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Phyto-Chemical, Antibacterial and Brine Shrimp Toxicity Studies of Green Banana Leaves

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Abstract Green banana or cooking banana plants have been of great importance to man, serving diverse needs from food, culture and religion. The biochemical, antimicrobial and cytotoxicity activity of the ethyl acetate fraction of the dried leaf powder of cooking banana is evaluated following fractionation, soxhlet extraction and chromatography. The TPC, TQC and TTC content of the fraction is recorded as 12.85 GAE mg/g, 6.91 QE mg/g and 2.74 mg/g of the dry weight respectively which is comparatively higher than the preceding chloroform fraction. The MIC of the fraction against gram negative anaerobic bacteria *E. tarda* is recorded at 625 μg/ml and radical scavenging strength with IC_{50} at 0.055 mg/ml with AAI of 0.0173. The cytotoxicity assay of the ethyl acetate fraction was examined on brine shrimp nauplii hatched from 250 μm cysts under suitable laboratory conditions with PHR at 92%. The threshold percent mortality was calculated from probit logistic regression analysis with LC_{50} at 0.845 μg/ml at 6 h exposure. The normal distribution of the data is assured by Shapiro Wilk significance.

Keywords *Musa paradisiaca*, *Edwardsiella tarda*, DPPH, IC_{50}, LC_{50}.

Introduction

There is a growing concept that population and ecology complement each other but this complementation is greatly hampered by use of precarious chemicals and microcidal, insecticidal and numerous synthetic chemicals (Wang et al. 2009). Demands for high yield and numerous pathogenic outbreaks have instigated the fields of aquaculture and agriculture to depend extensively on synthetic chemicals, a large portion of which reaches the non-target system. In view of this concept, allelopathy can be used as an efficient bio-intensive tool to address ecosystem level aspects. Allelopathy is the study of allelochemicals which are low molecular weight compounds mostly produced as secondary metabolites of plants which they exude to the environment. The phyto-antimicrobial resources exemplify a wide range of plant secondary metabolites (Tamokou et al. 2011, Gatsing et al. 2009, Al-Mariri 2008) asserting diverse effects from extreme deadly to immense beneficial. The plant products may be phyto-anticipins or phyto-alexins (Reuben et al. 2008, Al-Bayati 2009). Though finding therapeutics in plants is an ancient idea but in most cases lack defined protocol for formulations and quality control constrains scientific validation. With this

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Fig. 1. *Musa paradisiaca* in natural habitat.

state of fact an attempt has been initiated to study the antimicrobial activity of banana leaves against fish pathogens followed by cytotoxicity effect (Fabrega et al. 2011). A very frequent sight which we come across most of the waterbodies is rows of banana plants (Fig. 1) growing contiguously, with leaves drooping down to water. *Musa* or banana is an exotic plant with its origin in the tropics of South Eastern Asia. They are large woodless flowering plants belonging to the Musaceae family (Bilba et al. 2007). Amongst 100 other species, *Musa paradisiaca* is the most widely cultivated alkaloid rich vegetable banana or green banana owing to its high iron content of 0.33 mg/100 mg of banana (Garcia et al. 2015). There are numerous articles on ethno pharmaceuticals (González–Lamothe et al. 2009) of banana fruits, flowers and stem on human health management (Aysal et al. 2007 ; Kanrar et al. 2010). In this study we worked on the biochemical and antimicrobial activity of the leaf extract of the cooking banana against fish pathogen and their cytotoxicity on brine shrimp nauplii.

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Materials and Methods

Collection and processing of the plant sample

The banana leaves approximately collected 3.5 kg from both terrestrial and flood plains. Samples carried to laboratory in sterile polypropylene upon washing. The leaves were cleansed throughly with double distilled water in the laboratory and lyophilised. Following the preparative steps, the leaves were powdered using electrical mixer to obtain a dry weight of approximately 800 g. The dried leaves were processed preferably within 24 h.

Extraction and isolation of active fraction

500 ml–800 ml of fresh solvents are preferred for the process. The leaf powder is loaded in the soxhlet at least an inch below the siphon tube to prevent its

Table 1. Solvents / Solvent system used in column chromatography.

Fraction No.	Solvents/ Solvent System	Ratio	Quantity (ml)
F_1	Petroleum ether	–	250
F_2	Pet ether : Chlo	7 : 3	500
F_3	Chloroform	–	500
F_4	Ethyl acetate	–	300
F_5	Acetone : Water	7 : 3	250
F_6	Ethanol : Water	7 : 3	300
F_7	Ethanol : Water	3 : 7	250

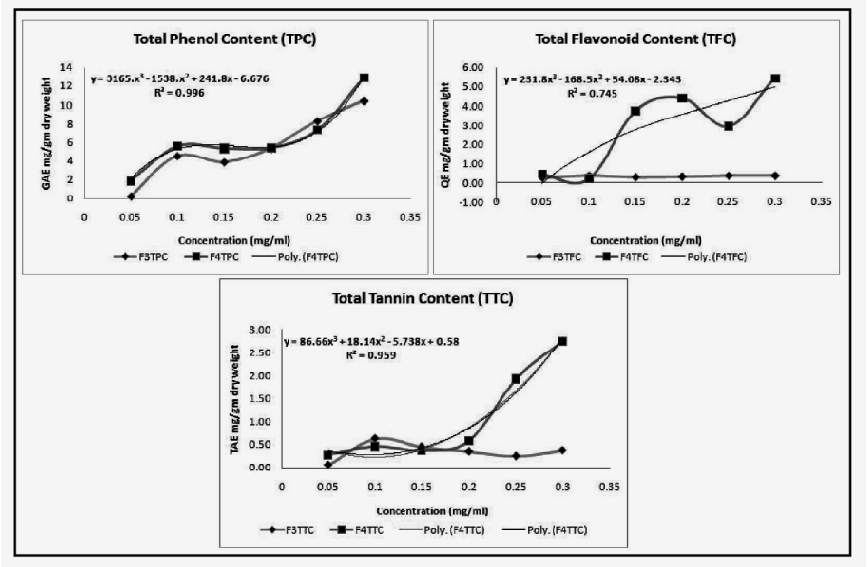

Fig. 2. Graphical representation of TPC, TFC and TTC of the F_3 and F_4 fraction of *Musa* leaves.

entry and blockage of solvent refluxing. The refluxing is done until there remains no deposit in the solvent that is being refluxed. Moisture removal is done by sodium sulfate. The process in brief is schemed below.

The powdered leaf sample was subjected to soxhlet extraction with Hexane for 24 h at 68°C for defatting.
↓
The defatted sample was now extracted in Methanol for 18 h at 65°C
↓
The extracted sample was concentrated in Rotary vacuum evaporator at 48°C–52°C ; 103 rpm
↓
Subjected to partitioning (v/v) with Ethyl acetate : Water :: 1 : 1
↓

a. Polar/Aqueous phase	b. Non-polar/Ethyl acetate
↓	↓
Partitioning with n-Butanol	Subjected to Column Chromatography

Schematic diagram of extraction procedure from *Musa* leaves

Chromatography

Column Chromatography

Glass columns (50 × 15 cm^2) are tightly packed with Silica gel with three different mesh sizes (69–120 initially and latter with 230–400) upto 30 cm. The sample coated in activated Silica gel is dried and poured into the column. Solvents / Solvent system with various combinatorial proportions following the eluotropic series was added which is as charted below (Table 1). The selection of suitable solvent system depends much on the target compound to be eluted.

Thin Layer Chromatography (TLC)

Each column fraction is subjected to thin layer chromatography. The standardization of the Retention factor (Rf) value and the ideal solvent system is done using pre–coated TLC plated on aluminium sheets Silica gel F_{254} with binder and latter the isolation of the sample is carried on preparative TLC with glass plates (20 × 20 cm^2) layered with slurry of uniformly homogenized Silica gel G and acetone with 1mm thickness with an applicator. The coated plates are allowed to dry at room temperature for 24 h. Thereafter, on the day of work the plates are activated in hot air oven at 110°C. Usually 10–15µl of the fraction is spotted on the plates using a glass capillary. The loaded plated are placed in a glass chambers of (25 × 100 cm^2) presaturated solvents. The spots are allowed to develop at a maximum height of 19 cm and minimum 15 cm

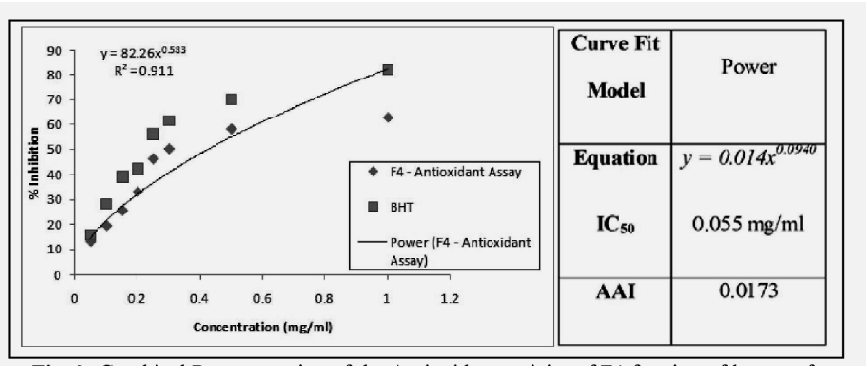

Fig. 3. Graphical representation of the antioxidant activity of F_4 fraction of leaves of *Musa paradisiaca*. Tabulated : Curve fit model, Equation, IC_{50} and Antioxidant Activity Index (AAI) of the antioxidant activity of F_4 fraction.

depending upon the number of compounds the fraction holds. Finally, the chromatographed plates are put in iodine chamber or observed under UV to detect fluorescent spots.

Biochemical analysis

The biochemical parameters of a plant extract helps asserting its bioactivity and prediction of functional groups. The role of the fraction on plant defence or fragrance or strong taste can be holistically be concluded by observing the biochemical composition (Shad et al. 2013). In this study we have worked on three primary biochemical parameters.

Total Phenol Content (TPC)

The total phenolic content was determined following Folin-Ciocalteu method (Baba and Malik 2015) using 0.1 ml of the extract with a concentration range of 0.05–0.3 mg/ml of the leaf leachate. The extracts were mixed with Folin–Ciocalteu reagent (Phosphomolybdate and Phosphotungstate) and sodium carbonate (Na_2CO_3) which forms an alkaline solution. A blue chromophore of Folin –Ciocalteu complex is formed following incubation for 30 mins at room temperature. The change of color was measured in spectrophotometer with absorbance reading at 765 nm. Gallic acid in the same concentration as the sample was used as positive control. The total phenol content was expressed as GAE in milligram per gram of dry material using the calibration curve, where X was the absorbance and Y was GAE (mg/g).

Total Flavonoid Content (TFC)

Flavonoid estimation was carried out following the method (Agati et al. 2012). The preferred concentration range for the leaf leachates were 0.2-1.2 mg/ml with 0.1 ml of the extract. Later the extract was added with 1.2 ml distilled water, 0.12 ml of 5% sodium nitrite ($NaNO_2$) with uniform intermixing. Following incubation for 5 minutes at 25°C temperatures the nitrification is enabled in acid labile medium using 0.12 ml of 10% $AlCl_3$ solution. The tubes were further incubated at room temperature for 5 minutes and added with 0.8 ml of 1 mm sodium hydroxide (NaOH) solution and 1.16 ml of distilled water. The absorbance was measured at 510 nm. Methodically, quercetin in the same concentration as the sample was used as positive control. Total flavonoids content as calculated as quercetin (mg/g) using the calibration curve, where X was the absorbance and Y was QE (mg/g).

Total Tannin Content (TTC)

The total tannins content using tannic acid as standard curve. Briefly, 200 µl of extracts of 0.05–0.3 mg/ml was mixed with 200 µl of ferric ammonium citrate (0.35%) prepared freshly and 200 µl of ammonia (0.8%). The change of color of Iron–Tannate complex was measured at 525 nm and expressed as TAE mg of per gram of extracts or fractions.

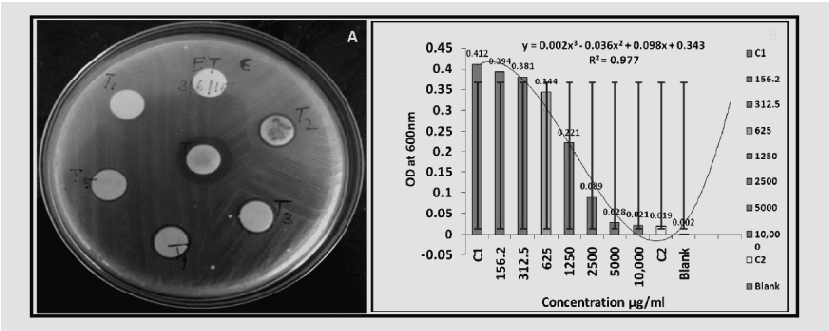

Fig. 4. A : Disc diffusion assay against *E. tarda* ; B : Graphical representation MIC and MBC of *E. tarda*.

Antioxidant assay

DPPH radical scavenging activity

The free radical scavenging activity of extracts and fractions for the radical DPPH was measured as described (Gulcin 2012). Freshly prepared DPPH solution 0.004% (w/v) in methanol was prepared and 3.9 ml of this solution was mixed with 0.1 ml of extract in methanol containing different concentration range (0.05–1 mg/ml) of the extract. After an incubation of 30 minutes, the absorbance was measured at 517 nm using spectrophotometer. The DPPH solution was assured with an absorbance of 0.50–0.60 at 517 nm. Butylated Hydroxy Toluene (BHT) in the same concentration as the sample was used as positive control. The capability to scavenge the DPPH radical was calculated using the following equation :

$$\text{DPPH radical scavenging activity (\%)} = \{A_c - A_t / A_c\} \times 100$$

Where A_c is the absorbance of the blank reaction and A_t is the absorbance in presence of the sample of the extracts. IC_{50} which defines the concentration of the plant extract that's needed to scavenge 50% of the radical present was calculated by the following equation.

$$IC_{50} = \{\text{Percentage inhibition/Concentration of the sample}\} \times 50$$

A second parameter for the antioxidant activity (Arteaga Figueroa et al. 2014) is expressed as the antioxidant activity index (AAI) which is formulated as :

$$\text{AAI} = \text{Final concentration of DPPH } (\mu g.ml^{-1}) \text{ in the control sample/ } IC_{50}\ \mu g.ml^{-1}$$

Microbiological screening

The plant fractions were subjected to antimicrobial assay against *Edwardsiella tarda* and *Streptococcus aureus*. The bacterial strains were confirmed using 16Sr RNA sequencing at molecular level. [Primers- 16S univ 5'-GAG TTT GAT CCT GGC TCA G-3' 27f, 5'- TAC GGT TAC CTT GTT ACG AC-3' 1492r]. Single pure colony was confirmed in R^2A medium. Antimicrobial activities of different extracts were

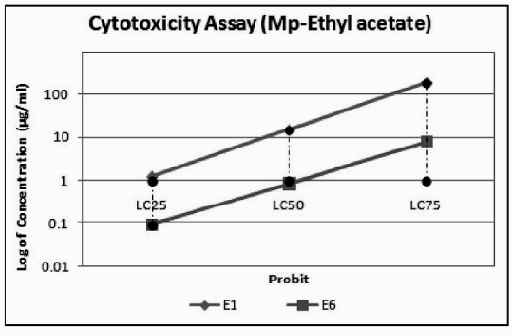

Fig. 5. Graphical representation of LC_{25}, LC_{50} and LC_{75} against log of concentration for *M. paradisiaca* at 1 h, 6 h and 12 h exposure time of brine shrimp lethality assay of Ethyl Acetate fraction.

Table 2. Statistical analysis of antioxidant assay and cytotoxicity assay ; Test of normality : Shapiro Wilk and Kolmogorov-Smirnov test and probit analysis of F_4 (Ethyl acetate fraction) of *M. paradisiaca* on brine shrimp lethality at 1 h and 6 h exposure time.

Model summary

R	R. square	Adjusted R square	Std. error of the estimate
0.953	0.908	0.893	0.179

The independent variable is concentration

Anova

	Sum of squares	df	Mean square	F	Sig.
Regression	1.907	1	1.907	59.449	0.000
Residual	0.192	6	0.032		
Total	2.099	7			

The independent variable is concentration

Correlations

		percentage of inhibition	concentration	BHT as positive control
percentage of inhibition	Pearson correlation	1	0.925**	0.948**
	Sig. (2-tailed)		0.001	0.000
	N	8	8	8
Concen-tration	Pearson correlation	0.925**	1	0.872**
	Sig. (2-tailed)	0.001		0.005
	N	8	8	8
BHT as positive control	Pearson correlation	0.948**	0.872**	1
	Sig. (2-tailed)	0.000	0.005	
	N	8	8	8

**Correlation is significant at the 0.01 level (2-tailed).

Tests of normality

		Kolmogorov-Smimov[a]			Shapiro Wilk		
Time	1 h-*Musa*	Statistic	df	Sig.	Statistic	df	Sig.
Mortality	1	0.192	5	0.200*	0.949	5	0.730

*. This is a lower bound of the true significance.
a. Lilliefors Significance Correction

Tests of normality

		Kolmogorov-Smimov[a]			Shapiro Wilk		
Time	6 h-*Musa*	Statistic	df	Sig.	Statistic	df	Sig.
Mortality	6	0.223	5	0.200*	0.879	5	0.304

*. This is a lower bound of the true significance.
a. Lilliefors Significance Correction

Table 2. Continued.

Parameter estimates

Time 1 h	Parameter	Estimate	Std. error	Z	Sig.	95% Confidence interval Lower bound	Upper bound
PROBIT*	Concentration	0.620	0.054	11.533	0.000	0.515	0.726
	intercept	– 0.730	0.107	–	6.809	0.000	–
.837	–	.622					

a. PROBIT model PROBIT (p) = Intercept+B × (Covariates ×are transformed using the base 10.000 Iogarithm)

Chi-Square tests

Parameter		Chi-Square	df	Sig.
PROBIT	Pearson goodness-of-fit test	9.336	3	0.025*

a. Since the significance level is less than .150, a heterogeneity factor is used in the calculation of confidence limits.
b. Statistics based on individual cases differ from statistics based on aggregated cases.

Parameter estimates

Time 6 h	Parameter	Estimate	Std. error	Z	Sig.	95% Confidence Interval Lower bound	Upper bound
PROBIT*	Concentration	0.699	0.83	8.453	0.000	0.537	0.861
	Intercept	0.051	0.111	0.460	0.646	0.060	0.162

a. PROBIT model PROBIT (p) = Intercept + B × (Covariates × are transformed using the base 10.000 Iogarithm)

Chi-Square tests

Parameter		Chi-Square	df*	Sig.
PROBIT	Pearson goodness-of-fit test	3.518	3	0.318*

a. Since the significance level is greater than, 150, no heterogeneity factor is used in the calculation of confidence limits.
b. Statistics based on individual cases differ from statistics based on aggregated cases.

evaluated by the agar well diffusion method with little modification and Minimum inhibitory concentration (Verma et al. 2012).

Sample and media preparation

The sample was concentrated to dryness and re-suspended in 1% DMSO. A dilution range of 1000 µg /ml to 1 µg /ml (T_1–T_6) was prepared with 1% DMSO as control. The 18 h old bacterial culture grown in Muller Hilton Broth (MHB) was transferred to 0.85% saline solution and the turbidity adjusted to 0.5 McFarland using a turbidimeter to ensure 5×10^5 CFU / ml. Barium sulfate was used as standard. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined by serial broth dilution assay (Coulidiati et al. 2011).

Disc diffusion method

The disc diffusion method was used to determine the antibacterial activity. The Muller Hilton Agar (MHA) plates were swabbed (sterile cotton swabs) with 50 µl of 0.5 McFarland adjusted culture. The plate extracts were adsorbed in the 10 mm cellulose sterile disc with 20 µl of sample placed in the plate and

allowed to stand in room temperature for 45 minutes along with the control plates. The plates were incubated at 35°C for 36–48 h. The diameter of the inhibition zone (mm) was measured.

Minimal Inhibitory Concentration (*MIC*) and *Minimal Bacterial Concentration* (*MBC*)

Broth dilution method was preferred to perform the MIC assay. The dilutions were chosen depending on the results of the disc diffusion assay (Sadeghian et al. 2011). 1 ml of broth was transferred in all the nine tubes ranging from 10000 µg/ml to 156 µg/ml of the extract and two controls one without the inoculum and the other without extract was maintained for experimental assurance. 20 µl of the inoculums was transferred from saline solution to Muller Hilton Broth (MHB) for clear reading and incubated for fifteen minutes. The extract and 5 µl of the inoculums was added all the eight tubes. The tubes were incubated in 35°C for 18 h and the OD reading was recorded at 600 nm with broth as the blank (Coulidiati et al. 2011).

Cytotoxicity assay

Brine Shrimp lethality Test (*BLT*)

The safety of the applicable plant sample in an ecosystem is assured by *in vitro* eco-toxicity assay brine shrimps (*Artemia salina*) are small crustaceans which are capable of laying dormant eggs also known as cysts. Brine shrimp lethality test is a quantal toxicity assay useful and significant especially when the target ecosystem is a continuous resource variable–water. We have tested our experimental compounds on the nauplii stage of the shrimps (Ghosh et al. 2015). The BLT procedure is simple, rapid and above all requires a very small amount of the compound. Brine shrimp (*Artemia salina*) cysts were obtained from Jadavpur University, Kolkata West Bengal. The plant sample was diluted for five concentration range from 10^4 ppm – 1 ppm with DMSO as control. The eggs were hatched in artificial sea water prepared by 38% of sea salt with salinity 25 – 32 ppt. The light intensity of 1200 lumen with a partition for dark (covered) and light effects for a period of 36 h and pH of 7.5–8.0 was used. Once hatched the nauplii were attracted towards light. 10 shrimps were added in each of the dilutions. After 24 h the mortality of the shrimps were recorded in each dilutions (Syahmi et al. 2010). The PHR (Percent Hatching Rate) was approximately 92%. The % mortality of hatched nauplii were converted to probit and plotted against concentration to obtain the LC_{25}, LC_{50} and LC_{75} to assess the toxicity curve of the sample.

Results and Discussion

Extraction and isolation of active fraction

The solvents/solvent systems used for column chromatography and the procured quantity are tabulated in Table 1. TLC was done with three different solvent systems. (a) Pet ether: Chloroform :: 5:5 ; (b) Hexane : Ethyl acetate :: 7:3 and (c) Acetone : Methanol:: (9.9:0.1.Rf of approx. 0.74 was calculated.

Biochemical screening

Total Phenol Content (*TPC*)

Phenol content was expressed as Gallic acid equivalents (GAE) in mg/g dry weight. The experimental data exhibits (Fig. 2) there was no proportionate increase along the concentration gradient with a sudden fall at 0.15 mg/ml with 5.44 GAE mg/g and at 0.20 mg/ml with 5.41 GAE with the highest noted at 0.3 mg/ml with 12.85 GAE mg/g of (0.05 – 0.3 mg/ml) extracts concentration. The result is validated with the coefficient of determination (R^2 = 0.963) which reads the data as best fit along the regression line.

Total Flavonoid Content (*TFC*)

The total flavonoid content was expressed as Quercetin equivalents (QE) in mg/g fresh weight. The flavonoid content showed a consistent data (Fig. 1) evenly throughout the concentration range. It increased throughly with raise in concentration and 1.2 mg/ml extract had the highest flavonoid content with 6.91 mg/gm QE. Presence of flavonoid may result as alkaloids and glycosides in the plant extract. The R^2 value 0.935 was found to be significant and defines the goodness of fit of the data along the regression line.

Total Tannin Content (*TTC*)

Tannic acid equivalents (TAE) in mg/g dry weight is preferred as a yard stick for the progress of tannin. The highest tannin content was recorded at 0.3 mg/ml of the leaf extract with 2.74 mg/g TAE (Fig. 2). The strength of the response variables and the model was found to be significant with $R^2 = 0.959$. Considering the above results, F_3 and F_4 fractions were further worked upon for antioxidant assay and bioactivity.

Antioxidant activity

DPPH Radical Scavenging Assay

DPPH assay (Fig. 3) was performed to evaluate the antioxidant activity. Following a similar trend with phenols, the highest was recorded at 0.3 mg/ml. The positive control BHT recorded the highest percent inhibition as 35.6 at 0.3 mg/ml concentration.

Microbiological screening

The diameter of the zone of inhibition is tabulated below. The ethyl acetate fraction of *M. paradisiaca* showed appreciable inhibition on *Edwardsiella tarda* (Fig. 4) but showed very negligible impact on *Streptococcus aureus*. Hence MIC and MBC (Fig. 4) was proceeded only with *E. tarda*. 1250 μg was found to be the MIC because turbidity appeared till 625 μg OD was taken at 600 nm. Following which four concentrations 1250 μg, 2500 μg, 5000 μg, 10,000 μg were plated and kept in incubator at 370 C for 24 h / 48 h. 5000 μg was found to be the MBC as no growth appeared at 5000 μg and 10,000 μg.

Cytotoxicity assay

Brine Shrimp lethality Test

M. paradisiaca showed uniformity with concentration dependent mortality. However graphical re-presentation showed the 1 h exposure time to be a better fit model than 6 h time interval. The LC_{50} for 6 h was 0.845 μg/ml and that of 1 h was 15.005 μg/ml. Nevertheless the extract showed a toxicity effect beyond 6 h. This suggested that the fraction could contain cytotoxicity compounds (Fig. 5).

Statistical analysis

The statistical analysis of the antioxidant assay and brine shrimp lethality test is evaluated to evaluate the experimental data considered for conclusive comparison by IBM SPSS 20. The relationship graph for % scavenging distributed through a range a concentration is statistically evaluated below where the shrunken R^2 read at 0.908 with standard error at 0.179 consequently the accuracy of prediction with the regression model is high with significant difference between the group means. There is significant correlation between scavenging activity of the plant sample and the positive control following concentration incline (Table 2). For cytotoxicity test, the normal distribution of the dependent variable is displayed by the stem and leaf model of Shapiro-Wilk significance test of 0.730 and 0.304 for 1 h and 6 h respectively ($p > 0.05$) which confirmed its normal distribution. Henceforth, probit analysis for normal distribution was persuaded. However chi-square test with $p < 0.05$ displayed the 1 h exposure time to be a better curve fit model than 6 h time exposure (Table 2).

The use of chemicals in aquaculture is a very common practise which not only devastates the aquatic health but also accounts for major diseases upon consumption. By virtue of plant based products this chemical could be substituted (Alnamer et al. 2013) in terms of antibacterial for fish and other disinfectants (Volpatti et al. 2014). The ethyl acetate fraction of M. paradisiaca contains phenol and antioxidants. The fraction showed inhibitory effect against *E. tarda*, causative for emphysematous putrefactive disease of catfish. The graphs for biochemical and antimicrobial activity (Najiah et al. 2011) follow a non-monotonic power model of data. The compound displays threshold toxicity at 6 h exposure with $LC_{50} < 1$ μg/ml beyond which there is complete mortality.

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