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Phytochemical and toxicity evaluation of *Phaleria macrocarpa* (Scheff.) Boerl by MCF-7 cell line and brine shrimp lethality bioassay

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ABSTRACT

Objective: To evaluate the cytotoxicity of *Phaleria macrocarpa* fruits extracts.
Methods: The cytotoxicity test was carried out by *in vitro* MCF-7 cell line and *in vivo* brine shrimp lethality bioassay.
Results: The preliminary phytochemical test showed the presence of alkaloids, carbohydrate, glycosides, saponin, terpene, steroids, phenols and flavonoids. The MTT-assay results showed that the highest percentage of cell viability was 106.23% at concentration of 1.25 µL and the lowest percentage was 13.04% at concentration of 10 µL.
Conclusions: The MTT-assay and brine shrimp lethality bioassay results showed that the extract was non-toxic and it would be consumable as a herbal remedy.

1. Introduction

For centuries, the native Indonesians have been using the fruit of *Phaleria macrocarpa* (Scheff.) Boerl. (*P. macrocarpa*) to cure, treat and prevent diabetes liver diseases, vascular problems, cancer and high blood pressure[1-3]. It is known as Mahkota Dewa, are most commonly used in Indonesian traditional medicine for the treatment of cancer, diabetes mellitus, and hypertension. From an ancient era, the native Boerl combat diabetes, liver diseases, vascular problems, cancer and high blood pressure[4].

Previous studies have also shown that *P. macrocarpa* contains some secondary metabolites that could combat not only cancers or infectious disease such as malaria but also the so-called lifestyle diseases including diabetes, hypertension and atherosclerosis[5]. With respect to anti-diabetic studies, it has reported that the ethyl acetate extract of *P. macrocarpa* had anti-diabetic activity against alloxan-induced diabetic rats which were assumed to be mediated either by preventing the decline of hepatic diabetes or hypertension[6].

Natural phytochemicals have been reported to possess a wide range of biological activities including antioxidant, antimicrobial and anti-inflamatory properties[7]. *P. macrocarpa* contained kaempferol, myricetin, naringin and rutin as flavonoids. These flavonoids showed high potency as antibacterial and antifungal agent[4]. The leaves extract of *P. macrocarpa* had antibacterial, radical scavenging activities and cytotoxicity properties[8].

Empirically, *P. macrocarpa* has proved to be capable of controlling cancer, impotency, haemorrhoids, diabetes mellitus, allergies, liver and heart diseases, kidney disorders, blood diseases, rheumatism, high blood pressure, stroke, migraine, various skin diseases, acne and so forth[9]. Therefore, traditionally, seeds are only used for the treatment of skin conditions and for ornamental cultivation purposes or as a traditional biopesticide[3]. In current study, results showed that *P. macrocarpa* had potently inhibited carbohydrate hydrolyzing enzymes[6].

The identification of phalerin, a new benzophenone glucoside

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(3,4,5, trihydroxy-4-methoxy-benzophenone-3-O-β-D-glucoside) from the methanol extract of *P. macrocarpa* leaves[10,11]. Studies on the fruits of *P. macrocarpa* resulted in the isolation and characterization of icariside C3 and mangiferin[9].

A new phenolic glycoside mahkoside A, together with six known compounds including mangiferin, kaempferol-3-O-β-D-glucoside, dodecanoic acid, palmitic acid, ethyl stearate and sucrose were isolated from the pit of *P. macrocarpa*[2]. Lignans, pinoresinol, lariciresinol and matairesinol were isolated from different parts of *P. macrocarpa*. Recent results showed that *P. macrocarpa* had potently inhibited carbohydrate hydrolyzing enzymes[12].

The seeds of *P. macrocarpa* are very toxic and have unpleasant taste due to the presence of 29-norcucurbitacin, desacetylfevicordin A and other 29-norcucurbitacin derivatives, fevicordin A, fevicordin A glucoside and fevicordin D glucoside[13]. Seed of *P. macrocarpa* also contained flavonoid known as *viz.* quercetin. Traditionally, seeds are only used for the treatment of skin conditions and for ornamental cultivation purposes or as a traditional biopesticide. However, the mesocarp and pericarp of this plant fruits are non toxic and safe for animal experiment and human consumption[14].

2. Materials and methods

2.1. Collection and Identification of plant material

The ripe fruit (835 g) of *P. macrocarpa* was collected from area in Kuantan, Pahang, Malaysia in September 2012. Taxonomic identification was made by Dr. Norazian Binti Mohd. Hassan, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, International Islamic University Malaysia and deposited in the Herbarium, Faculty of Pharmacy, International Islamic University Malaysia, Malaysia (Voucher specimen No.: PIIUM 0230). The mesocarp and pericarp of the fruits were sliced and dried in a normal room temperature at 25 ˚C for 10 days, then it was pulverized to powdered form (713 g) using FRITSCH Universal Cutting Mill-PULVERISETTE 19, Germany, and kept in at 4 ˚C until further use[15].

2.2. Preparation of ethanol extract

The sample (710 g) was extracted to cold maceration by ethanol for 72 h at room temperature, filtered into a sterile round bottom flask using adsorbent cotton wool and filter paper (Whatman No. A-1). The extraction procedure with the solvent was repeated for eleven times to ensure the highest percentage of yield of ethanol soluble compounds from the *P. macrocarpa* fruits (mesocarp and pericarp) powder. The ethanol extract was concentrated in vacuo (temperature at 45 ˚C, 175 mbar and rotation 80–85 r/min) 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 bench top freeze dryer (ALPHA 1-4LD-2), to give an ultimate yield of 213 g.

2.3. 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.

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

2.4. Qualitative phytochemical screening of crude extract of *P. macrocarpa* fruits (mesocarp and pericarp)

The extracts were evaluated by 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[16].

2.4.1. 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 the Mayer's reagent to the test tube. A formation of white or creamy precipitate indicated the presence of alkaloids[17].

2.4.2. Detection of carbohydrates and glycosides

A small amount of methanol and water extract of selected plant was dissolved in solvent and filtered. The filtrated samples were tested with following reagents[18].

2.4.2.1. 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 formation of red precipitate.

2.4.2.2. 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 precipitate.

2.4.3. Detection of saponins

An amount of 4 mL diluted methanol and water extracts in the test tube was shaken for 15 min. A formation of 2 cm foam indicated the presence of saponins[19,20].

2.4.4. Detection of protein and amino acids

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

2.4.4.1. Libermann-Burchard's test

A small amount of each sample was diluted by its corresponding solvent. Two drops of 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 steroid.

2.4.5. Detection of phenolic and flavonoids

2.4.5.1. Ferric chloride test for phenolic

Two drops of diluted sample were placed in a test plate and 1 drop of 5% ferric chloride solution was added to each sample. Formation of dark green colour indicated the presence of phenolic compounds[16].

2.4.5.2. Magnesium and hydrochloric acid reduction test for flavonoids

An amount of 2 mL of diluted sample was placed in test tube. Subsequently, 3 pieces of magnesium pellet were put into each tube. A few drops of concentrated hydrochloric acids were added slowly. The presence of flavonoids was detected by formation of pink to crimson colour[16].

2.5. 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 LD_{50}/ED_{50}. 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[21-23].

2.5.1. 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.

2.5.2. Hatching of brine shrimp

Brine shrimp (*Artemia salina*) eggs were hatched in artificial sea water prepared by commercial sea salt. Brine shrimp eggs were added in 1 000 mL of sea water in a 1 500 mL beaker[24]. 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 a fresh, clear sea water to increase visibility and 10 nauplii were taken care by micropipette[25].

2.6. Preparation of test solutions with samples of experimental plant

Twenty milligrams of the test sample [ethanolic extract of *P. macrocarpa* (EEPM)] was dissolved in 200 µL pure dimethyl sulfoxide to give a crude extract concentration of 20 mg/mL[26]. A two fold serial dilution was carried out with artificial sea water 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 sea water containing 10 nauplii. The number of maple was confirmed by colony counter[27].

2.7. 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.

2.8. 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).

2.9. Counting of nauplii

After 24 h, the test tube was inspected using a magnifying glass and the number of surviving nipple was counted in each tube. After that, the percentage of lethality of nauplii was calculated for each concentration. It is usually expressed as a LC_{50} value.

2.10. 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% CO_2, 95% air and complete humidity[28].

They were detached using 0.05% trypsin/ethylene diamine tetraacetic acid and counted by means of trypan blue and hemocytometer when reached ~90% confluence and then resuspended at a concentration of 4×10^4 cells/cm^2 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[29-31].

2.11. Statistical analysis

The mean results of 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 LC_{50} values for the test samples.

3. Results

3.1. Percentage of yield determination and qualitative phytochemical screening

The ethanol extract of *P. macrocarpa* gave 30% yield from 710 g of raw fruits and finally it's became 213 g of dry crude extract (Table 1). In ethanol extract, the qualitative phytochemical test of this plant extract showed the presence of different phytochemical groups like alkaloids, carbohydrate, glycosides, saponin, terpene, steroids, phenols and flavonoids (Table 2). In addition, previous study on *P. macrocarpa* also reported that it conatined flavonoids, alkaloids, tannins, steroids, phenol, terpenoids, saponins, glycosides, carbohydrates respectively (Tables 1 and 2).

Table 1

Percentage of yield determination of *P. macrocarpa* with same solvent under same conditions.

Plant sample (powder)	Extract process	Solvent	Rotary evaporator settings			Crude extract (g)	Yield (%)
			Temperature (°C)	Rotation (r/min)	Pressure (mbar)		
710 g	Cold maceration	Ethanol (1:7)	60	85	500–175	213	30

Table 2

Qualitative analysis of the phytochemicals of *P. macrocarpa* extracts.

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	Libermann-Burchard's test	Purple color	+
Phenols	FeCl test	Dark-green	+

3.2. The brine shrimp lethality bioassay study of EEPM fruits

The cytotoxicity study was done by using brine shrimp nauplii (*Artemia salina*) and it was considered as an *in vivo* toxicity test. In this cytotoxic activity study, the different mortality rate of the nauplii was observed in experimental groups, but the mortality rate was not more than 40% with the concentration of 800 µg/mL. However, the mortality rate was 100% when the EEPM concentration was 1600 µg/mL. This result indicated that the EEPM fruits were nontoxic (Figure 1).

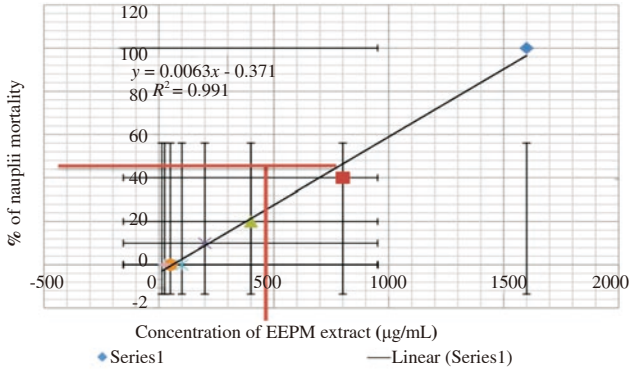

Figure 1. The mortality rate % of brine shrimp nauplii (*Artemia salina*) at 24 h, after being exposed to various concentrations of EEPM fruits.

The mortality rate of nauplii was increased when the applied test sample (EEPM) concentration was increased after the concentration reached EEPM 800 µg/mL. The IC_{50} value was determined. The administration of the EEPM fruits induced no significant cytotoxic activity where the LC_{50} was 2.93 µg/mL, whereas potassium dichromate was found IC_{50} with 0.92 µg/mL (Figure 2).

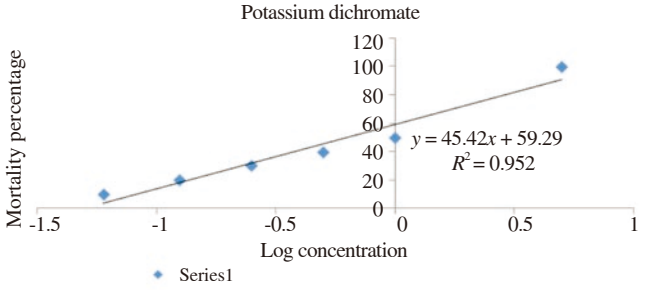

Figure 2. The mortality rate % of brine shrimp nauplii (*Artemia salina*) at 24 h, after treated with various concentrations of potassium dichromate.

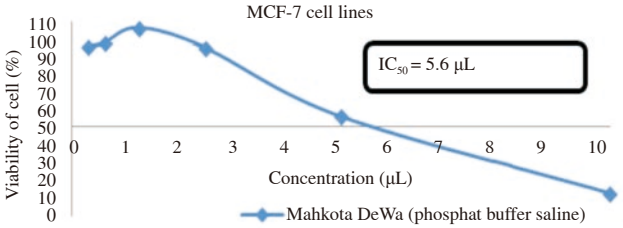

Figure 3. Percentage of cell viability at different concentrations of *P. macrocarpa* fruits extracts using MTT-assay.

3.3. The MTT-assay study of EEPM fruits

The MTT-assay results showed that the highest percentage of cell viability was 106.23% at concentration of 1.25 µL and the lowest percentage was 13.04% at concentration of 10 µL. However, rest of the concentration (0.31, 0.62 and 2.5 µL) showed a very well percentage of cell viability like 95.62%, 98.03% and 95.21%. Fruits extracts exhibited the IC_{50} values (56.65% cell viability) at the concentration of 5 µL. The overall results for *in vitro* MCF-7 cell line showed that the ethanol fruits extract was nontoxic (Figure 3).

4. Discussion

The extracts were found as a nontoxic substance against the brine shrimp nauplii. Therefore, the positive response obtained in this assay suggests that the plant fruits are nontoxic and it could be used as a traditional herbal remedy.

The EEPM fruits have been used as traditional herbal antidiabetic remedy for a long time in Association of Southeast Asian Nations countries, but until now there is not enough supporting scientific evidence that the fruits of this plant are not toxic. There were few toxicity studies related to the plant seeds that showed the strongest activity against cancer cells. The methanol extract of the plant seeds demonstrated cytotoxicity against different cancer cell lines like HeLa, colon cancer (HT-29), breast cancer (MCF-7), cervix cancer (CaSki) and malignant brain tumor (CGNH-89 and CGNH).

In this cytotoxic activity study, the different mortality rate of the nauplii was observed in both EEPM treated group and potassium dichromate treated group. The extract showed LC_{50} > 800 µg/mL and according to toxic dose level it clearly indicates that the extract is nontoxic. This indicated that the EEPM fruits are safe for further use or consumption as a herbal remedy.

Conflict of interest statement

We declare that we have no conflict of interest.

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