpublished):

% RFI= [(Area consumed in untreated-Area consumed in treated)/(Area consumed in untreated+Area consumed in treated)] × 100

Antifeedant activities were categorized based on the criteria of Huang et al. (2000), which was also used by Javier et al. (2018) to evaluate the antifeedant activity of ethanolic extracts against *S. litura*.

Statistical Analysis

Statistical Tool for Agricultural Research (STAR™) software was used for the statistical analysis. One-way ANOVA was done to test significant differences within replicates and between treatments. On the other hand, Tukey's HSD test was done to find means that are significantly different from one another.

Characterization of the Bioactive Isolate

The melting point of the crystalline isolate was determined using Fisher-Johns melting point apparatus. The mass spectrum (MS) data was obtained using positive electrospray ionization time-of-flight analysis on a Xevo G2-S QToF mass spectrometer at Pascual Pharma Corporation, UPLB Science and Technology Park, Los Baños, Laguna. The Fourier-transform infrared (FT-IR) spectrum was recorded using Thermo Scientific Nicolet 6700FT-IR spectrometer at De La Salle University, Manila.

RESULTS AND DISCUSSION

Extraction and Isolation of Bioactive Compounds

The green crude ethanolic extract was concentrated using rotary evaporator followed by air-drying; the extract then appeared as yellowish brown thick gel-like solid. The percentage yield was 1.67 ± 0.21%, which was comparable to previously reported yield using the same extraction solvent (Benjakul et al. 2014). Phytochemical screening of the crude extract suggested the presence of flavonoids, terpenoids, and saponins but the absence of alkaloids and tannins.

TLC analysis of the four major fractions collected from column chromatography of the crude extract showed that Fraction 1 (green solid) contained at least four components when developed using 1:2 (v/v) ethyl acetate: chloroform. Fraction 2 (green solid) consisted of at least seven components, four of which are similar to those of Fraction 1 when developed using the same solvent system. Fraction 3 (clear crystals) appeared as a single spot when developed using 2:1 (v/v) ethyl acetate: chloroform. Fraction 4, obtained from 1:4 (v/v) ethyl acetate: methanol, was of negligible mass when fully dried and was not subjected to further analyses.

Antifeedant Activity Assay

Among the test samples, only Fraction 2 did not show significant % RFI. Fraction 3 was found to be the most active, with % RFI comparable to citronella oil. Fraction 1 exhibited moderate antifeedant activity, which was better than the crude extract (Fig. 1). Citronella oil was used as the positive control since it is known to be an effective feeding deterrent against *S. litura* (Hummelbrunner and Isman 2001). The negative controls did not show any antifeedant activity (Fig. 2), and therefore did not contribute to the observed activities of the samples. Results of the antifeedant assay are summarized in Table 1. Based on ANOVA, there were no significant differences between the replicates within trials at 5% level of significance ($P>0.05$). On the other hand, the difference between treatments was highly significant ($P<0.01$). Based on Tukey's HSD, the activity of the crystalline isolate was comparable to the positive control.

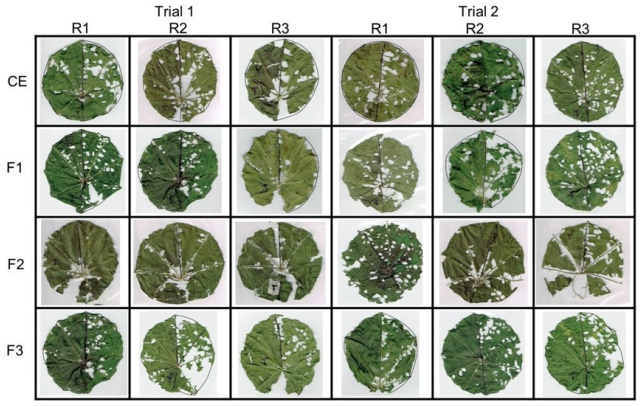

Fig. 1. Leaf discs from dual choice test on crude extract (CE), Fraction 1 (F1), Fraction 2 (F2), and Fraction 3 (F3). Two trials were performed with three replicates (R1, R2, R3) for each trial. Left side and right side of the leaves correspond to treated and untreated, respectively.

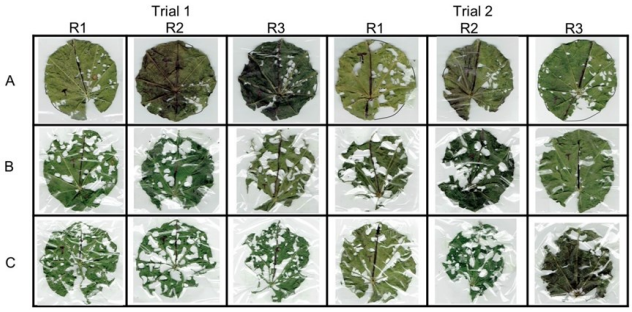

Fig. 2. Leaf discs from dual choice test on citronella (A), water (B), and acetone (C). Two trials were performed with three replicates (R1, R2, R3) for each trial. Left side and right side of the leaves correspond to treated and untreated, respectively.

Table 1. Antifeedant activity of crude extract and fractions from ipil-ipil (*Leucaena leucocephala*) seeds against common cutworm (*Spodoptera litura*) larvae. Controls are included for comparison.

Treatment	Antifeedant Activity	
	% RFI[1,2]	Category[3]
Crude extract	38.25 ± 2.31[c]	Low
Fraction 1	52.58 ± 2.74[b]	Moderate
Fraction 2	0.00 ± 0.00[d]	-
Fraction 3	79.93 ± 1.00[a]	High
Citronella oil	73.88 ± 7.61[a]	Moderate
Water	0.00 ± 0.00[d]	-
Acetone	0.00 ± 0.00[d]	-

[1]Based on two trials with 3 replicates per trial
[2]Values with the same superscript are not significantly different at 5% level of significance.
[3]Based on the criteria of Huang et al. (2000)

Characterization of the Bioactive Isolate

Melting point determination

The crystalline isolate was stable at 300°C, the highest melting point that can be measured using Fisher-Johns melting point apparatus.

Mass spectrum analysis

The MS peaks obtained were not sample peaks since the soft ionization technique was used (Niessen and Correa 2017). After subtracting the peaks obtained for the blank from that of the crystalline isolate, characteristic molecular positive ions were identified at *m*/*z* 275, 284, 463, 565, 685, and 686. The peak at *m*/*z* 275 is possibly an isotope of *m*/*z* 274 which was present in the blank. The peak at *m*/*z* 284 likely corresponds to Nylon 66 contaminant due to use of nylon filters in sample preparation for mass spectrometry. The peak at *m*/*z* 463 may correspond to polyethylene glycol contaminant. The remaining peaks which correspond to the crystalline isolate were at *m*/*z* 565, 685 and 686 $[M+Na]^+$, with the peak at *m*/*z* 685 being the most abundant. This corresponds to a molar mass of 662 g mol^{-1} for the major component of the crystalline isolate. The MS data revealed that, although in crystal form, the isolate was composed of more than one compound.

FT-IR spectrum analysis

The functional group assignments for the FT-IR peaks of the crystalline isolate are presented in Table 2. These were assigned according to characteristic peaks of saponins reported by Almutairi and Ali (2015). The FT-IR spectrum of the isolate is also comparable to those of other saponins reported by Luo et al. (2013) and Sharma and Paliwal (2013).

Possible Identity of the Bioactive Compound

The MS and FT-IR spectra indicate the presence of saponin with a molar mass of 662 g mol^{-1}. Based on phytochemical screening, the probable groups of compounds in the crude extract are flavonoid, saponin and terpenoid. However, TLC analysis under ultraviolet light at 254 nm showed absence of spots, suggesting that flavonoid was not present in the isolate (Mandal et al. 2015). Terpenoids could not be present as well since they are known to be quite insoluble in water (Liu 2011) whereas the crystals obtained were soluble to some extent. Therefore, the major component of the isolate was most probably a saponin, which is also supported by FT-IR data. Furthermore, many saponins are soluble in water, alcohol, chloroform and ethyl acetate (Razmovski-Naumovski et al. 2005), which were the solvents used in extraction and fractionation.

Comparison with other studies supports the possible presence of saponins in the crystalline isolate. In a study by Kraus et al. (1999, unpublished), white crystalline solids isolated from plants were found to be mixtures of saponins which cannot be separated from one another. In a patent by Holmes and Nygaard (1991), a crystalline saponin-containing complex was obtained from a mixture constituting saponin-containing aqueous plant extract solids soaked with methanol and acetone, which was allowed to stand at ambient temperature and pressure for a period of time. Plant fats and non-saponin carbohydrates remained substantially in the solution while the crystalline complex was being formed. This might have also occurred in our study since the fractions obtained were also air dried at ambient temperature and pressure, which possibly resulted in complex formation. Formation of crystalline saponin-containing complexes is further supported by the very high melting temperature

Table 2. Functional group assignments of the FT-IR peaks of the crystalline isolate.

Wave No. / cm^{-1}	Functional Group Assignment
3439	O-H
2958	
2924	C-H
2853	
1633	C=C
1076	C-O-C

since the melting point range for this type of compound was observed to be around 285 to 305°C (Holmes and Nygaard 1991).

Based on physical and chemical characterization, the most abundant component of the crystalline isolate could be a saponin without a carbonyl group. Morallo-Rejesus et al. (1999, unpublished) reported similar saponins from sunflower (*Tithonia diversifolia*) that exhibited toxicity against *Spodoptera exempta*. Their molar masses, however, were smaller than the molar mass of the major compound in the crystalline isolate.

RECOMMENDATIONS

A no-choice test should be conducted to further determine the effect of the bioactive compound and confirm its antifeedant activity against common cutworm. The insecticidal properties of saponins should be investigated especially on other major lepidopteran insect pests like the fall armyworm, which is now the most devastating insect pest of corn.

ACKNOWLEDGMENT

We are grateful to Dr. Pio A. Javier, Evangeline G. Punzalan and Bonifacio A. Navasero from the National Crop Protection Center, University of the Philippines Los Baños for their assistance in the antifeedant assays.

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