



ANTIBACTERIAL AND CYTOTOXIC ACTIVITIES OF *Carica papaya* L. (PAPAYA) SEEDS

Sheikh Ashikur Rahman and Md. Shamim Akhter*

Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna 9208, Bangladesh

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Abstract: The crude ethanolic and methanolic extracts of *Carica papaya* L. seeds were investigated to evaluate their antibacterial and cytotoxic potentialities. The average zone of inhibition for ethanolic extract of *C. papaya* seed ranged 6.25 – 9.75 mm for 500 µg/disc and 8 -14.75 mm for 1000 µg/disc. With methanolic extract the zone of inhibition ranged 7.75 – 8.50 mm for 500 µg/disc and 11.25 – 14.50 mm for 1000 µg/disc. Again from minimum inhibitory concentration (MIC) assay (Resazurin assay), the MIC was found to be 0.625 mg/ml for ethanol and 0.312 mg/ml for methanol extract. The minimum bactericidal concentration (MBC) was found to be 1.25 mg/ml for ethanol and 0.625 mg/ml for methanol extract. Compared to vincristine sulphate (with LC₅₀ of 1.20 µg/ml) both ethanolic and methanolic extract of *C. papaya* seeds showed toxicity higher than 100 µg/ml. The study affirmed potential antibacterial property of *C. papaya* seed extracts with mild cytotoxic activity. These findings could be correlated with the traditional medicinal uses of papaya seeds and showed the rationale for further investigation for screening out the possible bioactive constituents.

Keywords: Antibacterial activity, brine shrimp lethality bioassay, MIC, MBC, *Carica papaya*

Introduction

Papaya, originating in southern Mexico and Costa Rica was subsequently introduced into subtropical and tropical regions (Krishna *et al.*, 2008). Papaya contains a wide range of phyto-chemicals which have many medicinal and pharmacological properties. Leaf and flower have antioxidant properties (Devi *et al.*, 2011; Ayoola *et al.*, 2008). The most common use of papaya fruit is to expel worm from human body as it contains an enzyme called 'papain' having the potential activity against worms. Leaf, root, flower have antibacterial properties against bacteria like *Salmonella typhi*, *Klebsiella pneumonia*, *Bacillus subtilis*, etc. (Nirosha *et al.*, 2013; Devi *et al.*, 2011; Anibijuwon *et al.*, 2009). Papaya has wound healing properties (Nayak *et al.*, 2012) and due to this property it has been traditionally used by folk medicinal practitioners from the ancient period of time to heal severe wound. It has been reported that different parts of *C. papaya* have other medicinal applications like anticancer, hepatotoxicity reducing, anti-amoebic, hypoglycaemic, anti-fertility and anti-malarial activities (Udegbumam *et al.*, 2014; Juarez-rojop *et al.*, 2012; Kovendan *et al.*, 2012; Otsuki *et al.*, 2010; Adeneye *et al.*, 2009; Tona *et al.*, 1998;).

*Corresponding author: <shamim11akhter@gmail.com>

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The objective of this study was to investigate the antibacterial and cytotoxic properties of ethanolic and methanolic crude extracts of *C. papaya* L. Seeds to establish scientific relevance of the uses of papaya seeds in traditional medicine.

Materials and Methods

There are a number of methods available to determine antibacterial activity of any substance. Majority of the researchers used one of the following *in vitro* assays: disc diffusion, broth dilution and agar dilution method to determine antibacterial activity. But the most popular method is disc diffusion method (Bauer *et al.*, 1996). And in addition to agar disc diffusion assay, MIC and MBC assay are recommended (Sarker *et al.*, 2007).

Cytotoxicity of a sample can be determined by 'brine shrimp lethality' assay (Michael *et al.*, 1956) which has a good correlation with cytotoxic activity on tumors in human body (Meyer *et al.*, 1982).

Collection of sample: Seeds of *C. papaya* L. (Lal Teer Seed Limited, Dhaka, Bangladesh) (variety 'Red lady') were purchased from the local seed market of Daulatpur in Khulna, Bangladesh.

Preparation of sample: Seeds were washed three to four times consecutively with distilled water. Then seeds were dried 2 to 3 days under mild sunlight. Dried seeds were powdered using mortar and pestle.

Preparation of extract: Fifty gm of powdered sample was soaked in 100 ml of 50% ethanol and similarly 50 gm of the sample was soaked in 100 ml of 50% methanol. Contents were then kept in water bath at 50°C for one and a half hours. Then the contents were cooled and filtered through Whatman filter paper. The filtrates obtained were evaporated by rotary evaporator under reduced pressure at 40–45 °C and then air dried. The air dried extract were weighed and 3 gm and 2.75 gm of seed extracts were obtained from ethanolic and methanolic extractions, respectively. The extracts were then stored into a refrigerator at 4°C.

Antibacterial screening: Antibacterial testing of crude extracts was performed using agar disc diffusion method. Seven pathogenic bacterial strain including 5 gram negative and 2 gram positive bacteria (*Pseudomonas aeruginosa*- ATCC (American Type Culture Collection) 27833, *Staphylococcus epidermidis*- ATCC 12228, *Proteus vulgaris*- ATCC 13315, *Escherichia coli*- ATCC 8739, *Salmonella paratyphi*- ATCC 13311, *Vibrio cholera*- ATCC 14035 and *Staphylococcus aureus*- ATCC 25923) were chosen for testing. The bacteria were maintained on nutrient agar media by streak plate method (Waksman *et al.*, 1945).

The sterile filter paper disc of 5 mm were impregnated with 500 µg and 1000 µg of each of the test substances and dried under aseptic condition to evaporate residual solvent. Standard azithromycin (30µg/disc) were used as positive control and blank disc were used as negative control. The sample discs, antibiotic discs, negative control discs were gently placed onto nutrient agar plate which was pre-inoculated with test bacteria. The plates were inversely kept in incubator at 37° C for 24 hours. The antibacterial activity was determined by measuring the diameter of zone of inhibition (Wilkinson *et al.*, 2006).

Minimum Inhibitory Concentration (MIC) assay : For supporting and ensuring the results obtained from agar disc diffusion assay, MIC assay was performed using the Resazurin 96-well micro-titre plate based in vitro antimicrobial assay (Sarker *et al.*, 2007). All bacterial strains were cultured on nutrient agar and incubated for 24 hr at 37° C prior to MIC determination. Ciprofloxacin was used as a positive control. Resazurin solution was prepared by dissolving 4 mg of Resazurin in 20 ml of sterile distilled water and was used in this assay as an indicator of cell growth (Sarker *et al.*, 2017). A sterile 96 well plate was labelled. A volume of 100 µl of test material in 10% (v/v) Dimethyl Sulfoxide (DMSO) was pipetted into the first row of the plate. To all other wells, 50 µl normal saline was added. Serial dilutions were performed using a multichannel pipette. Tips were discarded after use as each well had 50 µl of the test material in serially descending concentrations. To each well of 30 µl nutrient broth and 10 µl of resazurin indicator solution was added. Finally, 10 µl of bacterial suspension (3.5×10^5 cfu/ml) was added to each well. Each plate was wrapped loosely with cling film to ensure that bacteria did not become dehydrated. Each plate had a set of controls: a column with a broad- spectrum antibiotic as positive control (usually ciprofloxacin in serial dilution), a column with all solutions with the exception of the test compound, and a column with all solutions with the exception of the bacterial solution adding 10 µl of nutrient broth instead. The plates were prepared in triplicates, and placed in an incubator set at 37 °C for 18–24 hrs. The color change was then assessed visually. Any color changes from purple to pink or colorless were recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value. The average of three values was calculated and that was the MIC for the test material and bacterial strain.

Minimum Bactericidal Concentration (MBC) assay: The MBC is the lowest concentration of an antibacterial agent required to kill a particular bacterium. It was determined from broth dilution MIC tests by sub culturing in agar plates that do not contain the test agent. The MBC was identified by determining the lowest concentration of antibacterial agent that reduced the viability of the initial bacterial inoculum by $\geq 99.9\%$.

Cytotoxic activity testing: The brine shrimp lethality test (BST) was used to observe the presence of cytotoxic activity in the extracts (Meyer *et al.*, 1982). For the experiment, 5 mg of each extracts were dissolved in 1 ml of sea water and one drop Tween-80 and adjusted to final concentration of 5 µg/µl. Then 4 ml of seawater was given to every test tube. With the help of micropipette, specific volumes of 5, 10, 20, 40, 80, 160 and 320 µl of samples were transferred from the stock solution to the test tubes by serial dilution, adjusted to 10 ml with saline water and the final concentration of 2.5, 5, 10, 20, 40, 80, 160 µg/ml, respectively was obtained. Finally, with the help of a Pasteur pipette 10 live brine shrimp nauplii were taken into each of the test tubes (Meyer *et al.*, 1982). Vincristine sulphate was used as positive control. After 24 hours, the test tubes were inspected to count mortality and a graph of % mortality and logarithm of concentration (Persoone *et al.*, 1980) was plotted and lethal concentration (LC₅₀) was calculated using Microsoft Excel 2007.

Results

Antibacterial activity: Both ethanolic and methanolic extracts of papaya seeds produced significant antibacterial properties against almost all of the tested bacteria (Table 1, Fig. 1 &

2) 1000 µg/disc of seed extract was found to be more potent against bacteria as there was significant ($p < 0.05$) differences among zone of inhibitions of the same extract at different concentration. At a concentration of 1000 µg/disc of ethanolic seed extract of papaya showed maximum activity against *Proteus vulgaris* which was 14.75 mm. On the other hand, 1000 µg/disc of methanolic seed extract of papaya showed maximum zone of inhibition against *Staphylococcus aureus* (14.50 mm). Both the extract showed good zone of inhibition against *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *S. paratyphi* and *Vibrio cholerae*. The results for positive control ranged from 28.5 to 34.5 mm for all the tests. Representative zone of inhibitions are as shown in Fig. 3 & 4.

Table 1: Significance test ($p < 0.05$) for mean of zone of inhibition of two different type of extracts

Bacteria	Statistical analysis					
	50% ethanol			50% methanol		
	t-value	p-value	Significant (yes/no)	t-value	p-value	Significant (yes/no)
<i>P. aeruginosa</i>	6.944	0.0004	Yes	4.426	0.0044	yes
<i>S. epidermidis</i>	2.176	0.0724	No	7.133	0.0004	yes
<i>P. vulgaris</i>	6.246	0.0008	Yes	5.620	0.0014	yes
<i>E. coli</i>	-	-	-	6.727	0.0005	yes
<i>S. paratyphi</i>	3.162	0.0195	Yes	6.092	0.0009	yes
<i>V. cholerae</i>	2.340	0.0568	No	5.174	0.0021	yes
<i>S. aureus</i>	6.929	0.0004	Yes	8.677	0.0001	yes

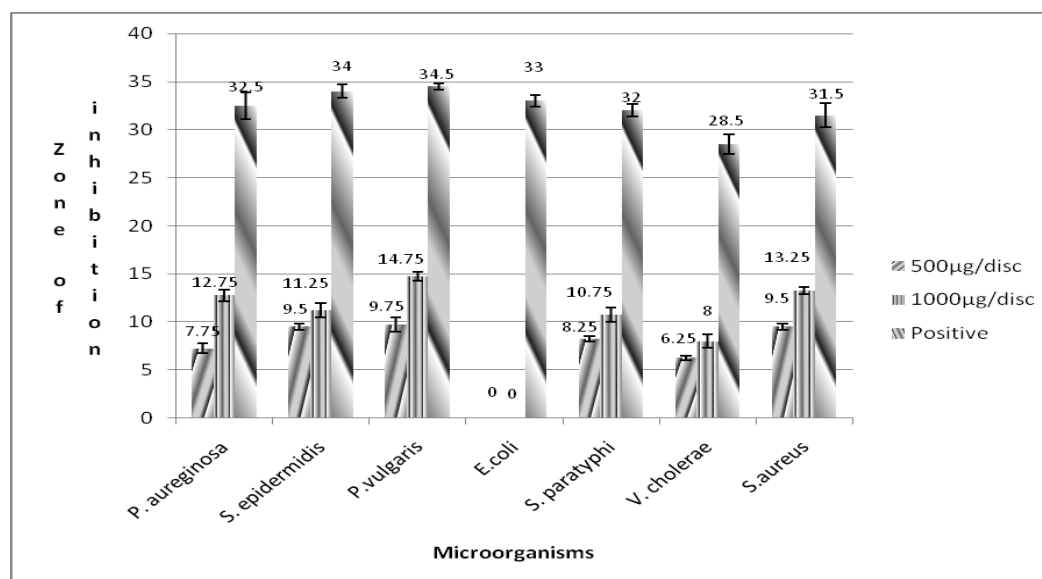


Fig. 1: Antibacterial activity of ethanolic extracts of *C. papaya* L. seed at different concentrations

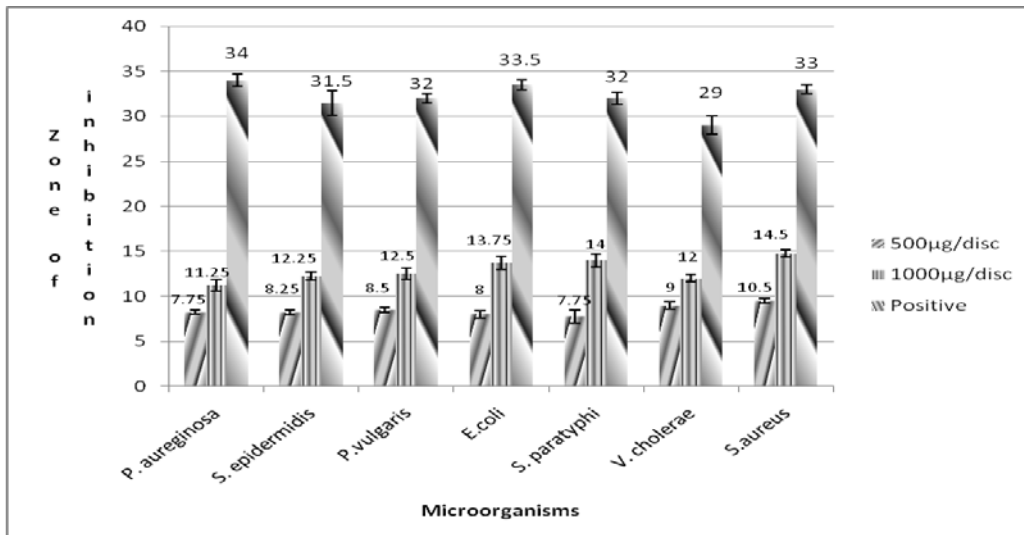


Fig. 2: Antibacterial activity of methanolic extracts of *Carica papaya* L. seed at different concentrations

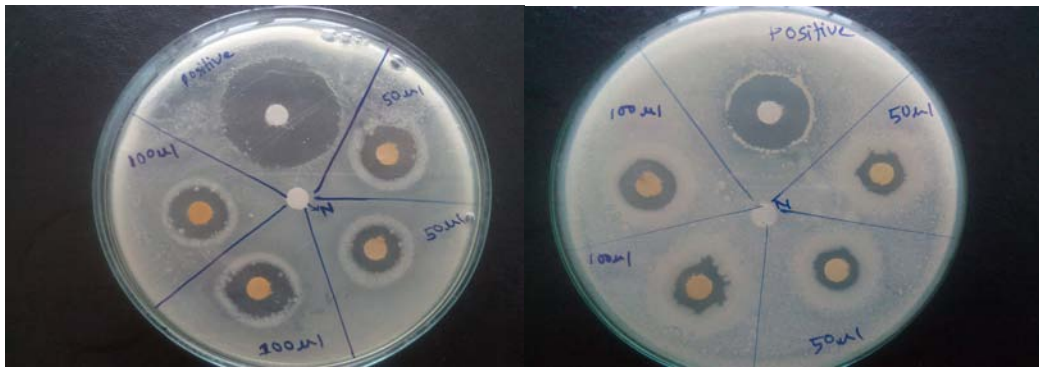


Fig. 3: Zone of inhibition for ethanol extract *P. vulgaris* (left) and *S. aureus* (right)



Fig. 4: Zone of inhibition for ethanol extract *S. aureus* (left) and *S. paratyphi* (right)

The result of MIC and MBC confirmed the results obtained from agar disc diffusion assay (Table 2 & 3). For ethanol extract the best result was obtained against *Proteus vulgaris* (0.625 mg/ml) and for methanol extract the best result was obtained against *Staphylococcus aureus* (0.312 mg/ml) from the MIC assay. MBC was 1.25 mg/ml for ethanol extract against *Proteus vulgaris* and in case of methanol extract it was 0.625 mg/ml for *Staphylococcus aureus*.

Table 2: Minimum inhibitory concentration of papaya seeds extracts

Microorganisms	Ethanol Extract mg/ml	Methanol Extract mg/ml	Positive Control mg/ml
<i>Staphylococcus aureus</i>	1.25	0.312	0.00013
<i>Pseudomonas aeruginosa</i>	2.5	5	0.00098
<i>Escherichia coli</i>	10	0.625	0.00049
<i>Proteus vulgaris</i>	0.625	2.5	0.00013
<i>Vibrio cholerae</i>	5	1.25	0.00065
<i>Staphylococcus epidermidis</i>	2.5	5	0.00027
<i>Salmonella paratyphi</i>	5	0.625	0.00014

Table 3: Minimum bactericidal concentration of papaya seeds extracts

Microorganisms	Ethanol Extract mg/ml	Methanol Extract mg/ml
<i>Staphylococcus aureus</i>	2.5	0.625
<i>Pseudomonas aeruginosa</i>	5	5
<i>Escherichia coli</i>	-	2.5
<i>Proteus vulgaris</i>	1.25	5
<i>Vibrio cholerae</i>	-	2.5
<i>Staphylococcus epidermidis</i>	5	-
<i>Salmonella paratyphi</i>	5	1.25

Cytotoxic activity by brine shrimp lethality test: Results of brine shrimp lethality tests (Table 4) showed that both the extracts of *Carica papaya* L. had low toxicity to brine shrimp as the LC₅₀ values were over 100 µg/ml (Meyer et al., 1982). The LC₅₀ values were 596.51 µg/ml and 487.23 µg/ml, respectively for ethanolic and methanolic extract of papaya seeds (Figure 5 and 6). For positive control, the LC₅₀ value was 1.20 µg/ml (Table 5). Regression equation of ethanolic, methanolic extracts of papaya seeds and vincristine sulphate, including value of R² is summarised in Table 6.

Table 4: Effect of ethanolic and methanolic extracts of *C. papaya* seed on brine shrimp

Concentration $\mu\text{g/ml}$	Log C	Mortality % of ethanolic extract	LC ₅₀ $\mu\text{g/ml}$	Mortality % of methanolic extract	LC ₅₀ $\mu\text{g/ml}$
2.5	0.39	0	596.51	3.33	487.23
5	0.7	6.67		6.67	
10	1	13.33		16.67	
20	1.3	20		23.33	
40	1.6	23.33		26.67	
80	1.9	30		33.33	
160	2.2	40		40	

Table 5: Cytotoxic activity of vincristine sulphate (positive control)

Concentration($\mu\text{g/ml}$)	LogC	% Mortality	LC ₅₀ ($\mu\text{g/ml}$)
0.060	-1.22	15	1.20
0.125	-0.903	20	
0.25	-0.602	30	
0.5	-0.30	45	
1	0	55	
5	0.7	60	
10	1	75	

Table 6: Regression equation and R²-values of ethanolic, methanolic extracts of papaya seeds and vincristine sulphate

Sample	Regression equation	R ²
Ethanol	y=20.956x-8.1659	0.9891
Methanol	y=20.57x-5.2867	0.9882
Vincristine Sulfate	y=26.497x+47.873	0.9532

Discussion

Traditional healers use plants to prevent or cure infectious diseases. The use of plant derived drugs and search for dietary supplements from plants have advanced in recent years (Cowan, 1999). Every plant does have some sorts of medicinal value. Extraction of the active components from different plant parts vary in the concentration. Parts having the highest concentration of the active compounds are preferred to therapeutic purposes and the parts can be the leaves, stems, barks, roots, bulks, woods, flowers, fruits or the seeds (Kafaru, 1994). Some active compounds from plants inhibit the life process of disease causing microbes. They inhibit microbial growth by binding protein molecules of microbes and altering their biochemical systems. Plants have the ability to produce cytotoxic agents, too (Garrod *et al.*, 1995).

Plant, for its own protection, accumulates an armory of antimicrobial secondary metabolites where some metabolites are constitutive and others are inducible antimicrobials (González-Lamothe *et al.*, 2009). Plant is rich of secondary metabolites, which have been found to have both *in vitro* and *in vivo* antimicrobial activities (Cowan, 1999).

Seeds, fruits, pulps, leaves of *Carica papaya* L. have been used in Ayurvedic medicine from early period of time Traditional healers use various parts of *C. papaya* L. for the remedy of various infections. (Krishna *et al.*, 2008). Benzyl-isothiocyanate is an active antibacterial compound (Sofrata *et al.*, 2011). It is also evident that myrosinase, which activates glucomoringin, has potential antibacterial activity (Galuppo *et al.*, 2013). Of note, Seeds of *C. papaya* L. contains different types of nutritional components like myrosinase, fatty acids, benzyl-isothiocyanate, glucose inolate, caricin, etc (Krishna *et al.*, 2008). So, it can be concluded that benzyl-isothiocyanate, myrosinase, etc. of *C. papaya* L. potentiates it's role as antimicrobial properties.

Seeds of *C. papaya* L. have the traditional use to treat food poisoning caused by bacteria. This study supports that use as the methanolic seed extract of papaya showed good zone of inhibition against *E. coli* which causes food poisoning (Hughey *et al.*, 1987). Again, it is believed that for the home remedy of bacterial infections seeds of *C. papaya* L. can be a good antibacterial agent (Aravind *et al.*, 2013). This study provides a scientific proof of that traditional practice and belief. *C. papaya* L. seeds can be used to treat skin ulcer as well as urinary tract infection as they are active against bacteria like *Proteus vulgaris* and *Staphylococcus epidermidis*. Again seeds of papaya can be useful to treat disease caused by *Staphylococcus aureus*. Thus, the study provided information about antibacterial activity of seeds of *C. papaya* L. The extract showed good zone of inhibition ($p < 0.05$) against the tested microbes. The LC_{50} values were found in this study as 596.51 $\mu\text{g/ml}$ and 487.23 $\mu\text{g/ml}$, respectively for ethanolic and methanolic extract of papaya seeds (Fig. 5 & 6), whereas LC_{50} value was found as 142.27 ± 40.35 $\mu\text{g/ml}$ and 357.26 ± 47.56 $\mu\text{g/ml}$ from ethyl acetate fractions and Hexane fractions of papaya seeds, respectively (Khor *et al.*, 2014). The difference could be due to variation of extraction solvents, process, origin of papaya variety, etc.

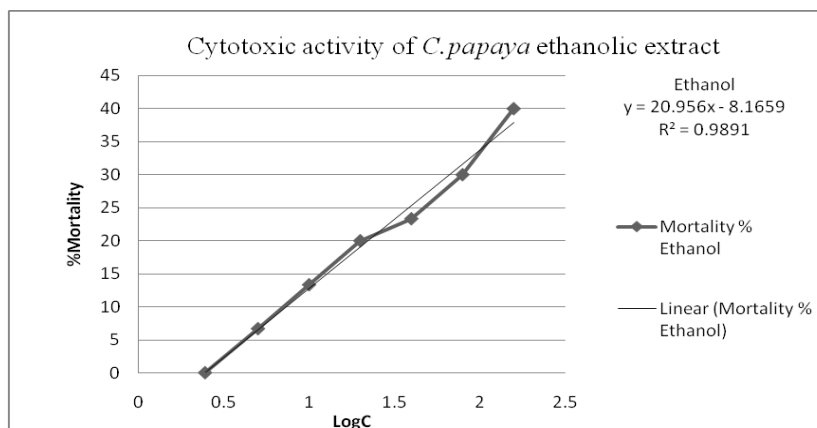


Fig. 5: Log concentration of ethanolic extracts of papaya seeds versus percent shrimp mortality

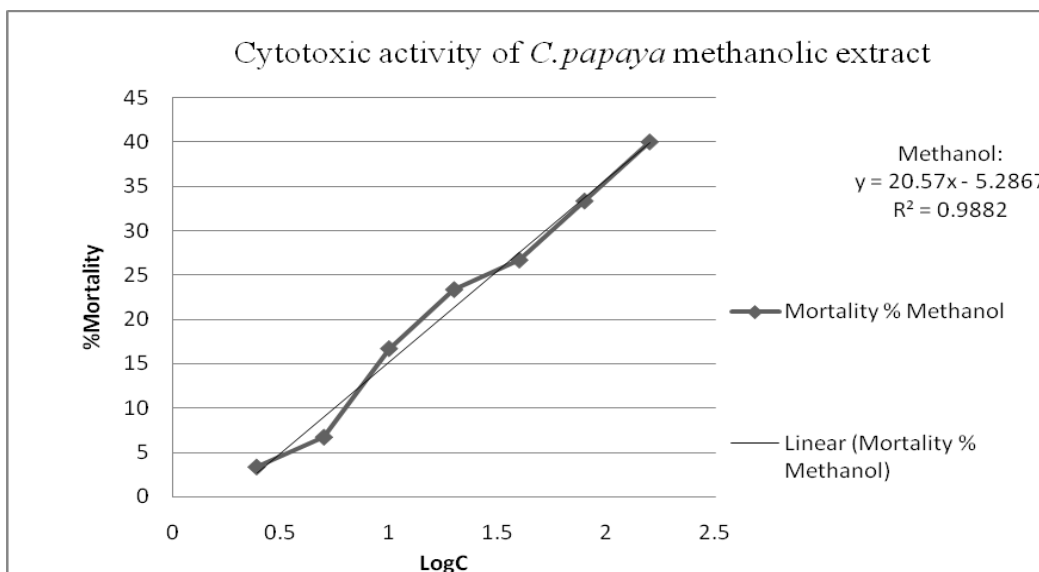


Fig. 6: Log concentrations of methanolic extracts of papaya seeds versus percent shrimp mortality

The synthesis of the active compounds in plant parts may vary due to environmental conditions thus the potentiality can be influenced due to the different experimental conditions, locations and extraction procedures. Another study tested the

papaya seeds from unripe fruit and found the seeds as bacteriostatic (Osato *et al.*, 1993). Taken together all these findings into consideration, it is suggested that papaya seed (both ripe and unripe) is an effective candidate as a potential drug against human pathogenic bacteria and demands isolation of active ingredients. It is also important to note that the seed extracts of papaya showed low toxicity to brine shrimp suggesting that the papaya seeds' preparation is suitable for treatment of bacterial infection. The toxicity was found low, indicating that direct consumption of papaya seeds might not be harmful to human body. Although further research, including *in vivo* trial are needed in this regard to make sure of its use.

Conclusion

Papaya seeds are usually thrown away but their proper utilization can lead to the development of novel drug. Here *in vitro* studies provide scientific footing to enhance confidence in the traditional use of *C. papaya* seeds. The antibacterial and cytotoxic activity of the *C. papaya* seeds extracts suggest isolation of active ingredients through bioassays. *In vivo* trials would help to sort out active compounds of the seed as pharmaceutical and therapeutic agents.

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