RESEARCH ARTICLE

Phytochemical and Cytotoxicity Evaluation of *Lagerstroemia speciosa* (L.) Leaves Extract by MCF-7 Cell Line and Brine Shrimp Lethality Bioassay

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ABSTRACT

This study aimed to investigate the cytotoxicity of *Lagerstroemia speciosa* (L.) leaves crude extract. It has been reported to show various bioactivities. The phytochemical screening of the extract has been carried qualitatively. The cytotoxic effect was determined through *in vitro* MTT assay of MCF-7 cell line, and brine shrimp lethality bioassay. The presence or absence of alkaloid, carbohydrate, glycoside, saponin, terpene, steroid, phenol, and flavonoid in the extract was determined through the qualitative tests. The extract showed cell viability of 100% (1.95–3.9 µL/mL), 96% (1.95 µL/mL), ≈ 95% (3.9–15.62 µL/mL) and 88% (250 µL/mL) while the mortality of brine shrimp nauplii was from 5% to 10% (7.8 – 125 µL/mL) respectively. For both assays, DMSO of 1 & 0.1% were used as vehicle controls, while the potassium dichromate as the positive control for the brine shrimp only. These results proved the leaves extract to be non-toxic.

Keywords: Brine shrimp nauplii, Crude extract; Cytotoxicity, *Lagerstroemia speciosa* (L.) leaves, MCF-7 cell line, MTT assay, Phytochemicals.

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INTRODUCTION

The *Lagerstroemia speciosa* (L.), leaves also known as crepe myrtle or *Banaba* locally belonging to Lythraceae family and *Lagerstroemia* genus, has been known as a natural remedy for diabetes mellitus worldwide, particularly in Southeast Asian countries including Malaysia. It is a blooming tree locally found in Malaysia, the Philippines, and India.[1] It has been reported to exhibit anti-hyperlipidemic, anti-diarrheal, analgesic, hepatoprotective, anti-oxidant,[2-3] antidiabetic and alpha-amylase inhibitory activity by earlier studies.[4] Some previous reports have shown several potential therapeutic effects, but there was no toxicity study similar to the current one. Therefore, it is necessary to investigate the cytotoxic effect of this commonly consumed medicinal leaves in Malaysia and other countries.

EXPERIMENTAL

Collection and Identification of Plant Material

The fresh and matured leaves of Lagerstroemia speciosa (L. speciosa) (15-20 cm) are collected from IIUM Kuantan Campus areas, Indera Mahkota, Kuantan-25200, Malaysia, in the month of February-March, 2016. It was authenticated by the Department of Pharmaceutical Chemistry of Pharmacy Faculty of IIUM with a voucher specimen no. PIIUM:0423.

Preparation of Ethanol Extract

The fresh leaves are collected for microscopic analysis. For the extraction process, matured leaves of L. speciosa are identified, collected, and shade dried up to 7-12 days; care taken to avoid direct sunlight contact. Then, the leaves are crushed and grinded into fine powder by a mixer or blender. The sieving was done repeatedly to remove the coarse parts. Defatting is done by immersing the leaf powder into petroleum ether (Pet-ether) for more than 12 hours by regular shaking and stirring. The defatted leaf powder was used for extraction. The ethanol extract was concentrated in vacuo (temperature at 45°C, 175 m bar, and rotation 80-85rpm) using a rotary vacuum evaporator (BUCHI R-205) to a final corrected volume of 500 mL. This was further frozen at −70°C and shifted instantly to three weeks' successive freeze-drying at −50°C using benchtop freeze dryer (ALPHA 1-4LD-2), to give an ultimate yield.[1]

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Percentage of yield determination

The extracts were kept in the fridge (4°C) from which aliquots were withdrawn for the test. The yield of extract was determined by the final extract weight over the dried plant powder.

$$\% \text{ of extract (yield)} = \frac{\text{Extract weight}}{\text{Powdered leaves weight}} \times 100$$

Qualitative Phytochemical Screening of Crude Extract of Lagerstroemia Speciosa (L. speciosa)

The extracts were evaluated by a standard phytochemical screening of different constituents for the presence or absence of secondary metabolites, such as alkaloids, carbohydrates, saponins, amino acids, phytosterols, phenols, and flavonoids.

Detection of Alkaloids

A small amount of selected plant extract was diluted in 2 mL methanol and stridden with 1 to 2 drops of hydrochloric acid. The test was done by adding 1 to 2 drops of Mayer's reagent to the test tube. A formation of white or creamy precipitate indicated the presence of alkaloids.[5]

Detection of Carbohydrates and Glycosides

A small amount of methanol and water extract of the selected plant was dissolved in a solvent and filtered. The filtrated samples were tested with the following reagents.[6]

- *Fehling's Test*

An amount of 1 mL Fehling solution A and B was added into 1 mL of filtrated and boiled solution. The presence of sugar was indicated by the formation of a red precipitate.

- *Benedict's Test*

An amount of 1 mL Benedict's reagent was added into 1 mL of filtrate. The mixture was boiled, and the presence of sugar was indicated by a formation of a precipitate.

Detection of Saponins

An amount of 4 mL diluted methanol, and water extracts in the test tube was shaken for 15 minutes. A formation of 2 cm foam indicated the presence of saponins.[7]

Detection of Protein and Amino Acids

Two drops of ninhydrin solution were added into 2 mL of diluted methanol and water extracts. A purple color indicated the presence of amino acids.[7]

- *Libermann-Burchard's Test*

Its corresponding solvent diluted a small amount of each sample. Two drops of the sample were placed in a test plate, and a drop of acetic anhydride was added to each sample. Later, 1 or 2 drops of concentrated sulfuric acid were slowly added. A blue color indicated the presence of terpene. On the other hand, the purple color indicated the presence of steroids.[6]

Detection of Phenolic and Flavonoids

- *Ferric Chloride Test for Phenolic*

Two drops of diluted samples were placed in a test plate, and 1 drop of 5% ferric chloride solution was added to each sample. The formation of dark green color indicated the presence of phenolic compounds.[7]

- *Magnesium and Hydrochloric Acid Reduction Test for Flavonoids*

An amount of 2 mL of the diluted sample was placed in the test tube. Subsequently, three pieces of magnesium pellets were put into each tube. A few drops of concentrated hydrochloric acids were added slowly. The presence of flavonoids was detected by the formation of pink to crimson color.[7]

Brine Shrimp Lethality Bioassay

This is a rapid and comprehensive bioassay to determine the cytotoxicity of bioactive compounds of natural and synthetic origin. Natural product extracts, fractions as well as the pure compounds, can be tested for their bioactivity and LD50/ED50.[8] It utilizes in vivo lethality in a simple biological organism (brine nauplii) as a convenient procedure for determining the compatibility of new bioactive plant extracts.[9]

Preparation of Seawater

Thirty-eight grams' sea salt was weighed, dissolved in 1 L of distilled water, adjusted to pH-8.5 using 1 mol/L NaOH and filtered off with cotton to get a clear solution.[10-11]

Hatching of Brine Shrimp

Brine shrimp (Artemia salina) eggs were hatched in artificial seawater prepared by commercial sea salt. Brine shrimp eggs were added in 1 000 mL of seawater in a 1 500 mL beaker. Two days were allowed to hatch the shrimp and to be mature as nauplii. Nauplii were transferred from the beaker by a pipette and diluted in fresh, clear seawater to increase visibility, and ten nauplii were taken care of by micropipette.[12]

Preparation of Test Solutions with Samples of Experimental Plant

Twenty milligrams of the test sample was dissolved in 200 µL pure dimethyl sulfoxide to give a crude extract concentration of 20 mg/mL.[6] A two-fold serial dilution was carried out with artificial seawater to obtain a test solution in the range of 800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 µg/mL. Then 2.5 mL of the plant extract solution was added in 2.5 mL of seawater containing ten nauplii. The number of maples was confirmed by colony counter.

Preparation of Control Group

Control groups were used in the cytotoxicity study to validate the test method and ensure that the results obtained were only due to the activity of the test samples and the effects of the other possible factors were nullified. Two types of control groups were used in the present study.

Preparation of the Positive Control Group

In the present study, potassium dichromate was used and was evaluated at very low concentrations (10, 5, 1, 0.5, 0.25, 0.125 and 0.06 µg/mL).[6]

Counting of Nauplii

After 24 hours, the test tube was inspected using a magnifying glass, and the number of surviving nipples was counted in

each tube. After that, the percentage of lethality of nauplii was calculated for each concentration. It is usually expressed as a IC50 value.

MTT-assay Procedure

MCF-7 cells were cultured in 25 t-flask and were maintained in Dulbecco's modified Eagle's medium supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 10% fetal bovine serum at 37°C with 5% CO2, 95% air and complete humidity. They were detached using 0.05% trypsin/ethylenediaminetetraacetic acid and counted through trypan blue and hemocytometer when reached ~90% confluence and then suspended again at a concentration of 4 × 104 cells/cm2 to add into a 96- well plate (i.e., 250 µL/well) via a channel pipette. Some wells were kept cell-free as blanks (i.e., controls) for background absorption and comparison.[9]

Statistical analysis

The mean results of the mortality percentage of the brine shrimp versus the log of concentrations were plotted using the Microsoft Excel (2010) spreadsheet application, which also formulated the regression equations. Then, it was used to calculate the LC50 values for the test samples.

RESULTS AND DISCUSSION

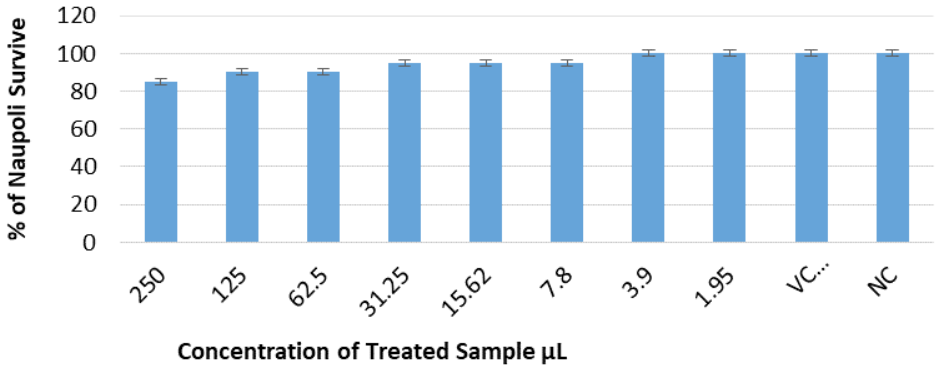
Percentage of Yield and Phytochemical Screening

According to extraction, the yield in percentage (%) obtained from *Lagerstroemia speciosa* (L.) leaves or *Banaba* of 0.52 kg in dry and raw using 75% ethanol was 34.23% (w/w) initially which was equivalent to 178 g of dried crude extract (Table 1).

While the preliminary qualitative phytochemical tests showed the presence of all the phytoconstituents targeted such as alkaloid, carbohydrate, glycoside, saponin, terpenoid, steroids, phenol and flavonoid (Table 2).

Brine Shrimp Lethality Bioassay

The cytotoxicity test on brine shrimp nauplii (*Artemia Salina*) is considered as an *in vitro* toxicity experiment. In this bioassay, the extract was dissolved 0.1% DMSO, which was used as the vehicle control, while potassium dichromate was the positive one.[13] In the experimental groups, a varying mortality rate of the sample at different concentrations was observed. The most noticeable survival rate ranged from 90 to 95 % at the concentration between 7.8 µL and 125 µL. In comparison, 100% survival or zero rate of mortality was found at the concentration of 1.95 µL and 3.9 µL (Figure 2).



*VC = Vehicle Control, NC= Normal Control.

Figure 2: The mortality rate % of brine shrimp nauplii (*Artemia Salina*) at 24 hour, after being exposed to various concentrations of *L. speciosa* leaves extract (Banaba).

Table 1: Percentage of yield determination of *L. speciosa* leaves extract

			Rotary evaporator settings				
Plant sample (powder)	*Ext. process*	*Solvent*	*T(°C)*	*R (rpm)*	*P (mbar)*	*Crude extract (g)*	*Yield (%)*
520g	Cold maceration	Ethanol (1:7)	60ºC	85 rpm	500-175	178	34.23

Table 2: Qualitative phytochemical analysis of the *L. speciosa* leaves extract.

Detection	*Test Reaction*	*Positive indication*	*Results*
Alkaloids	Mayer's test	White/creamy	++
Carbohydrate	Fehling's test	Red precipitate	+
Glycosides	Benedict's test	Precipitate	+
Saponin	Foam test	Foam	+
Terpenoids	Libermann-Burchard's test	Pink-purple	++
Steroids		Emerald-green	+
Phenols	$FeCl_3$ test	Dark-green	++
Protein and amino acids	Ninhydrin solution	purple color	+
Flavonoids	Magnesium and hydrochloric acid reduction test	crimson color	++

(+) indicated the presence/intensity of the phytochemicals group in the tested plant sample.

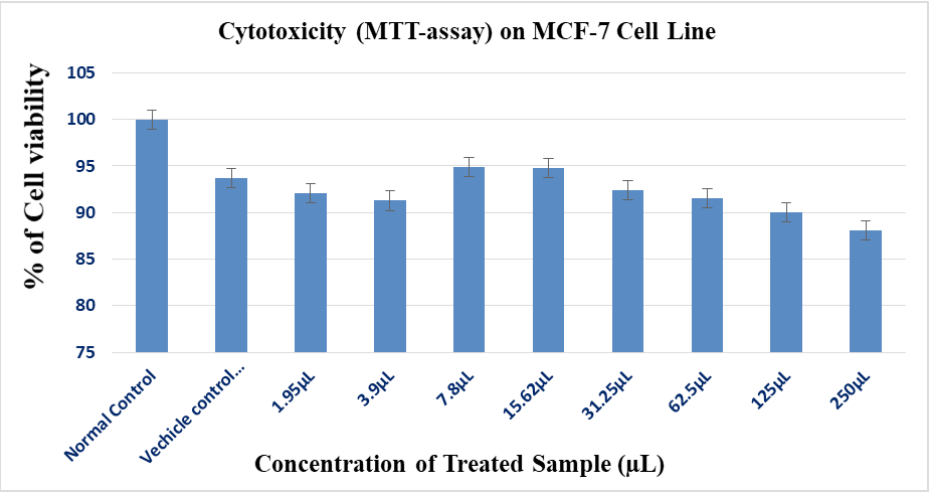


Figure 3: Percentage of cell viability at different concentrations of Lagerstroemia speciosa leaves extract (Banaba) using MTT-assay.

However, the mortality rate was not significantly ($p > 0.05$) different at the concentration 250 µL/mL (12%) compared to 1.95 µL/mL (0.00%), the lowest concentration. This shows that the extract of *Lagerstroemia speciosa* (L.) leaves is nontoxic.

The MTT-assay of MCF-7 Cell line.

The study was performed by partial modification of the previously described method.[14-15] The result of MT assay showed the highest percentage of cell viability was 96.0% at concentration 1.95 µL/mL whilst the lowest was 88.0% at 250 µL/mL. However, the concentrations 7.8 and 15.62 µL/mL showed the same cell viability of 94.82% when the vehicle control (MDSO; 1% v/v) was 93.7% At the same instance, the concentrations such as 1.95 µL/mL vs.31.25 µL/mL and 3.9 µL/mL vs. 62.5 µL/mL have shown almost the similar cell viability of 92.04% vs. 92.4% and 91.27% vs. 91.54% respectively. The overall results of *in vitro* MCF-7 cell line MTT assay showed that the ethanol extract of *Lagerstroemia speciosa* (L.) was not lethally toxic at the range of concentrations from 1.95 µL to 250µL/mL compared to normal control and vehicle control (Figure 3).

CONCLUSION

The current study suggested that the crude ethanol extract of *Banaba* is not lethally toxic to brine shrimp nauplii and MCF-7 cells as they are well- tolerated to the tested dose level from 1.95 to 250 µL/mL. Further studies are required to confirm LD_{50} and no-observed-adverse-effect level (NOAEL) of *Lagerstroemia speciosa* (L.) leaves extract, which might be predicted above the highest concentration used (250 µL/mL) while LD_{50} could be at 1,041.67 µL/mL.

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