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Allelopathic Ellagitannin from *Annona muricata L. (Guyabano)* Leaf Extract against the Rice Weed *Echinocloa crus-galli*

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Allelopathy is a biological phenomenon where one plant inhibits the growth of another. The application of the allelopathic properties of some plants is a natural and environment-friendly approach which increases crop yield, decreased dependence on synthetic herbicides, and conservation of the ecological environment. This study extracted the tannins from *Annona muricata* L. leaves and determined the allelopathic effect on the germination and seedling growth of the rice weed, *Echinochloa crus-galli*. The dried powdered leaves of *Annona muricata L.* were extracted using 80% ethanol. Tannins were isolated through column chromatography. The total tannin content was determined and the isolate characterized by UV-Vis and IR spectrophotometry. The allelopathic effect of tannin on the seed germination and seedling growth of *Echinochloa crus-galli* rice weed was also obtained.The total tannin content of the isolate was 25.33±00 mg GAE g^{-1}. The percent germination was 66.67±5.77% in the control and 0 in all the tannin treatments. Highest inhibitory effect on the seedling growth was observed in the 8 and 10 mg/mL tannin. Although 8 and 10 mg/mL exhibited the strongest inhibitory effect on the seedling growth, differences in effect among the tannin concentrations were similar but significantly different from the control. The tannin responsible for the inhibition of seed germination and seedling growth is an ellagitannin based from the UV-Vis and FTIR Spectroscopy. The ellagitannin from guyabano leaves has a strong inhibitory effect on the seed germination and seedling growth of the rice weed, *Echinochloa crus-galli* and has the potential as a weedicide.

Keywords: tannin, *Annona muricata L.*, allelopathy, *Echinochloa crus-galli* rice weed

Introduction

Allelopathy is a biological phenomenon where one plant inhibits the growth of another. The application of the allelopathic properties of some plants is a natural and environment-friendly approach which increases crop yields, decreases our dependence on synthetic herbicides, and improves the ecological environment. The concept of allelopathy was first studied in the forestry ecosystems where many of the species investigated had negative effects on

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food and fodder crops (Olofsdotter, 1998). Due to these findings, allelopathy has been defined as the direct influence of chemicals released by a plant on the development and growth of another plant. Here, we use the term allelopathy as the negative effect of one plant to another one through the release of chemical compounds into the environment and considered as just one form of non-resource interactions among plants (Callaway, 2002).

Potential allelopathic compounds identified in living and decomposing tissue of small grain-cover crops include phenolic acids, other organic and volatile substances. Among these, phenolic acids were identified most frequently as phytotoxins. The role of phenolic acids as allelopathic agents in no-till systems pertains to the fact that concentrations of individual phenolic acids recoverable from field soils are well below levels required for inhibition of germination and growth in vitro (Blum, 1995).

Crop allelopathy may be useful to minimize serious problems in the present agricultural production such as environmental pollution, unsafe products, human health concerns, depletion of crop diversity, soil sickness and reduction of crop productivity. Several crops including alfalfa, buckwheat, maize, rice, rye, sorghum, sunflower, wheat, etc. are affected either by their own toxicity or phytotoxin exudates when their residues decompose in the soil, that show strong suppression on weed emergences. Allelopathic crops when used as cover crop, mulch, smother crops, green manures, or grown in rotational sequences are helpful in reducing noxious weeds and plant pathogen, improve soil quality and crop yield. Thus, allelochemicals from allelopathic crops may aid in the development of biological herbicides and pesticides (Khanh *et al.*, 2005).

Weed infestation has become a problem. The weeds compete with the plant for nutrients and exhibit allelopathy resulting to decreased crop productions. Increase in the amount of herbicides applied led to an increase in the production cost as well as environmental problems such as water contamination. To avoid the detrimental effects of herbicides, researchers are searching for sources of plant allelochemicals in the development of natural herbicides to lessen the harmful effects of synthetic herbicides. Thus, this study determined the allelopathic effect of the leaves extracts of *Annona muricata L.* on the germination and growth performance of the rice weed, *Echinochloa crus-galli*. The total phenolic content and the tannin responsible for the allelopathic property were identified.

Materials and methods

The ethanol and acetone were bought locally from ChemLine Scientific Corporation. Analytical reagents such as $FeCl_3$, gelatin solution, NaCl, $FeSO_4$, sodium potassium tartrate, silica gel, hydrochloric acid, vanillin reagent, gallic

acid, Folin Ciocalteu, and Na_2CO_3 were obtained from Biochemical, Organic and Natural Products (BONP) Research Laboratory of the Department of Chemistry.

Sample Collection and Preparation

Leaves of *Annona muricata L.* were collected from Bantug, Science City of Munoz, Nueva Ecija. The leaves were cleaned using tap water and air dried for about one week. The samples were then chopped into small pieces, ground, and pulverized.

The plant material was identified by a taxonomist from the Department of Biological Science, College of Arts and Sciences, Central Luzon State University.

Preparation of the Plant Extract

About 300-grams of the ground and pulverized plant sample were placed in a container with approximately 1800 mL ethanol (80%). The bottle was covered and set aside for two days at room temperature. The mixture was then filtered using Whatmann filter paper no. 1. The plant residue was discarded after drying and weighing, while the filtrate was concentrated using rotary vacuum evaporator set at 40^oC. The concentrated crude extract was stored in a tightly covered amber bottle and kept in a refrigerator until analysis.

Test for Tannins

One milliliter portion of the extract was placed in a test tube and five drops of 1% $FeCl_3$ solution in methanol was added. Formation of green to black solution indicated the presence of tannins. In another test tube one milliliter of the extract was placed and added with one milliliter of 1% gelatin and NaCl solution. The formation of a white precipitate indicated further the presence of tannins. Identification of tannins on the thin layer chromatogram (TLC) was also done by spraying with the $FeCl_3$ solution.

Thin Layer Chromatography of the Annona muricata L. Leaves Extract

A 50-mL beaker with a watch glass as cover was used as a developing chamber. The beaker was lined with filter paper around the sides to prevent "edge effect." The chamber was filled with the appropriate solvent system and equilibrated for five minutes.

The tannin fraction was spotted on the thin layer plate using micropipette. The spotted plates were placed inside the equilibrium chamber. The chamber was tightly covered and the solvent allowed to travel until the fronts just reached about 1cm before the edge of the coating. The plates were then removed from the chamber and allowed to dry.

Thin layer chromatography was performed on pre-coated TLC plates (Merck F_{254}) layer thickness of 0.2 mm and dimensions of 5 x 8 cm. A 0.4-µL of the tannin fraction was drawn using a micropipette and applied as spots on the silica-coated plate, about 1 cm from the base.The TLC plates were developed with the solvent systems. The band separations were detected under UV light, followed by visualization using iodine chamber and spraying with $FeCl_3$ solution.

The Rf values of each spot were computed as follows:

$$Rfvalue = \frac{\text{Distance traveled by each spot from the origin (mm)}}{\text{Distance traveled by the solvent from the origin (mm)}}$$

Extraction and Characterization of Tannin from Annona muricata L. Leaves Extract

The crude extract (10 g) was dissolved in 20 mL of ethanol and applied on a column (5 × 40 cm) packed with silica gel (70 – 230 mesh). One liter of 50% acetone (v/v) was used to elute tannins. The method of Tambe (2014) was adopted to determine the total tannin content of the eluate. About 0.1 mL of the eluate was added to a volumetric flask (10mL) containing 7.5 mLof distilled water, 0.5 mL of 10% (v/v) Folin-Ciocalteu reagent, 1 mL of 35% Na_2CO_3 solution and diluted to 10 mL with distilled water. The mixture was shaken and kept at room temperature for 30 minutes. A set of reference standard solutions of gallic acid (20, 40, 60, 80, and 100 µg/mL) were prepared in the same manner as described earlier. Absorbance for the test sample and standard solutions were measured against a blank at 725 nm using UV-Visible spectrophotometer. The tannin content was expressed in terms of mg GAE/g of extract.

Solvent from the tannin isolate was removed using a rotary evaporator. The tannin isolate was characterized using UV-Vis (Hitachi U-2900 UV-Vis Spectrophotometer) and IR spectrophotometer (ABB MB300 FTIR). The spectra were compared with references from literatures and standard gallic acid.

Weedicide Bioassay of Tannin from Annona muricata L. leaves

In order to determine the allelopathic property of *Annona muricata L.* leaves, the extracted tannin was oven dried. The dried samples were measured to prepare 10 mg/mL as stock solution. Concentrations of 2, 4, 6 and 8 mg/mL were prepared from the stock solution. The concentrations used to observe the allelopathic property of *Annona muricata* L. was adopted from Arowosegbe *et al.* (2012).

Test For Seed Germination

Echinochloa crus-galli seeds were planted in ELISA plates and treated with 0.1 mL of each concentration of the tannin. The prepared 50% acetone was used as control. Ten seeds were used for each treatment in three replicates. The percent germination was recorded after 5 days.

$$\%\ Germination = \frac{Number\ of\ seeds\ germinated}{Total number of seeds planted} x\ 100$$

Test For Seedling Growth

Echinochloa crus-galli seeds were planted in microplates for 5 days until shoots were developed. The initial lengths of the shoots were measured using a Vernier caliper. The shoots of *Echinochloa crus-galli* were placed in ELISA plate. The wells were treated with 0.1 mL of each concentration of the tannin. Fifty percent acetone was used as control. Three replicates were made in each treatment. The seed growth was recorded by measuring the length of the shoots (mm) after 5 days. The whole experiment were performed thrice to fully confirm the results.The experimental results were expressed as mean ± SD. A one-way analysis of variance (ANOVA) on the seed bioassay test was used to analyze the data.

Results

Crude extract of Annona muricata L.

The crude extract obtained from 200 grams *Annona muricata L.* leaves was 13.46 grams. Percent recovery was 6.73%. The extract has a sticky texture with strong odor. The crude extract of *Annona muricata* L. contains hydrolysable tannin based on the results for tannin tests.

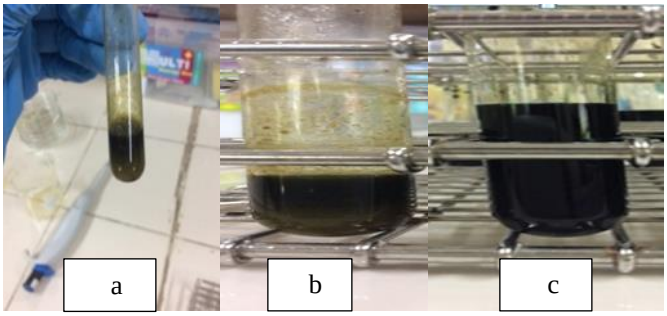

Figure 1. Tannin tests (a) crude extract (b) white precipitate (c) blueblack solution

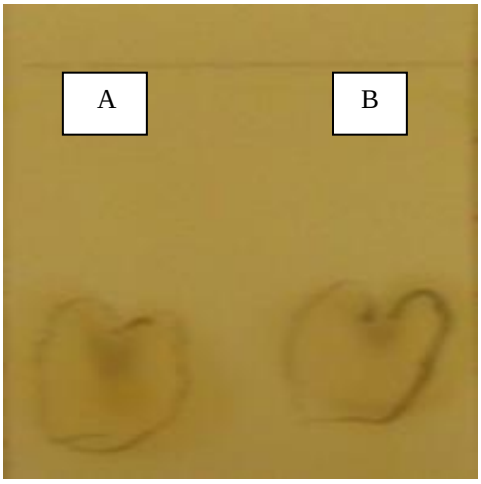

Figure 2. Thin layer chromatogram of (A) gallic acid (B) tannin isolate

The R_f value of gallic acid was 0.65 and 0.66 for tannin in *Annona muricata* (Figure 2). Almost similar R_f values indicate that tannin in *Annona muricata* has the parent structure of gallic acid.The total tannin content in *Annona muricata L.* leaves was found to be 25.33±0.00 mg GAE g^{-1} sample.

Weedicide Bioassay of Tannin in Annona muricata L. leaves

No seed germination was recorded in all the concentrations of tannins applied while 66.67%±5.77 germinated in the control (Figure 2). All test concentration of tannin inhibited the germination of *Echinocloa crus-galli* seeds. The result only implies that even at lowest concentration of 2 mg/mL, tannin from *Annona muricata* can inhibit the germination of this rice weed.

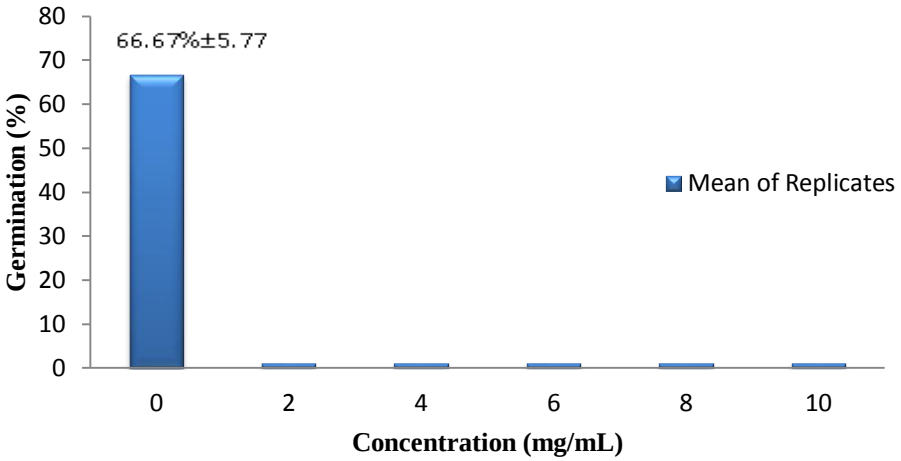

Figure 2. Effect of different concentrations of tannin in *Annona muricata L.* leaves on the seed germination of *Echinochloa crus-galli*

Moreover, the tannin of *Annona muricata* L leaves. inhibited the seedling growth of *Echinochloa crus-galli* (Table 1). Measured effect of tannin on the seedling growth was obtained by getting the difference of the final and initial length of the roots and shoots. The tannin of *Annona muricata* L leaves significantly inhibited the seedling growth of *Echinochloa crus-galli* compared to the control. However, similar inhibiting effect on the seedling growth were noted in concentrations 2, 4 and 6 mg/mL and between 8 and 10mg/mL. Stronger inhibiting effect was observed in 8 and 10 mg/mL concentrations. The results obtained can be attributed to the amount of tannin applied which caused a corresponding stronger inhibitory effect on the growth of *Echinochloa crus-galli* seeds. An increase in the concentration of the extract can increase the growth inhibition of the plant as reported by Chung and Miller (1995).

Table 1. Effect of tannin from *Annona muricata* L. on the seedling growth of *Echinochloa crus-galli* seed at different concentrations.

Concentration (mg/mL)	Initial (mm)	Final (mm)	Difference (mm)
0	50.80 ±0.56	56.91 ±3.36	6.11 ±3.06[a]
2	32.30 ±2.09	31.21 ±1.15	-1.11 ±0.55[b]
4	33.57 ±2.74	31.57 ±0.75	-2.00 ±1.00[b]
6	24.00 ±4.24	21.56 ±3.70	-2.44 ±1.22[b]
8	34.37 ±7.91	30.15 ±6.54	-4.22 ±2.11[c]
10	34.23 ±5.00	28.90 ±1.65	-5.33 ±2.67[c]

Means with the same letters are not significantly different and negative values indicate decrease in the final length

UV-Vis and Infrared spectrophotometry of tannins from Annona muricata L

The absorption peak for the leaf tannin extract of *Annona muricata L.*was identified at about 255 nm (Fig. 3). Infrared spectrum of the isolated tannin revealed the presence of hydroxyl group (-OH) at around 3371 cm^{-1}; symmetric and anti-symmetric -C-H- stretching vibrations of $-CH_2$ and $-CH_3$ groups with sharp peak at 2923 $cm^{-1}$and a small shoulder at 2854 cm^{-1}, respectively; stretching vibrations of $-C-C_{aromatic}$ groups at around 1442 $cm^{-1;}$ aromatic esters at 1064 $cm^{-1}$and carbonyl groups. C-O stretching vibration at 1100-1300 cm^{-1}.

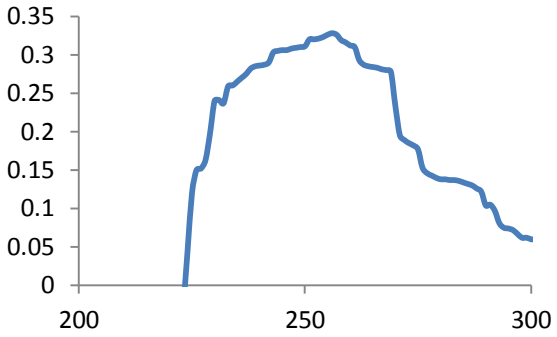

Figure 3. UV-Vis spectrograph of tannin from *Annona muricata L.*

Detailed informations are listed in Table 2. These results are comparable to those reported in the literature for polyphenolic compounds as tannic acid.

Table 2. Infrared positions of tannin in *Annona muricata* L. leaves.

Group	**Vibration**	**Approach frequency/experimental (cm^{-1})***	**Tannin of *Annona muricata* L. (cm^{-1})**
Arom-CH2-OH	ν OH	3400/3379	3371
Arom-CH2-OH	ν_aCH2	2920/2923	2923
Arom-CH2-OH	δ CH2	1420/1458	1442
Arom-C-O-	ν C-O	1630	1604
Arom-O-	ν_aC-O	1050/1164	1064
Arom-O-	νC-O	800/825	817

Data with (*) are based from Socrates (2004)

Discussion

The leaves of *Annona muricata* L. contains hydrolysable tannin which was confirmed by the tannin tests. A classic way to detect simple phenol extract like tannin is to add a solution of 1% $FeCl_3$ in water. The Fe^{3+} ions form complexes with the tannins in the extract forming a green or blue-black color (Harbourne,1973).Tannins, acetogenins, alkaloids, phenolics, cyclopeptide, flavanol triglycoside, steroids, essential oils, and cardiac glycosides were reported to be the major compounds in *Annona muricata* L.(Moghandamtousi *et al*. 2015). The qualitative phytochemical screening of *Annona muricata* L. in this study is in agreement with the works of Falodun *et al*, (2011), Foong and Hamid, (2012), Vijayameena *et al*. (2013), Solomon-Wisdom et al.(2014) and Usonobun *et al*. (2014). The almost similar R_f values on the thin layer chromatogram of the tannin from *Annona muricata* L. leaves and the standard gallic acid further confirms similar chemical structure.

Carotenoids especially phenolic compounds were higher in the leaves of older trees (14 years) than the young ones (4 years). According to Silva *et al*. (2016), the total phenol content they obtained was 562.35 mg GAE g^{-1} while condensed tannins was 343.98 mg 100 g^{-1} GAE. The low tannin content obtained in this study could be attributed to the age of the tree where the leaves. The low amount of tannin in the leaves of *Annona muricata* L. obtained from this study could have come from a young tree where the content of tannin is lower. There is a presence of very trace amounts of secondary metabolites like tannins, steroids, cardiac glycosides, etc. the leaves of *Annona muricata* L (Gajalakshmi *et al*., 2012).

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Allelochemicals such as phenolic acids and associated compounds are the most common growth inhibitors of plants (Macias *et al*., 2008). Tannins belong to the phenolic secondary metabolites. The tannin extracted from *Annona muricata* L. has the ability to inhibit the seed germination of *Echinochloa crus-galli*. In the seeds, hormones and proteins are present for survival. Hormones, like gibberellins whose mechanism is to affect enzyme production that mobilizes food production used for growth of new cells (Tsai *et al.*, 1997) and proteins in which they play a role in equipping the seed for survival, maintaining

a minimal level of hydration in the dry organism and preventing the denaturation of cytoplasmic component (Bray *et al.*, 1993) are being affected by the tannin in the leaf extract and causes the seeds not to germinate. The result is supported by Muscolo *et al.* (2001) who explained that phenols, like tannins, may cause inhibitory effect in the germination of seeds.

The tannin from *Annona muricata* L. leaves also inhibited the seedling growth of *Echinochloa crus-galli*. The inhibition effect is concentration dependent. This means that inhibition in the growth of the seedling took effect with increasing concentration. In the control, the seedling grew by 6.11 mm after five days. On the other hand, negative values were obtained in the treatments indicating that the seedling ceased to grow which may have led to the impairment in the metabolic activities of the seedling resulting to a decrease in their root and shoot length (Abu-Romman *et al*., 2010). Cell division also stopped because no shoots grew in all the treatment. In the study of Arowosgabe (2012), similar concentrations of 2,4,6,8 and 10 mg/mL were used in observing the allelopathic effect of *Aloe ferox mill* root extract on tomato. Inhibition in the seedling growth of *Echinochloa crus-galli* was observed using the same concentrations. Phenolic compounds like coumarin, had inhibitory effects both on seedling growth and seed germination on lettuce (Li *et al.,* 2010).

Ellagitannin belongs to a group of tannin. The other one is gallotanin. Moilanen *et al.* (2013) recorded an absorption peak of 250 nm for ellagitannin. More complex tannins can form by oxidative transformations which can yield macrocyclic ellagitannins (Okuda *et al*., 1989, Okuda, 2005). Functional groups in tannin showed the presence of hydroxyl groups, carbonyl groups, aromatic esters and CH2 stretching characteristics of the structure of tannins (Hagerman, 2010; Moilanen *et al.*, 2013). According to Okuda (1999) hydrolyzable tannins grouped as gallotanins and ellagitannins are composed of gallic acid or hexahydroxydiphenic acid esters, respectively, linked to a sugar moiety. The tannin responsible for having an allelopathic weedicide property against the rice weed, *Echinochloa crus-galli*, is an ellagitannin.

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