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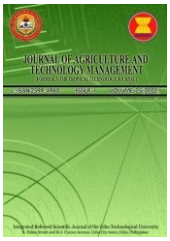

Original article

Phytochemical screening and toxicity testing of mature sugarcane leaf extract, saccharum officinarum (Linnaeus, 1753)

Raamah C. Rosales[1*], Cristina Ramas[1] and Maria Eden Rosales[2]

[1]College of Arts and Sciences, Cebu Technological University-Main Campus, Cebu City 6000, Philippines
[2]Gun-ob National High School, Gun-ob, Lapu-Lapu City 6015, Cebu, Philippines

ABSTRACT

Sugarcane is an important agricultural crop in the Philippines. Massive volume of plant leaves is produced annually that can provide a considerable volume of samples for natural drug studies. This study aimed to determine the secondary compounds and biological effect of the mature sugarcane leaf extract. The phytochemical screening was conducted for the detection of secondary metabolites, namely: alkaloids, flavonoids, tannins, saponins, cardiac glycosides, carbohydrates and anthracene glycosides. Brine shrimp biological assay was used to determine the toxicity of the crude extract while LC_{50} was utilized to analyze the toxicity of the different concentrations. The phytochemical screening positively detected the presence of the secondary metabolites except for saponins. The lethality assay on brine shrimp nauplii indicated high mortality on the highest concentration with no deaths recorded on the negative control using seawater. The LC_{50} was determined at 34.06 that means 50% of the mortality rate occurred at this concentration. The LC_{50} has classified the 100 µL and 50 µL concentrations as extremely toxic while 10 µL, 1 µL, and 0.1µL concentrations were slightly toxic. Isolation and purification of the secondary metabolites may be conducted in the future to specify the compounds with its biological effects.

KEYWORDS: *phytochemical screening, secondary metabolites, biological assay, and toxicity*

1 INTRODUCTION

Sugarcane, *Saccharum officinarum* L., is a widely cultivated agricultural crop in the Philippines. The annual production of sugar contributes around P70 Billion to the national economy. Sugarcane plantation was estimated to cover 414, 980 hectares of the entire archipelago with about 62,000 farmers identified as dependent on the sugar industry (Philippine Sugar Statistics, 2019). The Philippines produced a million metric tons of raw sugar annually that also resulted in a massive volume of sugarcane leaves being produced with no other significant use. This study aimed to determine the pharmacological properties of the mature sugarcane leaf extract to explore its potential medicinal value.

Plant leaves developed adaptive strategies throughout its life history against predation and other environmental pressures (Masa et al., 2016). This includes production of secondary metabolites that are essential for plant growth, survival and development. The biosynthesis of these compounds largely depend on the capacity of the plant to respond to environmental signals and some biotic attacks that induce complex transduction pathways (Okada et al., 2015). Pathogens and presence of herbivores are among the signals which can modulate these pathways.

Developmental stages of plant leaves can greatly affect the composition of secondary metabolites (Achakzai et al., 2009; Azam et al., 2013; Watanabe et al., 2016). The study of Achakzai et al (2009) on phytochemical screening on the leaves of different plants at different ages showed that mature leaves contain higher levels of phenolic contents than the young leaves. Masa et al (2016) quantified flavonoids and diterpenes in *Cistus ladanifer* L. and the result showed varying contents in young and mature leaves. The plant secondary metabolites are known to have pharmacological effects (Abbas, 2013). Plants have been used as natural medicines by different cultures as a remedy against various diseases (Singh et al, 2015). However, development of natural drugs from plants would require a massive amount of samples to obtain a viable amount of compounds.

Sugarcane leaf is generally considered as waste for most of the sugarcane plantation. Mature leaves are normally removed during the entire planting season to prevent fire from occurring. The sheer volume of sugarcane leaves that are available annually would make this plant a viable choice for natural product development. This study aimed to determine the secondary compounds and biological effect of the mature sugarcane leaf extract through phytochemical analysis and toxicity testing

*corresponding author: epoch7era@gmail.com
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2 MATERIALS AND METHODS

Collection of mature leaf samples

This study was submitted for ethical review by the IACUC of the University of San Carlos-Talamban Campus. Plant leaf samples were collected in a sugarcane plantation located at the northern tip of Cebu Island, Philippines (11.1491° N, 123.9861° E) from March to June 2018. Sugarcane leaves are alternate and numbered from top to bottom with the uppermost leaf designated as leaf +1 (Fig. 1) (Kujiper, 1915). The collected mature leaf samples were described as fully expanded and highly exposed to sunlight specifically on leaf +2, +3, and +4 (Gianotto et al., 2011; Zhao et al., 2012).

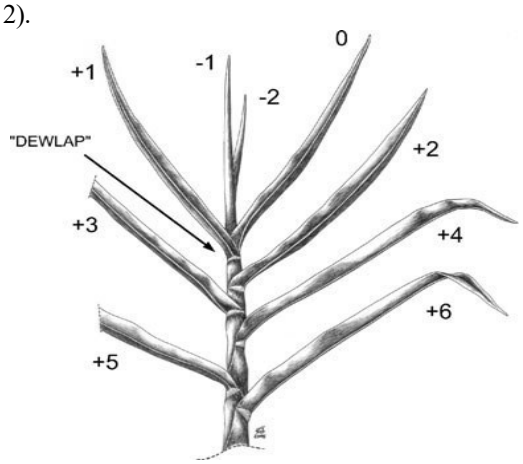

Figure 1. Kujiper's leaf numbering system on sugarcane plant (1915).

Preparation and extraction plant samples

A total of 10 kg air-dried and finely chopped leaves macerated with 95 % ethanol were placed in glass containers. The containers were thoroughly shaken and allowed to stand at room temperature with occasional manual agitation using a stirring rod. The mixture was allowed to stand for 72 hr to ensure complete extraction, then strained and the marc discarded. The menstruum was filtered using sterile Whatman no. I filtered paper and concentrated using a rotary evaporator (Heidolph Hei-VAP Platinum 2 Rotary Evaporator, Germany) at 60-65ºC temperature range. The filtrate was placed in a water bath at 75-80oC temperature range to evaporate to dryness and obtain solvent-free crude extract—a total of 430 g with a percent yield of 4.2 % extracted after the entire concentration process.

Qualitative Phytochemical Analysis

Phytochemical analysis for qualitative detection of alkaloids, tannins, saponin, flavonoids, cardiac glycoside, carbohydrates and anthracene glycosides were carried out on crude extract using the methods enumerated below.

Determination of Alkaloids

The extract was diluted with 10ml of acid alcohol, boiled and filtered. 2ml of diluted ammonia was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10ml of acetic acid. This was divided into two portions. Meyer's reagent was added to one portion and Wagner's reagent to the other. The formation of a cream (with Meryer's reagent) or reddish-brown precipitate (with Wagner's reagent) was taken as positive for the presence of alkaloids (Oluduro, 2012).

Test for Saponins

The ability of saponins to produce frothing in aqueous solution was used as a screening test for saponins. The plant extract was shaken with 10ml distilled water in a test tube. Frothing which persisted on warming was taken as evidence for the presence of saponins (Sofowora, 1982).

Determination of Tannins

The extract was stirred with 10ml of distilled water, filtered and ferric chloride reagent added to the filtrate. A blue-black precipitate will indicate the presence of tannins (Trease & Evans, 1978).

Determination of Flavonoids

The plant extract was added with 10 ml boiling distilled water. The mixture was filtered while hot. The filtrate was allowed to cool and 5 ml of 20% sodium hydroxide was added to equal volume of the filtrate. A yellow solution indicates the presence of flavonoids (Trease & Evans, 2002).

Test for Cardiac Glycosides (Keller-Killani Test)

The plant extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. It was underlaid with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicates a deoxysugar characteristic of glycosides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout the thin layer (Morsy, 2017).

Detection of Carbohydrates

The plant extract was divided into 2 portions and used to test the presence of carbohydrates using the following reagents.

Molisch's Test. The plant extract was treated with 2 drops of alcoholic α-naphthol solution in a test tube. The formation of the violet ring at the junction indicated the presence of carbohydrates (Usman et al., 2009).

Fehling's Test. Filtrate was hydrolysed with dil. HCl, and then neutralized with alkali and warmed with Fehling's A & B solutions. The formation of brick red precipitate indicated the presence of reducing sugars

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(Usman et al., 2009).

Test for Anthracene Glycosides (Borntrager's Test)

About 10 ml of the plant extract was placed in a dried test tube and 10 ml of chloroform was added. The mixture was shaken for 5 min and filtered with Whatman No. 1 filter paper. To 3 ml of the filtrate equal volume of ammonia solution was added and shaken. Formation of a bright pink-red colour in the upper aqueous layer indicated the presence of free anthracene glycosides (Trease & Evans, 2002).

Brine Shrimp Lethality Bioassay

Substituting animals with alternative models such as brine shrimp is important to address ethical issues in toxicity studies. The utilization of *Artemia salina* bioassay for predicting toxicity is widely used to elucidate biological activity of the detected phytochemicals present in plant extracts (Parra et al., 2001; Sharma et al., 2013). This study did not include the determination of specific compounds that affect toxicity and its mechanism of actions. The determination of toxicity of sugarcane leaf extract in varying concentrations is highly limited to mortality of *A. salina* and did not include physiological effects to animal tissues and organs.

Brine shrimp eggs were hatched in an improvised hatching container filled with filtered seawater. Two containers were fed with an aerator to enrich the seawater with oxygen. The eggs (ca. 50mg) were sprinkled into the containers illuminated with a lamp. After 48 hours, the phototropic nauplii were collected by pipette from the lighter side, having been separated by the divider from their shells.

Ten shrimps were transferred to each sample vial using a 9in disposable pipette containing different concentrations of extracts and artificial sea water was added to make 5ml. The nauplii can be counted macroscopically in the stem of the pipette against a lighted background. The vials were maintained under illumination. Survivors were counted, with the aid of a 3x magnifying glass, after 24hr, and percent deaths at each concentration and control was determined. The test groups include seawater as the positive control and crude extracts with concentrations of 1mg/1ml (100 µL), 1mg/2ml (50 µL), 1mg/10ml (10 µL), 1mg/100ml (1 µL) and 1mg/1000ml (0.1 µL) (Hamidi et al., 2014). The reference standards for LC50 based on the rate of mortality were 0 for non-toxic, 1-30% slightly toxic, 31-60% moderately toxic and 61-100% extremely toxic.

Statistical Treatment

LC50's and 95% confidence intervals were determined from the 24hr counts using probit analysis method described by Finney for brine shrimp lethality assay. Tukey-Honest Significant Difference (HSD) for post-hoc analysis, including one-way analysis of variance, tested the statistical difference between the treatment and the controls to elucidate the variation between test groups using an excel of MS office.

3 RESULTS AND DISCUSSION

Phytochemical Screening of Crude Extract

Qualitative screening was conducted to detect the presence and absence of compounds. Active compounds that were screened include alkaloids, saponins, tannins, flavonoids, cardiac glycosides, carbohydrates, and anthracene glycoside (Table 1). All the target active compounds were tested positive except for saponins due to the absence of frothing.

Table 1. Active compounds positively detected from the crude extract.

Active Compounds	Description	Result
Alkaloids	Formation of a cream (with Meyer's reagent) or reddish-brown precipitate (with Wagner's reagent) was taken as positive for the presence of alkaloids	Positive
Saponins	Frothing which persisted on warming was taken as evidence for the presence of saponins	Negative
Tannins	A blue-black precipitate indicated the presence of tannins	Positive
Flavonoids	A yellow solution indicated the presence of flavonoids	Positive
Cardiac Glycosides	A brown ring at the interface indicated a deoxy sugar characteristic of glycosides	Positive
Carbohydrates	*Molisch's Test* formation of the violet ring at the junction indicated the presence of carbohydrates	Negative
	Fehling's Test The formation of brick red precipitate indicated the presence of reducing sugars	Positive
Anthracene Glycosides	*Borntrager's Test* Formation of a bright pink-red colour in the upper aqueous layer indicated the presence of free anthracene glycosides	Positive

Characteristics and Functions of Plant Active Compounds

The success of sugarcane plants could be attributed to the regulatory functions of the detected secondary metabolites although its mechanisms are still unclear. Secondary metabolites are known to facilitate plant developmental signals, such as changes associated with flowering and fruit process including leaf growth (Nascimento and Fett-Neto, 2010; Roepke et al., 2010). Secondary metabolites are also responsible for some pharmacological properties of plant leaves against various diseases (Babbar, 2015).

Alkaloids are ubiquitously present in plants and considered as one of the largest groups among plant active compounds (Usman et al., 2009; Abdelrahman et al., 2017). It is classified by the presence of a nitrogen

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atom at any position in the molecule except in amide or peptide bond (Bribi, 2018). The alkaloid in sugarcane leaf functions as a storage reservoir of nitrogen essential for the development of plant tissues and cell structures such as cell membrane and chlorophyll (Abdelrahman et al., 2017). Plant tannins belong to a large and diverse group of polyphenolic compounds covering the outer layer of leaves, fruits, seeds and barks. The astringent nature of tannins due to high polyphenolic content makes it the first line of defense for the sugarcane plant against herbivores (Furlan et al., 2014). Flavonoids are categorized as phenolic compounds and classified by the presence of benzo-γ- pyrone structure (Kumar et al., 2013). Flavonoids act as a unique UV- filter in plants and help in the transport of plant hormones. The presence of flavonoids in sugarcane leaves helps the plant become more resilient against harmful UV radiation and assist in the acclimatization process against direct exposure to sunlight in sugarcane plantation.

Cardiac glycosides normally occur in small amounts in leaves, seeds, roots, stems and barks of plants. Cardiac glycosides are classified either as C23 or C24 steroids and distinguished by a 14-hydroxy group with peculiar sugar attached in their skeleton (Melero et al., 2000). It functions as a potent poison against predators due to its ability to exert powerful effects on cardiac muscle (Morsy, 2017). Carbohydrates make up the major constituents of plants. Carbohydrates are essential in sugarcane physiological processes including cellulose formation for structural plant components, production of starch as reserve for energy source, and production of gums and mucilage that prevents tissue desiccation (Tharanathan et al., 1987). Anthracene glycosides or anthraquinone have been detected in some plant leaves in different genera including *Senna*, *Aloe*, and *Hypericum*. Anthracene glycosides are organic compounds from plants classified based on the presence of sugar in the form of β-D-glucose. The ingestion of plant leaves with high levels of anthracene glycosides can cause nausea, carcinogenesis, diarrhea and renal failure (Chien et al., 2015). These adverse effects may evolve into a defense mechanism that enhances plant survival against predators.

Pharmacological Potentials of Detected Compounds

Most of the related alkaloid compounds possess potent pharmacological effects including papaverine as muscle relaxant, apomorphine for Parkinson's disease, and berberine as antimicrobial agents (O'Connor, 2010; Cushnie et al., 2014). Tannins in plants are known for their biological activities, especially as anticarcinogens and antioxidants. Other studies have shown that tannins also possess pharmacological effects as anti-HIV, anti-inflammatory and cicatrizant (Gutierrez et al., 2008; Furlan et al., 2014). The pharmacological interest in flavonoid is in its antioxidant activities. The functional hydroxyl group of flavonoids exhibit antioxidant effects by scavenging free radicals or chelate metal ions (Kumar et al., 2013). Several flavonoids such as catechin exhibits hepatoprotective activity while its anti-inflammatory activity is on cell activation processes for the biosynthesis of cytokines that mediate adhesion of leukocytes to sites of injury (Tunon et al., 2009). Saponins possess many pharmacological properties such as anthelmintic, antifungal, anti-inflammatory, insecticidal, immunostimulant, hypocholesterolemic, and cytotoxic (Marelli et al., 2016)

Cardiac glycosides are primarily valuable medicine for congestive heart failure. Cardiac glycosides can increase the rate of heart contraction while maintaining low oxygen consumption. This pharmacological activity of cardiac glycosides helps the myocardium become a more efficient pump and sustain normal operation of the circulatory system (Morsy, 2017). Sorensen and Giese (2013) tested three derivatives of carbohydrates such as monosaccharides, disaccharides and polysaccharides as substrates to determine its capacity in the production of secondary metabolites. The study showed that all three substrates produced aurofusarin while moniliformin and enniatins were produced except in disaccharide (lactose). The presence of carbohydrates in mature sugarcane leaf extract may have influenced the production of the detected secondary metabolites. The anthracene glycosides possess a broad spectrum of pharmacological activities applicable in many diseases including anticancer, cathartic, antimicrobial, anti-inflammatory, vasorelaxant, and other phytoestrogen activities (Dave and Ledwani, 2012; Chien et al., 2014).

Toxicity Testing

The highest total number of dead nauplii was recorded at 100 µL concentration with a total of 88 dead nauplii with the lowest at 0.1 µL with 11 dead nauplii (Table 2). Negative control has no dead nauplii recorded.

Table 2. Total number and Percentage (%) mortality of brine shrimp nauplii treated with *F. officinarum* leaf extract.

Concentrations (µL)	Total # of nauplii	Total # of dead	% mortality
Negative Control	90	0	0±0%
0.1	90	11	12.22±9.72%
1	90	15	16.67±10%
10	90	18	20±11.18%
50	90	77	85.56±12.36%
100	90	88	97.78±6.67%

The graph on Figure 2 presents the % mortality of all the concentrations including negative control. The 100 uL has the highest % mortality of 97.78±6.67%with the lowest at 0.1 µL of 12.22±9.72%. The negative control

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has 0% mortality since no deaths were recorded.

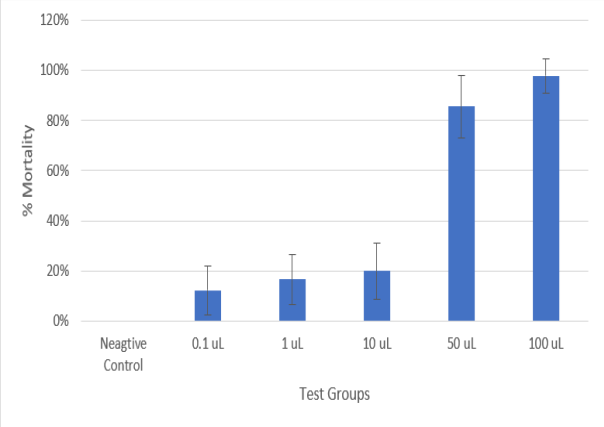

Figure 2. Mean percent mortality of dead nauplii versus test groups.

regression with the equation: $y=mx + b$
Where:
y= 50% of the population
m= slope of the dose effect line
b= represents y- axis
x= median lethal concentration
50= (0.9165) x + 16.917
X= 34.06uL

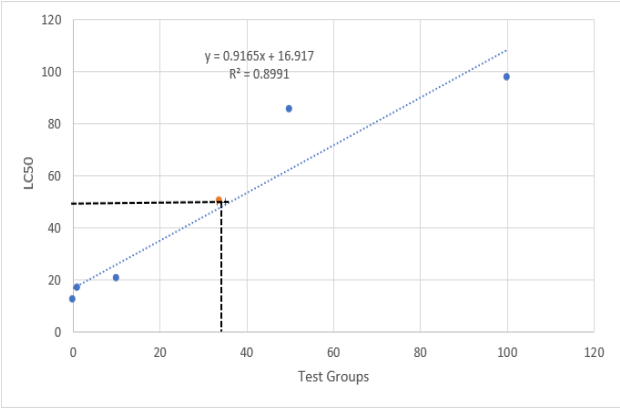

Figure 3. Determination of LC_{50} of the brine shrimp lethality assay.

The lethality assay based on the LC_{50} at 34.06 µL indicates that concentration beyond this level is extremely toxic. The result showed that 100 and 50 µL concentrations are extremely toxic while the rest concentrations are slightly toxic (Table 3). The percentage mortality is concentration-dependent because the percentage mortality increases in relation to the increase of plant leaf extract concentrations.

Table 3. The toxicity based on the LC_{50} of the different concentrations.

Concentrations (µL)	Total # of nauplii	Total # of dead	% mortality	LC_{50}
Negative Control	90	0	0±0%	non-toxic
0.1	90	11	12.22±9.72%	slightly toxic
1	90	15	16.67±10%	slightly toxic
10	90	18	20±11.18%	slightly toxic
50	90	77	85.56±12.36%	extremely toxic
100	90	88	97.78±6.67%	extremely toxic

Significant difference between groups in the results was tabulated (Table 4) indicating the p-level. For this set of statistical data, the corresponding p -level is 0.000 which is statistically significant based on the confidence level $p< 0.01$.

Table 4. One-Way ANOVA.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	78475.926	5	15695.19	182.27	0.000	2.41
Within Groups	4133.3333	48	86.11			
Total	82609.259	53				

To further analyze the difference between each test group, Table 5 shows the Tukey HSD Test results. It shows that there is an insignificant difference between the 100 µL vs 50 µL, 10 µL vs 0.1 µL and 1 µL vs 0.1 µL. All other comparisons with groups were found to be significant.

Table 5. Post – Hoc Analysis between each test group.

Tukey HSD (µL)	Concentrations (µL)	Mean Difference	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
100	50	12.2222	3.5799	0.0189	-27.8511	3.4066
	10	77.7778	12.7822	0.0000	-93.4066	-62.1489
	1	81.1111	12.9758	0.0000	-96.74	-65.4823
	0.1	85.5556	13.5097	0.0000	-101.1844	-69.9267
	Negative	97.7778	15.0756	0.0000	-113.4066	-82.1489
50	10	65.5556	10.3322	0.0000	-81.1844	-49.9267
	1	68.8889	10.5049	0.0000	-84.5177	-53.26
	0.1	73.3333	11.0326	0.0000	-88.9622	-57.7045
	Negative	85.5556	12.5202	0.0000	-101.1844	-69.9267
10	1	3.3333	3.1282	0.0000	-18.9622	12.2955
	0.1	7.7778	3.3616	0.1348	-23.4066	7.8511
	Negative	20.0000	3.4451	0.0000	-35.6288	-4.3712
1	0.1	4.4444	3.0977	0.3532	-20.0733	11.1844
	Negative	16.6667	3.0464	0.0001	-32.2955	-1.0378
0.1	Negative	12.2222	2.8758	0.0016	-27.8511	3.4066

Toxicity of Mature Sugarcane Leaf Extract

The plant extract has shown toxicity effects to brine shrimp even at its lowest concentration of 0.1 µL. The highest concentration of 100 µL is classified as extremely toxic while the lethality assay based on the LC_{50} was determined at 34.06 µL (Table 3). The determination of toxicity at varying concentrations is important in order to achieve safe treatment levels (Parra et al., 2001). Toxicity can be described as the totality of adverse events in relation to the administration of a substance such as the plant extract. It is generally influenced by the duration of

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exposure, dose, physiological factors, chemical structure, hereditary factors and many others (Theoduloz et al., 2012).

The toxicity of the sugarcane leaf extract can be indicative of biological activity of secondary metabolites. All of the detected compounds have been determined to have toxic effects due to its pharmacological effects. However, anthracene glycoside is considered as highly toxic among active compounds in plants due to its specific powerful actions to cardiac muscle. (Morsy, 2017). It is historically used as poison in arrows for hunting of animals in Asia, South America and Africa.

Mechanism of Action

The effect of toxicity may vary depending on the mechanisms of action on particular secondary metabolites present in the sugarcane leaf. One example is alkaloids which possess respiratory inhibition effects. Other compounds like isoquinolines, such as berberine, benzophenanthridine, protoberberine, and sanguinarine, can inhibit cell division by perturbing the Z-ring in a bacterial cell. The phenanthridine isoquinoline alkaloid ungeremine can inhibit nucleic acid synthesis. The pergularinine and tylophorinidine, which are indolizidine alkaloids, acts by inhibiting nucleic acid synthesis by targeting dihydrofolate reductase (Cushnie et al., 2014).

The mechanisms of action of toxicity of the different secondary metabolites may also vary considerably on the different metabolic systems in animals feeding them. Toxicity of these compounds may arise by enzymatic alterations that affect physiological processes, inhibition of DNA synthesis and repair mechanisms by intercalating with nucleic acids, or affecting the nervous system. Several secondary metabolites may also affect multiple functions of biological organisms (Mithöfer and Boland, 2012).

4 CONCLUSIONS AND RECOMMENDATION

The result clearly showed the presence of different secondary metabolites in the sugarcane leaf extract including alkaloids, tannins, flavonoids, cardiac glycosides, carbohydrates, and anthracene glycosides. These active compounds will have an effect on the toxicity as most of these compounds possess toxic effects as part of the defense mechanisms of sugarcane plants. Toxicity effects indicated an LC_{50} at 34.06 µL and the results indicated that concentration beyond this level is extremely toxic. Isolation and purification of the secondary metabolites may be conducted in the future to specify the compounds with its biological effects.

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