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Comparative Cytotoxic Properties of Two Varieties of *Carica papaya* leaf extracts from Mindanao, Philippines using Brine Shrimp Lethality Assay

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ABSTRACT
Phytotherapy has currently led to a resurgence of scientific interest in treating diseases. Carica papaya (papaya) leaf extract has been traditionally used to cure inflammatory and microbial illnesses however recently, it is taken orally to cure dengue, an epidemic viral disease in the tropics like the Philippines. Despite its extensive use, there is still little information about its cytotoxic properties. In vivo toxicity study of the C. papaya leaf extract is still lacking and only a few in vitro studies have been conducted for its anti-dengue efficacy and cytotoxic effect. In terms of its safety for consumption, only the sekaki variant of Malaysia has been notably tested. Thus, this current study sought to evaluate the comparative cytotoxic properties of the two varieties of C. papaya ('Cavite Special' and 'Sunrise Solo') popularly grown in the Philippines, using Brine Shrimp Lethality Assay. The degree of percent lethality was found to be directly proportional to the concentration of the extract in both papaya varieties. Maximum mortalities took place at a concentration of 1000 µg/ml and minimum percent mortality was at 10 µg/ml ('Cavite Special': decoction 92.86%, ethanolic 57.1%, hydroethanolic 10%; 'Sunrise Solo': decoction 93.94%, ethanolic 66.66%, hydroethanolic 71.43%). Decoction extracts from both variants exhibited lowest LC_{50} values ('Cavite Special' LC_{50} = 421 µg/ml;'Sunrise Solo' LC_{50} = 132 µg/ml). 'Sunrise Solo' papaya variant exhibited higher brine shrimp mortality and lower LC_{50} value compared to 'Cavite Special', hence it is more cytotoxic.This study confirms the easy and convenient use of brine shrimp (Artemiasalina) on assessing bioactivity and cytotoxicity of medicinal plants.
Keywords: *Carica papaya, Artemiasalina, phytotherapy, cytotoxicity, leaf extract, dengue, LC_{50} values*

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INTRODUCTION

Natural products have long been recognized since ancient times as potential sources of human drugs. Recently, phytotherapy or the use of medicinal plants in curing illnesses was given more attention since it is effective, safe and non-toxic[1, 2, 3, 4, 5, 6 and 7]. According to World Health Organization (WHO) fact sheet dated December 2008, 80% of the population in some Asian and African countries depend on traditional medicine as their primary health care due to economic and geographical constraints [8].

Carica papaya, commonly known as papaya of the Family Caricaceae has been used for centuries to remedy inflammatory and microbial ailments and has demonstrated anti-cancer activity[9, 10]. Currently, fresh papaya leaf extract is taken orally in 2 tablespoons every 12 hours by most of the people in dengue-epidemic localities in the Philippines. This practiced herbal remedy is supported by the significant results in increasing blood platelet in dengue fever patients in current studies performed in Malaysia, Pakistan, Indonesia and Sri Lanka[11, 12, 13, 14, 15, and 16]. However, toxicity studies of the *C. papaya* leaf extracts are still lacking and accordingly, there is still no *in vitro* studies in terms of its cytotoxic effect. Further, esophageal perforations, mucosal irritation and laxative effect are reported if used excessively and at high doses. If these are the documented effects, appropriate application must be undertaken in patients with dengue fever especially that only the *sekaki* variant of Malaysia which is noted to be safe for oral consumption [17, 18].In this study, the two most commonly grown papaya variants in the Philippines, the

'Cavite special' and *'Sunrise Solo'* were used to evaluate its cytoxicity potential [19]. It is hypothesized in this paper that other varieties of the papaya as well as its geographical location could be dissimilar in terms of its toxicity effect from the initially identified safety level of the *sekaki* variant as there's a need to substantiate the ethnopharmacological use since dosage forms and efficacy of these herbal plant preparations are usually not clearly defined despite the common use for therapy. Brine Shrimp Lethality Assayis considered a useful tool for preliminary assessment of toxicity and could provide basis for the preferable concentration of the papaya leaf extract depending on the therapeutic need of the individual, be it for dengue fever or for cancer [20].

MATERIALS AND METHODS

Collection and Identification of the Plant Material

For the purpose of this study, a private plantation was identified to ensure similar source of authenticated raw material used. *'Cavite special'* papaya leaves were taken from a plantation in Naawan while *'Sunrise Solo'* was collected from Liboran, Baongon (Figure 1). Both areas are part of Misamis Oriental province, the topmost papaya-producing province in the Philippines [21]. The papaya variety was identified and validated by an expert.

a **b** **c**

Figure 1. Papaya plantation (a) *'Cavite special'* papaya plantation intercropped with coconut trees in Naawan, Misamis Oriental; (b) papaya leaf structure and (c) *'Sunrise Solo'* papaya plantation in Liboran, Baongon, Misamis Oriental.

'Cavite Special' papaya is a popular semi-dwarf and hermaphrodite type, with yellow-orange fruit weighing 3-5 kilos. Papaya-growing companies usually processed their fruits in fruit cocktail cans. Most local consumers and growers prefer these large-fruited papayas hence this variety is widely dispersed and mostly run on a backyard scale. On the other hand, *'Sunrise Solo'* papaya is also a hermaphrodite type and is so-called because one fruit (about 0.45 kg) is enough for one individual's consumption. It produces high quality fruits with excellent flavor, which is suited for the export market. In fact, the Philippine export variety for fresh papaya is based mainly on *Solo* papaya, which is grown in large farms owned by multinational companies [21].

Preparation of the Leaf Extracts

Fresh and dried leaves of each papaya variant were utilized. Decoction was done to 500 grams of blended fresh leaves in 1L of distilled water. It was allowed to boil for 5 minutes and allowed to cool before filtering. Freeze-drying was done to the filtered samples.

For the air-dried papaya variants, 500 grams were separately submerged in two different extracts: 95% ethanolic extract and hydroethanolic extract (50% EtOH:50% water). It was soaked for 3 days in a freezer before being subjected to rotary evaporation. Filtration was done afterwards. For the 50:50 hydroethanolic extract, freeze drying was done after filtering.

The stock solution was prepared by dissolving 0.03 g extract in 3 mL sterilized seawater to make 10, 000 µg/ml solution, to which subsequent concentration of 10, 100, 500 and 1000 µg/ml were obtained by dilution. This chosen wide range concentration of the plant extract is based on WHO standard protocol. For decoction and hydroethanolic extract, sterilized sea water was added. Sonicator and magnetic stirrer were used to better dissolve the solution. For the ethanolic extract however, 3 ml of 95% ethanol was added to dissolve the extract. Purging of the ethanol was done with the use of nitrogen gas. One to three

microdrops of dimethyl sulphoxide (DMSO) was then added. Figure 2 shows the documented preparation of the leaf extracts.

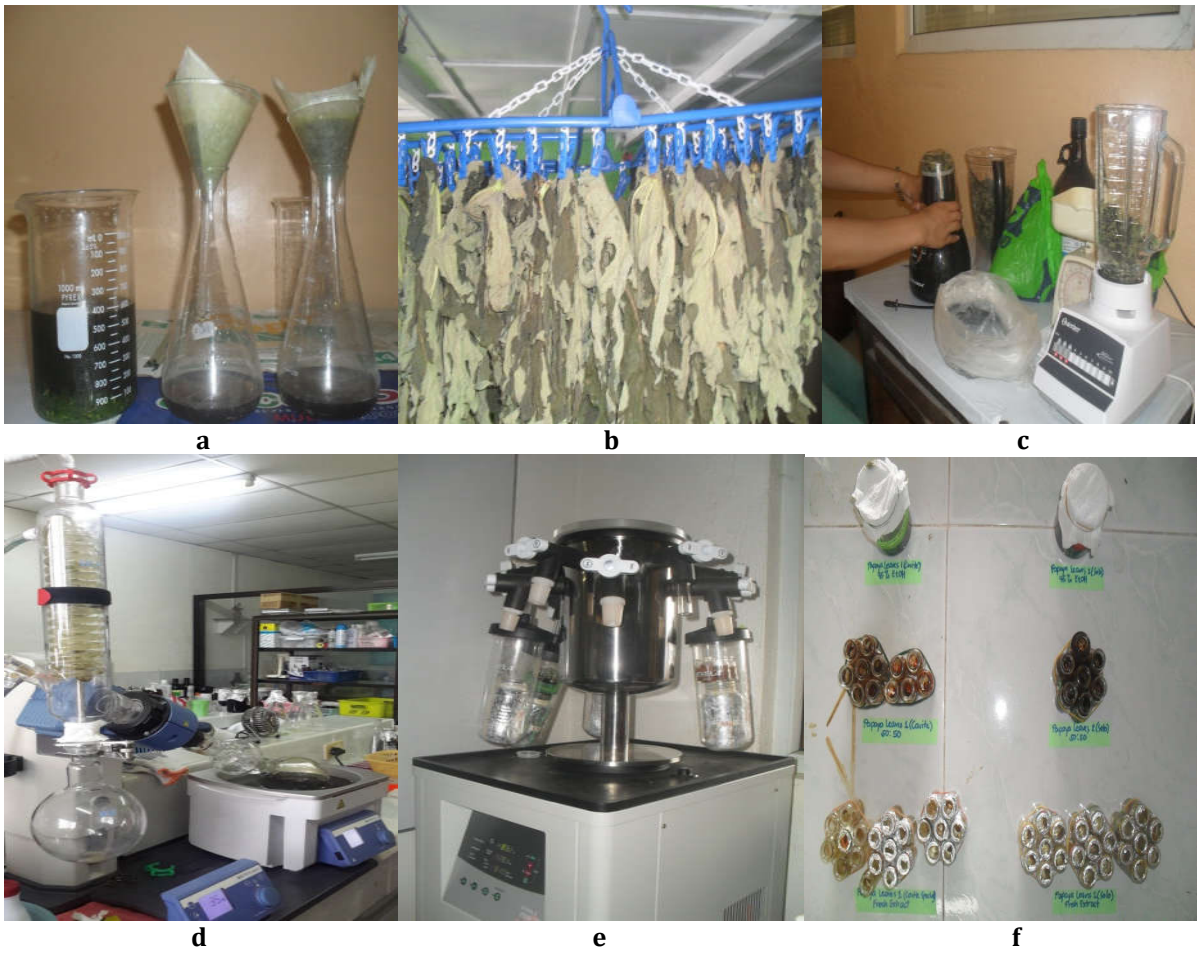

Figure 2. Preparation of the Papaya Leaf Extracts. (a) filtration after decoction of fresh leaves; (b) air-drying for 2 weeks; (c) blending of the air-dried leaves; (d) rotary evaporation; (e) freeze-drying; (f) separate vials ready for BSLT.

Brine Shrimp Lethality Assay

Artemiasalina cysts were activated in an improvised tank with sterilized sea water for 24 hours. Ten nauplii (< 48 h old brine shrimp larvae) were drawn through a glass capillary and placed in each calibrated test tube containing varying diluted solutions of the leaf extracts. The final volume was made up to 5ml using sterilized sea water. Experiments were conducted along with control, which include test tubes containing sterilized seawater only, and sterile seawater with 1 drop of DMSO, as well as 2 and 3 drops of DMSO.

These brine shrimp nauplii were then exposed to sample solutions for 24 hours in a warm ventilated room under a light source. Frequencies of immobility of the 10 nauplii in 5 ml solutions were scored. Each sample was tested in triplicate and incubated for 24 hours and finally, the tubes were examined under a magnifying glass and the number of dead shrimps was counted. The brine shrimp lethality from papaya leaf extract exposure was determined using the procedure of Meyer *et al.* (1982).

Lethal Concentration Determination

The median lethal concentration (LC_{50}) of the brine shrimp nauplii to different extracts was derived from two graphical procedures. The procedure plots the number of the accumulated survivors and the number of accumulated deaths on the same axes (number of animals) against log dose. The 2 curves will cross at the dosage (log concentration) where the number of survivors equals to the number of deaths (22].

RESULTS AND DISCUSSION

Percent lethality and the LC_{50} values of the brine shrimp obtained after chronic exposure to varying concentrations of the leaf extracts (decoction, 50:50 hydroethanolic extract and ethanolic extract) of *'Cavite Special'* and *'Sunrise Solo'* papaya variants are shown in Table 1.

Table 1. Percent mortality and Chronic LC_{50} Values of the Brine Shrimp exposed to varying leaf extract concentrations of the *'Cavite Special'* And *'Sunrise Solo'* Variants.

Papaya leaf extract variants	Concentration of the Extract (ppm)	Brine shrimp mortality after 24 hrs. (%)	Chronic LC_{50} (ug / ml after 24 hrs)
'Cavite Special'	Decoction		
	1000	92.86	421
	500	68.42	
	100	9.3	
	10	1.45	
	Ethanolic		
	1000	57.1	444
	500	11.11	
	100	0	
	10	0	
	Hydroethanolic (50:50)		
	1000	10	>1000
	500	0	
	100	0	
	10	0	
'Sunrise Solo'	Decoction		
	1000	93.94	132
	500	72	
	100	28.57	
	10	17.14	
	Ethanolic		
	1000	66.66	442
	500	31.58	
	100	14.86	
	10	5.38	
	Hydroethanolic (50:50)		
	1000	71.43	439
	500	12.5	
	100	0	
	10	0	

The degree of percent lethality was found to be directly proportional to the concentration of the extract. The least mortalities were at 10 µg/ml concentration, both in the two varieties of papaya. Maximum mortalities took place at a concentration of 1000 µg/ml in both papaya variants. Results further reveals that at 1000 µg/ml, *'Sunrise Solo'* papaya variant exhibited higher brine shrimp percent mortality compared to *'Cavite Special'* (mortality: 93.94% vs92.86%; 66.66% vs57.1%; and 71.43% vs 10%) in decoction, ethanolic and hydroethanolic extracts respectively. This means that *'Sunrise Solo'* exhibited greater toxicity and may contain more active cytotoxic components than *'Cavite Special'*. Decoction extracts show highest mortality among brine shrimp nauplii in both papaya variants at 1000 µg/ml. Fresh, green leaves as specified in the works of Ayoola & Adeyeye [24] and Nguyen *et al.* [23] contain saponins. Accordingly, the presence of saponins, which give the green leaves its bitter taste support the fact that it has cytotoxic effects such as permealization of the intestine as saponins are cytotoxic [23, 24].

Computation of the LC_{50} values after 24 hours for each of the variants shows that the decoction extract of *'Cavite Special'* exhibits higher cytotoxic activity than the other two extracts of the same variant having an LC_{50} value of 421 µg/ml. The same was observed in *'Sunrise Solo'* for its decoction extract having an LC_{50} value of 132 µg/ml. According to Meyer et al.(1982), crude plant extract with LC_{50} value of less than 1000 ppm is considered toxic (active) while value higher than 1000 ppm is considered non-toxic (inactive).

Further, other researchers state that the cytotoxic activity is considered weak when the LC_{50} is between 500 and 1000 µg/ml, moderate when the LC_{50} is between 100 and 500 µg/ml, strong when the LC_{50} ranged from 0 to 100 µg/ml and designated as non-toxic when the LC_{50} value is greater than 1000 µg/ml [25, 26]. Hence, all extracts except that of *Cavite Special* hydroethanolic leaf extract possessed moderate cytotoxicity towards the brine shrimp nauplii. However, it is remarkable to note that decoction extracts of both variants exhibited lowest concentrations in their LC_{50} values, with decoction extract of *'Sunrise Solo'* having the lowest LC_{50} thus, has greater cytotoxic potential compared to *'Cavite Special'*. These are supported by the works of Halim, *et al.* (2011), Owoyele*et al.* (2011) and Tarkang*et. al.* (2010) which reveals that the ethanolic extract of C. papaya leaves is less toxic than the other two extract types tested (decoction and hydroethanolic). It should be noted also that each of the variant's ethanolic and hydroethanolic extracts have lower cytotoxic properties and *'Cavite Special'*ethanolic and hydroethanolic leaf extracts possess much lower cytotoxicity than *'Sunrise Solo'*[10, 17 and 25].

In a recent phytochemical examination of the cytotoxic properties of *C. papaya* leaf extract constituents by Nguyen *et al.* (2014), it was reported that ethanolic and chloroform extract contain alkaloids, flavonoids, tannins and saponins, while water extract contains tannins and saponins only. These secondary metabolites are considered to be cytotoxic, which are metabolized by plants in response to herbivory [23]. *'Sunrise Solo'* papaya leaves may contain greater amount of these secondary metabolites. This may help to explain its higher cytotoxicity than the *'Cavite Special'*. Methanolicextract containing triterpenoids and flavonoids also exhibits cytotoxic effects against DENV2 growth in *in vitro* conditions [18]. Further, air-dried papaya leaves are the best as a tonic and blood purifier as stated by Ayoola and Adeyeye (2010), which supports the belief that it can increase blood platelet count in dengue fever patients. Alkaloids present in air-dried ethanolic extracts can be an effective anti-malaria or anti-dengue since alkaloids consist of quinine and quercitin [24].

This present study shed scientific light to the initially recent practice on consuming papaya leaf extract by dengue fever patients in the Philippines. The finding in this study suggests that the extract may possess some cytotoxic activities at higher dose (1000 µg/ml) and therefore may have a potential for therapeutic investigation. However, as to the safety level of consumption based on consuming fresh extract is still to be explored further.

Further work, including confirmation via authentic standard comparison and tandem mass spectrometry, as well as cytotoxicity studies of pure compounds will confirm the identity and the therapeutic activities of the bioactive compounds in *Carica papaya* leaves.

CONCLUSION

This study investigated on the cytotoxic properties of the extracts of two varieties of *Carica papaya* specifically the '*Cavite Special'* and '*Sunrise Solo'* variants. Based on computations of percent mortality and LC_{50}, the *'Sunrise Solo'* variant exhibited the highest cytotoxic potential particularly on its decoction extract which remarkably draws more interest since several evidences have shown that ethanolic extracts exhibit higher cytotoxicity compared to decoction and hydroethanolic extracts. Hence, further investigation is required to explore its cytotoxic components and the factors causing such phenomenon. Moreover, the use of pure and concentrated papaya leaf extracts as medicine needs to be examined more thoroughly since this study's findings show that even the decocted extract elicits moderate toxicity which may have health hazards that can be overlooked or whether it may have long term effects. Additionally, this study confirms the easy and convenient use of brine shrimp (*Artemiasalina*) on assessing bioactivity and cytotoxicity of medicinal plants.

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