Journal of Advanced Research in Applied Sciences and Engineering Technology

Journal homepage:
https://semarakilmu.com.my/journals/index.php/applied_sciences_eng_tech/index
ISSN: 2462-1943

Phytochemical Screening of *Muntingia Calabura* Fruit for Antioxidant and Cytotoxic Activities

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ABSTRACT

Muntingia calabura (*M. calabura*) is an indigenous fruit species that is widely distributed in Malaysia and traditionally believed for constituting medicinal properties, yet little-known and underutilized. Hence, this study aimed to perform an extraction of *M. calabura* fruit to identify phytochemicals potential by carrying out several analysis such as Total Phenolic Content, Fourier Transform Infrared Spectrum (FTIR), High Performance Liquid Chromatography (HPLC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Besides, the antioxidant and cytotoxicity properties of *M. calabura* were also identified from Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and brine shrimp lethality tests. The finding suggested that further study on the modification of *M. calabura* extraction should be carried out to explore maximal potential of *M. calabura* as natural medicine and commercial use.

Keywords:
Muntingia calabura, phytochemicals, antioxidant, cytotoxicity

Received: 3 July 2022 | **Revised: 30 August 2022** | **Accepted: 3 Sept. 2022** | **Published: 21 Sept. 2022**

1. Introduction

Natural constituents (bioactive compounds) from plant-based can be found from any part of plants such as leaves, flowers, fruits, seeds and roots [1]. Bioactive compounds that typically present in plant-based fruits, for instance polyphenols and antioxidants that can provide numerous health benefits over basic of nutrition and chronic disease prevention [2]. Previous reported that the bioactive compounds can regulate the metabolic processes for better health [3]. However, it has been a great challenge for the study on the evaluation of bioactive compounds towards the identification of biological activities of plants or fruits [4]. The use of organic solvents such as ethanol aqueous, methanol or acetone could establish methods for extracting the bioactive compounds [5]. To date, there are several extraction methods have been introduced, such as

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https://doi.org/10.37934/araset.28.1.116125

solvent extraction [6,7], distillation method [8] and pressing extraction [9]. Among the method, the solvent extraction has reported as the best extraction method due to better separation effect, forms higher degree of selectivity and faster mass transfer [10]. Several advantages of solvent extraction such as fast in action, large production capacity and low energy consumption [11].

Meanwhile, *Muntingia calabura* (*M. calabura*) is an indigenous fruit that globally known as Jamaican cherry which belong to genus Muntingia [12] and originated from tropical South America. It is widely cultivated in the east coast of Peninsular Malaysia [13] that can be seen throughout the year and its leaves are particularly lanceolate in shape. The taste is similar to ripe berries containing high content of soluble solids (10.24˚Brix), low total titratable acidity (0.11 g citric acid per 100 g fruit) with pH 5.64 [14]. Moreover, the fruit has been reported has 14.64% carbohydrates, 2.64% proteins, 2.34% lipids, 1.75% fibers and 1.28% minerals sources [14]. Various potential of *M. calabura* fruit were reported elsewhere for anti-inflammation, anti-diabetic, anti-oxidants, anti-ulcer and anti-proliferative [12,13]. Figure 1 shows the image of *M. calabura* fruits.

Fig. 1. *Muntingia calabura* fruits

In this study, *M. calabura* fruit was extracted in different solvents and its phytochemical properties were identified using range of qualitative and quantitative methods include Total Phenolic Content, Fourier Transform Infrared Spectrum (FTIR), High Performance Liquid Chromatography (HPLC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The antioxidant and cytotoxicity properties of *M. calabura* were also identified from Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and brine shrimp lethality tests.

2. Methodology

2.1 Solvent Extraction of Muntingia Calabura

Fresh fruits of *M. calabura* were collected from Jeli, Kelantan, Malaysia. The fruits were washed under running tap water and dried before weighing. The fruits were cut into smaller parts and put in oven to dry at 60℃ for approximately 12 hours to remove water content. Later, the fruits were grounded into powder form and weighed. The dried fruits were macerated in 70% methanol for about 70 hours before finally filtered and evaporated using rotary evaporator for about 12 hours. Figure 2 shows the fresh fruits and its final powder form.

Fig. 2. *M. calabura* of fresh fruits and powder form

2.2 Characterization

Functional groups and spectral properties of *M. calabura* were analysed by using Fourier Transform Infrared Spectroscopy (FTIR) (Thermo ScientificTM Nicolet iZ10) with single bounce attenuated total reflectance (ATR) technique in the scan range of 400 cm^{-1} until 4000 cm^{-1}. High Performance Liquid Chromatography (HPLC) analysis of the secondary metabolite of *M. calabura* was performed using UltiMateTM 3000 (Thermo Scientific Dionex, Bannockburn, IL, USA) that has photodiode array detector [15] and Chromeleon® software version 6.8 (Thermo Scientific) used for data reading. Chromatographic separation was done at 30 °C using 5-μm C18 EC column (4.6 × 250 mm) with respective parameter; flow rate of 1.0 mL min^{-1}, 5 μL sample injection, solvent A (water acidified with phosphoric acid, pH = 3.00) and solvent B (methanol) as mobile phase. Detection was observed at 280 nm and identification of the phenolic compounds was made based on the highest absorption wavelength of gallic acid at 272 nm.

2.3 Total Phenolic Content

0.5 ml of 1 mg/ml of M. calabura and gallic acid were added into respective test tubes with 2 ml of diluted 10% Folin-Ciocalteu reagent was put into each tube. Then, 4 ml of sodium carbonate (Na_2CO_3) was added into the test tubes and continue with the incubation at room temperature for 30 minutes. The absorbance of final solution was measured at 765 nm using spectrophotometer with methanol as blank solution. Quantitative analysis was carried out based on the standard calibration curve of different concentration of methanolic gallic acid. Total phenolic content of the extracts was expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g).

2.4 DPPH Assay

The antioxidant activity was carried out according to previous literature [16]. 5 ml of 0.1 mM DPPH solution was added to different concentration (100 to 500 µg/ml) of extract respectively and ascorbic acid was used as standard solution. The extract solution and ascorbic

acid were tested for the absorbance at 517 nm with 80% methanol as blank. The DPPH radical scavenging activity was calculated based on equation:

$$\% \text{ scavenging} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100 \quad (1)$$

which Abs_{sample} = absorbance of sample solution; $Abs_{control}$ = absorbance of control

2.5 Minimum Inhibition Concentration and Minimum Bactericidal Concentration

Escherichia coli (E. coli) and *Bacillus Subtilis* (B. subtilis) cultures were streaked on Mueller Hinton (MH) agar plates. Minimum inhibitory concentrations (MIC) was based on serial dilution method with some method modification [17]. 1 mL of MHA were added into every well in first row of the plate. Later, 0.08 mL of 1 mg/mL methanolic extract and another 0.92 mL of MH were added to the first well of first row and the mixtures were suspended. A two-fold serial dilution was then performed to achieve final concentration between 0.63 – 20 mg/ml by transferring 1 mL of the solution from the 1st well to the 2nd, 3rd, 4th, 5th and 6th well. In this method, Chloramphenicol was used as positive and DMSO as negative controls. Once the agar have solidified, 2 μL of inoculum was added to each well before the plates were put incubated at 37°C for 24 hours before turbidity and non-turbidity were observed.

2.6 Brine Shrimp Lethality Bioassay

Commercial sea salt (38 g sea salt per 1 little of deionized water) was prepared as artificial seawater. Then, the brine shrimp eggs were hatched in the seawater with constant light source and oxygen supply after 24 hours of incubation. A serial dilution of *M. calabura* methanolic extract was carried out to achieve final concentration between 1.96 to 1000 µg/mL prepared in seawater respectively. After serial dilution, ten hatched nauplii were added into each concentration and were adjusted to 3 mL sea water. The brine shrimps were then incubated for 24 hours under room temperature and the release of living nauplii at the next day was counted to determine % mortality or the lethal concentration (LC_{50}) for *Artemia nauplii*, based on following equation.

$$\% \text{ Mortality} = \frac{\text{Number of dead nauplii}}{\text{Initial number of live nauplii}} \times 100 \quad (2)$$

3. Results and Discussion

The moisture content of the fruit was calculated as 79.8% that is considerably high and almost similar obtained by [18]. This is a good indication that *M. calabura* has abundance of nutrients and quenching properties. The alcoholic solvent was used for the extraction of phytochemicals of *M. calabura* was chosen considering the polarity of the solute [19,20]. Thus, in this study 70% methanol was used for extraction and separation of wide range of components that present in the fruits. Powdered-dried fruits was first macerated in 70% methanol to extract polar compounds which constitutes the bulk compounds present in the samples. The extract of *M. calabura* yielded 26.7%.

The major absorption peaks that were observed from FTIR spectrum (Figure 3) almost similar finding that also reported previously according, denoted based on different modes of vibrations. The first peak at 3280.74 cm^{-1} was assigned to free and hydrogen bonding O-H stretching [21]. Whilst, two sharp peaks appeared at 2923.30 cm^{-1} and 2853.44 cm^{-1} were assigned to methylene group and C-H stretching respectively. The absorption peaks of the stretching C=O of acetyl group, and amide C=N at 1742 cm^{-1} band [22] and 1628.36 cm^{-1} [22], respectively. The absorption band at frequency 1082.33 cm^{-1} demonstrated the presence of C-O group from carbohydrates.

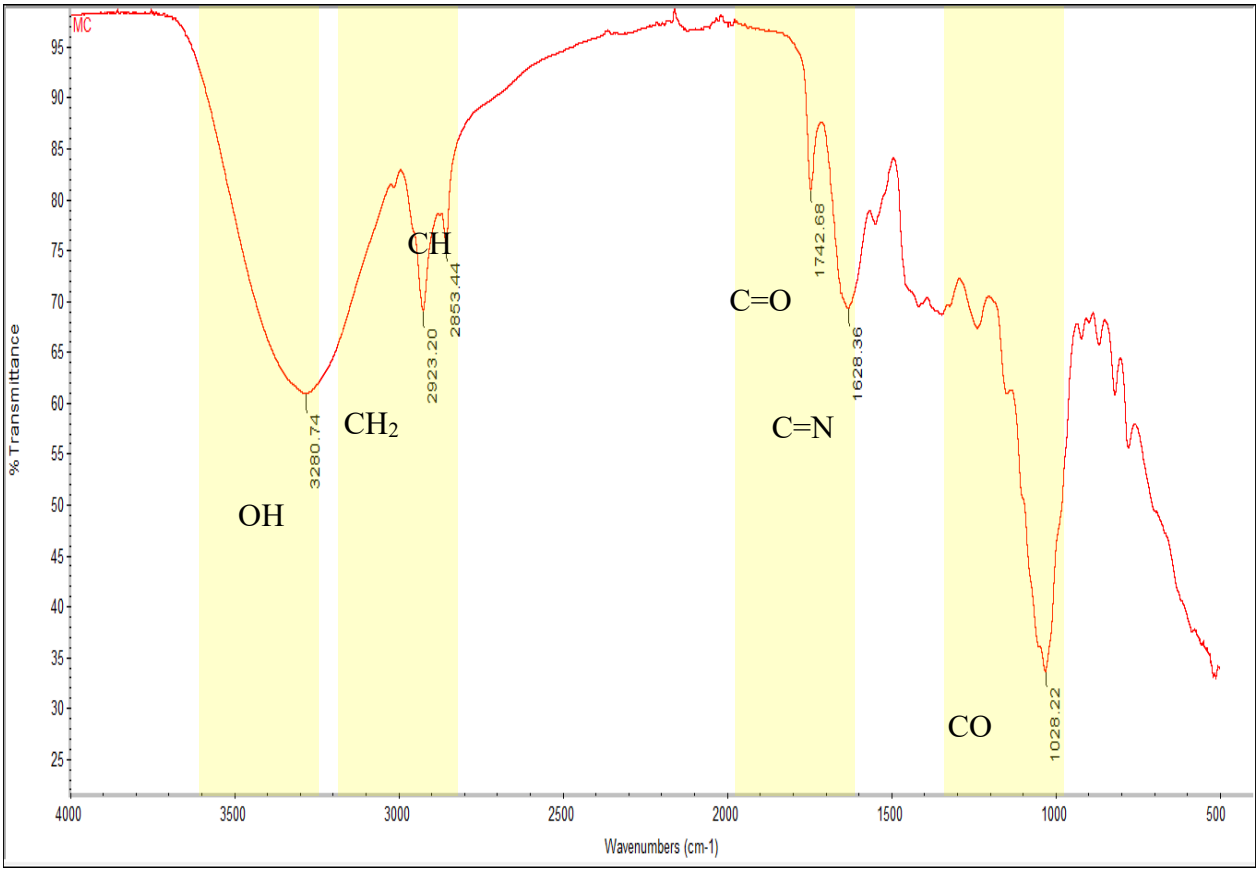

Fig. 3. FTIR spectrum of *M. calabura* methanolic fruit extract

The isolation and determination of individual phenolic content in fruits have been gaining huge interest for food and pharmaceutical applications [23]. The potential of *M. calabura* as antioxidant was identified based on the presence of gallic acid in fruit was determined using High Performance Liquid Chromatography (HPLC). Figure 4 (a) and (b) shows the chromatogram of gallic acid separation as standard and *M. calabura*extract respectively. By comparison of a and b, the presence of gallic acid in *M. calabura* extract was identified at 3.622 min retention time. Gallic acid is a natural phenol that possess strong antioxidant that exhibits antimutagenicity and more effective than water-soluble antioxidants. In addition, gallic acid derivatives were studied to be effective against *Staphylococcus aureus* that could attribute to antimicrobial properties. The presence of gallic acid proven its antioxidant properties in *M. calabura* fruit [24].

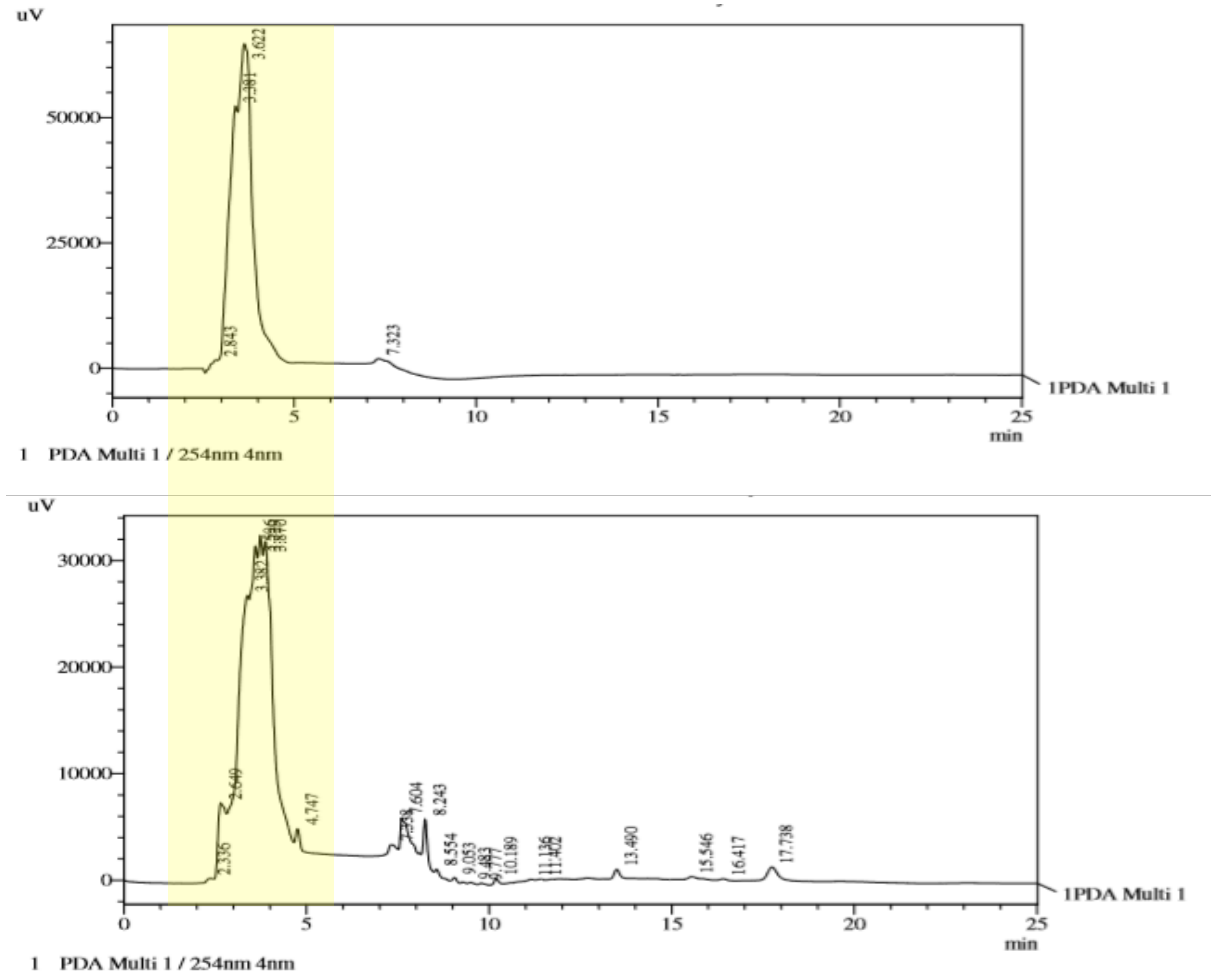

Fig. 4. Chromatograms of **a)** Gallic acid standard (above) and **b)** *M. Calabura* (below)

Meanwhile, Folin-Ciocalteu (F-C) method was used to identify total phenolic content (TPC) of *M. calabura* based on the formation of blue coloured complexes, $(PMoW_{11}O_{40})^{-4}$ which indicated the presence of phenolic compounds [25]. Total phenolic content was calculated based on the regression equation of calibration curve (Y = 0.0448x + 0.1637), with value of R^2 = 0.9975 and expressed 8.77 ± 0.91 mg GAE/g sample in dry weight. The presence of phenolic is in good agreement to the phenolic HPLC profile characterization and the TPC in the free form reported higher than that of bound form in other fruits [14], indicating that the *M. calabura* is actively contributing to the antioxidants of plant bioactivities. The antioxidant activity of *M. calabura* extract was determined via DPPH radical scavenging assay expressed in terms of percentage of inhibition (%) and IC_{50} values (µg/ml). Ascorbic acid was used as standard compound proportional to the examination of the antioxidant activity of *M. calabura.* Ascorbic acid was chosen as standard due to its reductive properties which is vital for therapeutic purposes and biological metabolism. In addition, ascorbic acid plays crucial role in activating the immune reaction and other metabolic responses [26]. Figure 5 shows linear representation of the antioxidant assay for both standard and *M. calabura.* From the obtained result, the antioxidant activity of ascorbic acid and *M. calabura* obtained at IC_{50} value of 95.86 µg/ml and 166.63 µg/ml, respectively. T-test analysis between ascorbic acid with *M. calabura* exhibited *p*-value of 0.045 which is depicted as significant difference as $p \leq 0.05$. Thus, it can be deduced that *M. calabura* plant is moderate antioxidant compared to ascorbic acid.

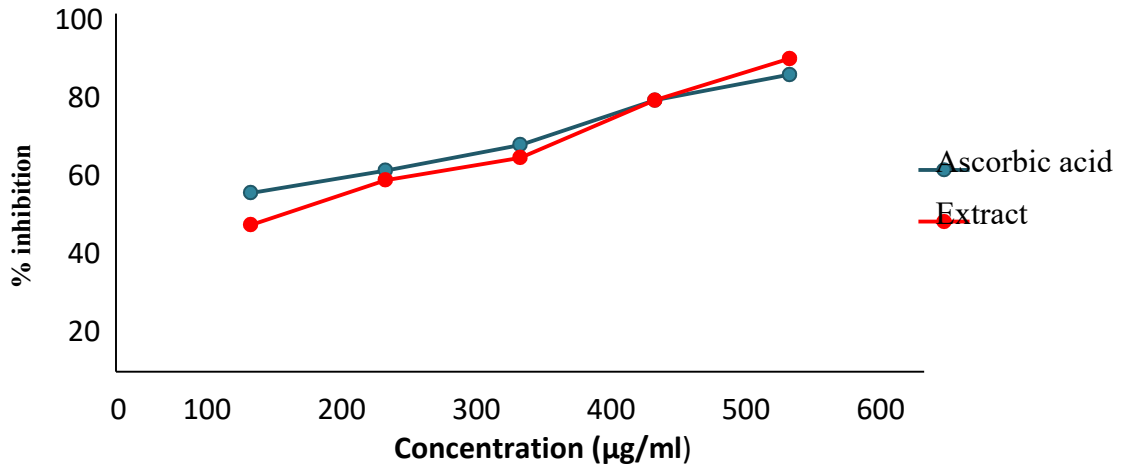

Fig. 5. The antioxidant activity of *M. calabura* over different concentrations

The antimicrobial study was carried out using Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays. The MIC assay signifies the lowest concentration to inhibit the growth of microbes whereas the MBC assay was used to reconfirm the results of MIC. In general, MBC represents the concentration at which 99% of the bacteria were killed. In this study, MIC and MBC values of *E. coli* and *B. subtilis* were 10 mg/mL and 20 mg/mL respectively. From the finding, *M. calabura* fruit extract can be considered as weak bacteria inhibitor [27]. Low value could be explained because of the presence of various phytochemical compounds, for example polyphenolics which interferes the antimicrobial activity of the extract. Moreover, the compounds might exert antagonistic effect on the bioactive compounds that perform antimicrobial activity [27]. Further optimization on *M. calabura* extraction is suggested to ensure pure target phytochemicals could be obtained for efficient antimicrobial activity.

The brine shrimp lethality bioassay was used to identify the cytotoxic activity of *M. calabura*. The purpose of this assay was to test the bioactivity of extracts which in most cases correlates with cytotoxic and anti-tumor properties [28]. The LC_{50} value of *M. calabura* (455.93 µg/ml) was derived from the plot of percentage of the shrimp nauplii killed against the concentrations of *M. calabura* extract, as shown in Figure 6. To the best of our knowledge, this is the first study on cytotoxicity test of *M. calabura.* From the bioactivity evaluation, LC_{50} value was recorded less than 1000 µg/ml which classified as cytotoxic. Although the fruit is edible, LC_{50} value of *M. calabura* indicated the presence of cytotoxic compounds which is responsible for the observed toxicological activity [29]. The observed lethality of the *M. calabura* extracts to brine shrimps might indicate the presence of antitumor components.

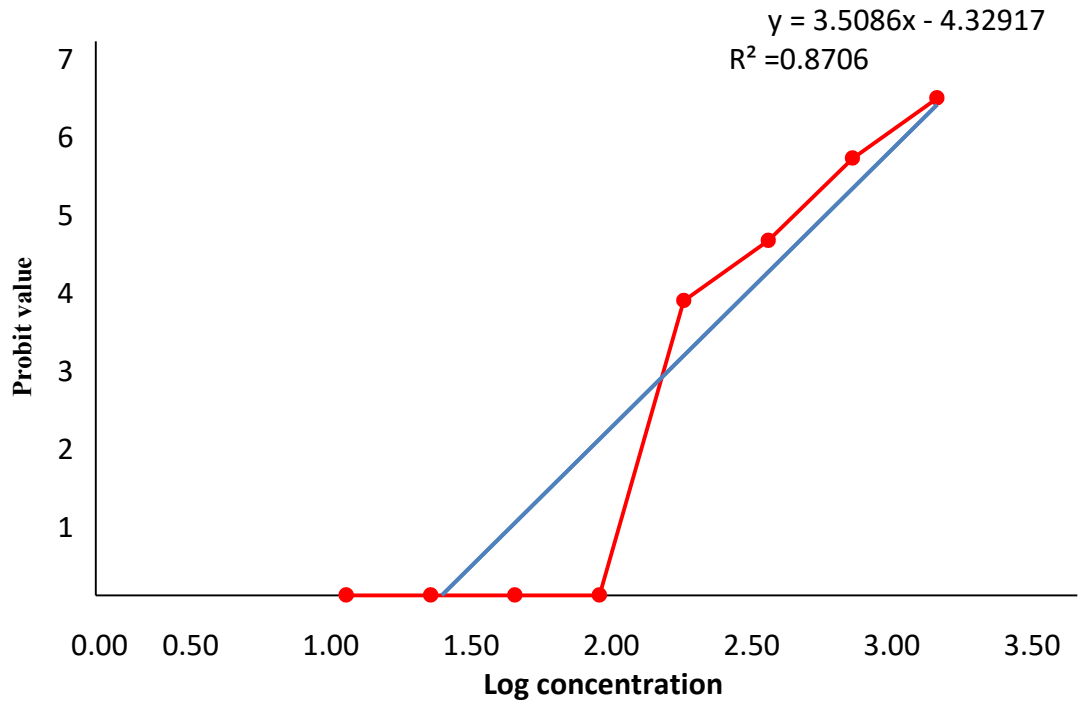

Fig. 6. Determination on LC_{50} of *M. calabura* extract

4. Conclusion

FTIR spectrum shows all important peaks of *M. calabura* extract was present. HPLC analysis also highlighted the presence of gallic acid, a phenolic compound that shows the potential antioxidant activity. This was further amplified by total phenolic content analysis which demonstrated high value of 8.77 ± 0.91 mg GAE/g sample in dry weight, indicating *M. calabura* has active antioxidant properties. Lastly, the result of brine shrimp test showed moderate toxicity (LC_{50} value of 455.93 µg/ml). Based on overall results, it can be concluded that *M. calabura* has rich source of phenolic compounds and further study should be carried out to explore its potential for commercialization. With some modification on the fruit extraction, it can be expected that the antioxidant and cytotoxicity activities of the fruit can be fully explored.

Acknowledgements

Author would like to thank Institute for Research in Molecular Medicine (INFORMM), USM for great collaboration and instrumentation facility.

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