PHYTOCHEMICAL SCREENING, CYTOTOXICITY, ANTIOXIDANT, AND ANTHELMINTIC PROPERTY OF THE VARIOUS EXTRACTS FROM *Crescentia cujete* Linn. FRUIT

Merell P. Billacura*, Glenn Caezar R. Laciapag*
Mindanao State University-Main Campus, Marawi City, Lanao del Sur 9700, Philippines
*For correspondence; Tel. + (63) 9978292612, E-mail: merelljohn@gmail.com
*For correspondence; Tel. + (63) 9101289578, E-mail: glenncaezar@gmail.com

ABSTRACT: The fruit of *Crescentia cujete* Linn. is widely used by locals throughout certain parts of the Philippines who claim to have observed some of the health effects of its fruit such as antimicrobial, anthelmintic, and anti-cholesterol. This study was done to investigate some of these health effects. Phytochemical screening was done to support the results of the following tests: toxicity test by calculating LC_{50} using brine shrimp lethality test; antioxidant screening using thin-layer chromatography; and purgative test by anthelmintic assay using *Eudrilus eugeniae* as test organisms. Phytochemical screening revealed the presence of alkaloids, flavonoids, cardiac glycosides, reducing sugars, saponins, tannins, phytosterols, and terpenoids in the fresh *C. cujete* fruit. BSLT results showed that ethyl acetate extract is the most toxic and exhibits the highest bioactivity having LC_{50} of 1.50ppm during the first 6 hours of treatment. Thin-layer chromatography exhibited the presence of phenolic antioxidants in *C. cujete* fruit crude ethanolic extract. Anthelmintic assay shows that ethyl acetate extract at 20,000ppm has the least time to paralyze and kill the test organisms, 1.39 minutes and 2.39 minutes, respectively. This effect is also observed to be dosage dependent and is significantly comparable with the positive control Levamisole (Latigo-50) as referred to the statistical analysis using ANOVA and Tukey Test. The fruit of *C. cujete* Linn. is cytotoxic and bioactive, it is also a potential source of components which exhibit antioxidant and anthelmintic effects.

Keywords: Miracle fruit, *Eudrilus Eugenia, Artemia salina*, Levamisole

1. INTRODUCTION

Helminthic infections are among the most common infection in human beings, affecting a large proportion of the world's populations. Diseases caused by helminth parasites in livestock continue to be a major productivity constraint, especially in small ruminants in the tropical and subtropical countries. Anthelmintics or antihelmintics are drugs that expel parasitic worm (helminths) from the body. They may also be called vermifuges (stunning) or vermicides (killing). An anthelmintic will destroy or expel worms from the digestive system [1].

Looking back upon the last 2000 years of the history of medicine mankind has mainly used plants as the best source of medicine [2]. The importance of plant-derived medicines in modern medicine is often underestimated [3]. Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs [4].

The selection of the plant species to be studied is crucial for the reason that this step does not guarantee that the selected plant species exhibit a specific biological activity. Through theoretical evaluation such as medicinal folklore, ethnobotany, or primary experimentation, this selection is made. The fruit of *Crescentia cujete* Linn. was the result of this selection which is based primarily on the fact that not much experimentation has been done for this plant species. The fruit of *Crescentia cujete* Linn. also has a reputation of being used as traditional medicine to cure many diseases and other health related issues in the Philippines. Its effects include laxative, diuretic, purgative, anti-inflammatory, hypertension and diarrhea.

In this study we determine the presence of phytochemicals, cytotoxicity, antioxidant and purgative effect of the fruit using *Eudrilus eugenia* as test subjects.

2. MATERIALS AND METHODS

Preparation of the extracts

The fruits of *Crescentia cujete* Linn. were collected at Tibanga, Zamboanga del Norte (07'28'N 123'N'25'E). The fresh fruit of *C. cujete* Linn. was directly strained and filtered to get the fresh fruit extracts. Different ratios of the decoction were made; 3:1, 2:1, and 1:1 (m/v) ratios, respectively. For the ethanolic extract, the fruits were soaked in 95% ethanol and stirred to ensure proper distribution of ethanol. The crude extract was then concentrated *in-vacou* using rotary evaporator.

Solvent partitioning

A volume of 100mL of the crude ethanolic extract was taken and placed in a separatory funnel. The same volume of the solvent hexane was also added to the set-up. It was shaken for 15mins and allowed to stand. The flasks were opened and after a couple of minutes, after the separation is visible, the lower portion (aqueous portion) of the set-up was withdrawn and collected (labeled aqueous extract). The upper portion (hexane extract) was also collected in a separate flask. The aqueous extract was extracted with hexane repeatedly until the hexane layer was clear enough and that no particles are present on the hexane layer. The aqueous extract was again partitioned but this time using ethyl acetate as the extracting solvent. These different extracts were again concentrated *in vacou.*

Phytochemical screening

The phytochemical screening of the fresh fruit, decoction, crude ethanolic, aqueous, hexane, and ethyl acetate extracts was carried out to determine the presence of alkaloids, flavonoids, reducing sugars, cardiac glycosides, saponins, phytosterols, tannins, and triterpenes with the procedures described by Tiwari et al [5].

Detection of alkaloids (Dragendroff's Test)

Two milliliters of the aqueous, hexane and ethyl acetate extracts were dissolved individually in 2M hydrochloric acid and filtered. Two milliliters of the filtrates were treated with

6 drops of Dragendroff's reagent. Formation of orange precipitate indicates the presence of alkaloids.

Detection of saponins (Foam Test)

Two milliliters of the extracts were shaken with 2mL of distilled water. If foam produced persists for ten minutes it indicates the presence of saponins.

Detection of terpenoids (Salkowski's Test)

Two milliliters of the extracts were treated with chloroform. The extracts were treated with few drops of concentrated sulfuric acid, shaken and allowed to stand. Appearance of golden yellow color indicates the presence of terpenoids.

Detection of phytosterols (Libermann Burchard's Test)

Two milliliters of the extracts were treated with 2mL chloroform. The extracts were treated with few drops of acetic anhydride, boiled, and cooled. Then concentrated sulfuric acid was added. Formation of brown ring at the junction indicates the presence of phytosterols.

Detection of tannins (Ferric Chloride Test)

Two milliliters of the extracts were treated with 3-4 drops of 5% ferric chloride solution. Formation of brown color indicates the presence of tannins and phenols.

Detection of flavonoids (Alkaline reagent Test)

Two milliliters of the extracts were treated with few drops of 0.1N sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicates the presence of flavonoids.

Detection of cardiac glycosides (Keller Killiani's Test)

Two milliliters of the extract was dissolved in 1mL glacial acetic acid containing 1 drop of 5% ferric chloride solution and was underlayered with concentrated sulfuric acid. A brown ring obtained in the interface indicated the presence of cardiac glycosides.

Detection of reducing sugars (Fehling's Test)

Two milliliters of the extracts was added with 1mL of both Fehlings A, and B and was then shaken and heated in a water bath for ten minutes. Formation of a brick-red precipitate indicates the presence of reducing sugars.

Brine shrimp lethality test

Ten nauplii were counted macroscopically in the steam of the pipette. Three milligram of dry yeast was placed in 3mL artificial seawater to make a suspension as a food for the nauplii. The nauplii were transferred to the test tubes previously prepared and the control vial by means of a pipette. Artificial seawater was placed in each sample and control vials to make a total volume of 5mL at a concentration of 10, 100 and 1000ppm. A drop of the yeast suspension was added to the vials as a food for the nauplii and the vials were placed under illumination. The number of survivors was counted after 6hrs and 24hrs. Then percent deaths were determined for each extracts and for each concentration along with the control vials.

Determination of the median lethal concentration (LC_{50})

The LC_{50} was determined using probit analysis method described by Finney [6]. If deaths occur in the control vials, the data was corrected using Abbots formula:

$$\%death = \frac{\%death\ in\ test\ vial - \%death\ in\ control\ vial}{100 - \%death\ in\ control} x\, 100 \quad (1)$$

Graph was obtained by plotting %deaths against concentration for each extracts. Then the LC_{50} was derived from the best-fit line obtained by linear regression analysis.

Antioxidant screening of the crude ethanolic extract

The screening of the antioxidants present in the crude ethanolic extract using thin-layer chromatography was carried out using the procedures described by Guevarra [7]. The developing chamber was first saturated with solvent in the dark for 30 minutes. The following solvent systems were used: ethyl acetate- ethanol, and hexane-ethanol (v/v). Two milliliters of the extracts was evaporated to incipient dryness over a steam bath. Then the extract was spotted on the TLC plates along with the standard Myra-E 400. The chromatogram was developed in the developing chamber equilibrated with the solvent. The plate was removed after the solvent reached the solvent front. The chromatogram was then sprayed with the spraying reagent (mixture of 1% Iron (III) Chloride and 1% Potassium Ferricyanide). The blue spots produced signify the presence of antioxidants.

Anthelmintic assay

Five *Eudrilus eugenia* of 3-5cm in length and 0.1-2cm in width were placed in labeled petri dishes, and subjected to the extracts at 5,000, 10,000, and 20,000ppm concentrations. Observations were made for the time taken to paralyze and death of individual test organism. Paralysis is marked by decrease in vigorous wriggling of the worm or slow movement of the worm after being pricked with pin. No movement indicates death, as well as the change in the flesh color from light red to pale. Levamisole and distilled water were used as controls.

Fourier transform infrared spectroscopy (FTIR) – Agilent Cary 630

The samples were prepared by obtaining a drop of the extracts and then letting it evaporate to incipient dryness by exposing it in the atmosphere and was then subjected to FTIR Agilent Cary 630 spectrometer. The resulting spectra were printed and analyzed.

3. RESULTS AND DISCUSSION

Phytochemical screening

Table 1 shows that the fresh fruit sample exhibited the presence of all tested secondary metabolites namely: alkaloids, cardiac glycosides, flavonoids, phytosterols, reducing sugars, saponins, tannins, and triterpenes in slight and moderate appearances.

Legend: (-) absence, (+) slight appearance, (+)(+) moderate appearance. FFE-fresh fruit extract, DFE-decocted fruit extract, CEE-crude ethanolic extract, AE-aqueous extract, HE- hexane extract, EAE- ethyl acetate extract.

The decocted fruit, exhibited the presence of all the tested secondary metabolites except saponins. The extracts showed varying results in terms of what secondary metabolites are present. Although it is difficult to establish definitive relationship between structure and biological activity due to the occurrence of a vast number of phytochemicals with similar chemical structures, and to the complexity of physiological reactions, as stated by Saxena et al [8], many papers describe some of the definitive functions of the phytochemicals present in plants. The presence of tannins

Table 1: Phytochemical screening of the fresh, decocted, and various extracts of *Crescentia cujete* Linn.

Secondary metabolites	Results					
	FFE	DFE	CEE	AE	HE	EAE
Alkaloids	++	++	+	+	+	+
Cardiac glycosides	+	+	+	+	+	+
Flavonoids	++	+	-	-	-	+
Phytosterols	+	+	+	-	+	+
Reducing sugars	+	+	++	+	+	-
Saponins	+	-	-	-	-	-
Tannins	+	+	+	+	-	++
Terpenoids	+	+	-	-	+	-

showed that the sample is a good potential source of components having anthelmintic and antioxidant properties [8-9]. The presence of phytosterols showed that the sample is a good cholesterol-lowering agent [10]. The presence of alkaloids, saponins, flavonoids, and terpenoids showed that the Crescentia fruit has antimicrobial as well as antioxidant effects [8]. Because of this, protective and antimutagenic effects of *Crescentia cujete* Linn. fruit can also be tested. The presence of reducing sugars also has a hypoglycemic effect for the reason that the aldehyde, in the form of glucose in this case is oxidized into carbonyl group.

Brine shrimp lethality test

Shown in Table 2 the results of the brine shrimp lethality test of the fresh, decocted fruit, and various extracts of the fruit of *Crescentia cujete* Linn. Death counts at 6 hours and 24 hours after treatment are noted and converted to %mortality and corrected if the control (artificial seawater) exceeds 10% mortality. Percent mortality was then converted to probits using the methods described by Finney [6].

Observations within 6 hours allow us to evaluate which extract exhibited the highest bioactivity or which extract has the most bioactive components. In other words, it allows us to see which extracts took effect the fastest. The faster the brine shrimps began dying, the more bioactive the extract is.

Observations within 6 hours implies that ethyl acetate extracts exhibited the most bioactivity having an LC_{50} value of 1.50ppm. This indicates that it takes 1.50ppm of the ethyl acetate extract to kill half of the brine shrimp population. After 24hours, calculation of the LC_{50} value for all extracts resulted in 0.00 for hexane and ethyl acetate extract, depicting that all of the test organism have died. The crude ethanolic extract has an LC_{50} value of 0.529ppm. The aqueous extract exhibited an LC_{50} value of 4.64ppm. Thus, all extracts showed LC_{50} values lower than 1000 which means that all are bioactive and toxic.

Antioxidant screening using thin-layer chromatography

Figure 1 shows that hexane-ethanol solvent system exhibited more separation when hexane is in larger amount. The ethyl acetate-ethanol solvent system also exhibited separation but not as hype as compared to the hexane-ethanol solvent system.

Table 2: Brine shrimp lethality test data

Extracts	Conc, Ppm	After 6 hours		After 24 hours	
		% Death	LC_{50}	% Death	LC_{50}
Crude ethanolic extract	1,000	97%	19.8	100%	0.529
	100	80%		100%	
	10	36.7%		84%	
Aqueous extract	1,000	100%	19.9	100%	4.64
	100	96.7%		100%	
	10	20%		68%	
Hexane Extract	1,000	100%	2.49	100%	0.00
	100	93.3%		100%	
	10	80%		100%	
Ethyl acetate Extract	1,000	100%	1.50	100%	0.00
	100	100%		100%	
	10	73.3%		100%	
Decoction	3:1	100%	0.00	100%	0.00
	2:1	100%		100%	
	1:1	100%		100%	
Fresh		100%	0.00	100%	0.00
Seawater (38,000ppm)		0%		26.7%	
Seawater (Control for 10ppm)		0%		16.7%	

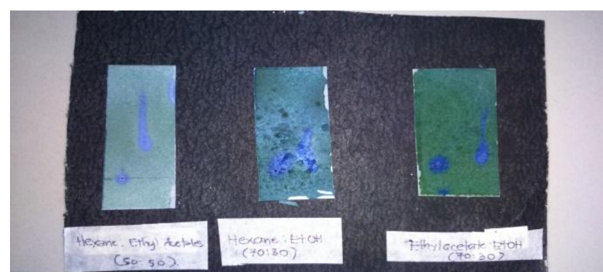

Fig (1) Antioxidant screening of crude extract of *Crescentia cujete* Linn fruit using different solvent systems. *The right spot found in each plate is from the standard Myra-E 400, while the left spot is from the crude extract of the Crescentia cujete Linn. fruit*.

The crude ethanolic extract shows positive result indicated by the blue coloration on left side of the TLC plate in every solvent systems used. The right side of the TLC plate shows the elution pattern of the standard used which is Myra-E 400. Based on the study of Al-jaber et al [11] the most important free radical in biological systems is radical derivatives of oxygen that is why phenolic compounds possess the largest group among any other phytochemical groups in such activity. Antioxidants protect the cells and organs systems of the body against reactive oxygen species [12].

Anthelmintic assay

Table 3 shows that among the extracts, ethyl acetate at 20,000ppm has the least time to paralyze the test organism having average paralysis time of 1.39 minutes. For the positive control, Levamisole (Latigo-50) at 20,000ppm was

used and has an average paralysis time of 2.93 minutes. The fresh fruit of *C. cujete* Linn. has an average paralysis time of 7.03 minutes. For the decoction, the 3:1 (m/v) ratio between sample and water has the shortest paralysis time of 29.37 minutes.

The crude ethanolic, aqueous, and hexane extracts, along with the decocted fruit, and distilled water have significantly different time to paralyze the test organism, *Eudrilus eugeniae*, which indicates that they do not have comparable effects as compared to the positive control, Levamisole. While DMSO and ethyl acetate have significantly the same paralysis time. Based on the results, DMSO has significant effects on the paralysis of the test organisms which means that the effects of ethyl acetate extract in paralyzing the test organism is not solely due to its bioactive components but also with DMSO. DMSO and ethyl acetate extract may have exhibited synergistic effects in paralyzing the test organisms as showed by the paralysis time of 1.39 minutes which is faster than the positive control.

Table 3: Mean average paralysis and death time using comparison among the different treatments

Treatments	Conc., ppm	Paralysis time, minutes	Death time, minutes
CEE	5,000	22.20	54.92
	10,000	13.75	22.48
	20,000	8.73	16.67
AE	5,000	20.61	**
	10,000	13.31	148.15
	20,000	9.38	11.71
HE	5,000	21.79	**
	10,000	14.64	**
	20,000	5.12	561.81
EAE	5,000	2.77	9.64
	10,000	1.67	3.78
	20,000	1.39	2.59
DFE	1:1	62.25	**
	2:1	38.97	72.05
	3:1	29.37	78.99
FFE	Crude	7.03	52.94
Levamisole (Latigo-50)	5,000	4.29	7.48
	10,000	4.48	7.42
	20,000	2.93	6.66
DMSO	5:50	7.18	118.57
Distilled water		*	**

Legend: (*) indicates no paralysis after 10 hours, (**) indicates no death after 24 hours. CEE – Crude ethanolic extract, AE – Aqueous extract, HE – Hexane extract, EAE – Ethyl acetate extract, DFE – Decocted fruit extract, FFE – Fresh fruit extract. For the decoction, (1:1) indicates that 100g of sample was dissolved per 100mL of distilled water, (2:1) indicates that 200g of sample was dissolved per 100mL distilled water, and (3:1) indicates that 300g of sample was dissolved per 100mL of distilled water. For the control, (5:50) indicates that 5mL of dimethyl sulfoxide (DMSO) was dissolved in 50mL of distilled water the same as the one used in preparation of the ethyl acetate extract.

Table 3 also shows that among the extracts, ethyl acetate at 20,000ppm has the shortest death time at 2.59 minutes. The positive Levamisole at 20,000ppm has a death time of 6.69minutes. The fresh fruit has a death time of 52.94 minutes and for the decoction, the 2:1 (m/v) ratio displays the shortest death time of 72.05 minutes.

The statistical results show that as compared to the positive control, the crude ethanolic extract, ethyl acetate, decoction, fresh, and control (DMSO) are significantly the same showing significant values greater than 0.05. The statistical results do not align with the death time data. This is because the treatments that show no death of the test organisms within 24 hours are marked 0.00 which is close to the mean difference death time value of Levamisole which is 7.12 minutes.

In general, the fruit of *C. cujete* Linn and its extracts exhibited anthelmintic properties except for the hexane extract. This was due to the absence of tannins in the hexane extract. These anthelmintic properties were found out to be dosage dependent which means that as the concentration increases, the time it takes to paralyze and kill the test organisms shortens.

Fourier-transform infrared spectroscopy - Agilent (FTIR)

The functional group of the chemically active compound present in the extracts as well as the fresh and the decocted fruit of *C. cujete* Linn. was determined using Fourier-transform infrared spectroscopy (FTIR). The FTIR spectrum was obtained at wavenumber range of 4000nm to 650nm. All spectra showed the presence of an alcoholic –OH functional group as well as an alcoholic C-O at absorbance 1000cm-1. Alcohols have a characteristic band in the range 3400 to 3650 cm-1 that is usually broad and intense. If present, it's hard to miss this band or to confuse it with anything else [13]. Shown in Figure 2 the spectra of ethyl acetate extract of the fruit of *C. cujete* Linn.. The spectra shows a characteristic –OH functional group at 3309.9 cm-1 and a sharp, intense peak at 1684.8 cm-1, probably caused by a C=O carbonyl group. Also apparent is the presence of a benzene ring as noted by the weak absorption at 1513.3 cm-1 and a medium absorption at 1449.9 cm-1. This indicates that aromatic compounds are present in the ethyl acetate extracts of *Crescentia cujete* Linn.

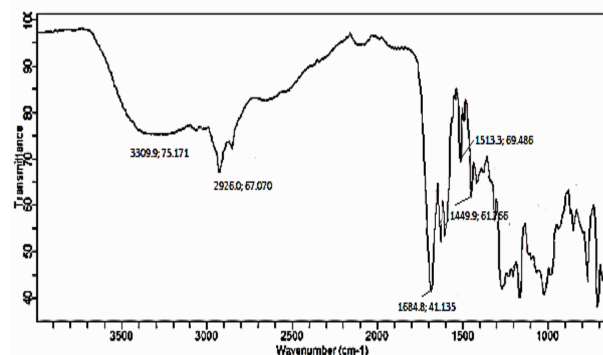

Fig (2) Infrared spectra of the ethyl acetate extract of the fruit of *Crescentia cujete* Linn.

4. CONCLUSIONS

Phytochemical screening of the fruit of Crescentia cujete Linn revealed the presence of all the tested extracts namely; alkaloids, flavonoids, cardiac glycosides, tannins, reducing sugars, phytosterols, terpenoids, and saponins. BSLT showed that all extracts are bioactive and caused death to the nauplii after 24 hours of treatment. The crude extract of the fruit of

Crescentia cujete Linn. was tested for its antioxidant activity using thin-layer chromatography and exhibited the presence of antioxidants in every solvent system used. The anthelmintic assay showed that ethyl acetate extract has the least mean paralysis time and mean death time for the highest concentration tested-20,000ppm. Using FTIR analysis it has been found out that *Crescentia cujete* Linn. contains alcohols, alkanes, and aromatic compounds.

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