A PRELIMINARY STUDY OF 'DUKUNG ANAK' (*PHYLLANHUS NIRURI* LINN) PLANT EXTRACTS ON HAEMATOLOGICAL PARAMETERS

by

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CERTIFICATE

This is to certify that the dissertation entitled "A PRELIMINARY STUDY OF 'DUKUNG ANAK' (*PHYLLANHUS NIRURI* LINN) PLANT EXTRACTS ON HAEMATOLOGICAL PARAMETERS"

is the bonafide record of research work done by

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ABSTRACT

The aim of this study was to assess the effect of 'dukung anak' plant (*Phyllanthus niruri* Linn) on human blood particularly on mean platelet volume (MPV) based on *in vitro* experiment. The effect of the plant extracts were studied based on observation and analysis of changes in mean platelet volume (MPV) and other blood parameters like haemoglobin within two hours following treatment with 'dukung anak' plant extracts. The blood parameters analyses were done on automated haematology analyzer (Abbott Cell Dyn. Model: 4000). Three doses of water and ethanol extracts of 'dukung anak' plant were used to determine their effect on blood MPV in order to assess the ability of the extracts to prevent platelet aggregation.

'Dukung anak' plants were harvested around Pasir Tumboh area of Kelantan. Subsequently all parts of 'dukung anak' plants were dried and extracted by organic and non-organic solvents. The organic solvent used was ethanol, while non-organic was distilled water. The extracts were dried into powdered form.

From the powdered extract obtained, calculation of three dosages of each type of extract was done based on the amount of extract powder, volume of solvent and amount of blood to be used for testing. The extract was then dissolved in phosphate buffer saline (PBS) of pH 7.3 to form an extract solution. The extract solutions obtained were tested on five fresh human bloods from different healthy donors. The effect of extracts on blood

were studied at 0 minute to 120 minutes following treatment with the intervals of every half an hour at 30 minutes, 60 minutes, 90 minutes and 120 minutes respectively.

In the entire research, the dosage of extracts used were at 0.001mg, 0.01mg and 0.1mg of extracts per 1 milliliter (ml) of blood and the tests were done within 2 hours following treatment with the extracts. The data obtained were then analyzed with the SPSS program version 11.5 for Windows.

The results of this study showed that 0.1mg of ethanol extract of the 'dukung anak' plant for every 1ml blood did not give any significance changes on mean platelet volume (MPV). The result also showed that the all three doses of the water and ethanol extracts of 'dukung anak' plant did not cause any changes on haemoglobin value. This observation means that the 'dukung anak' plant did not cause any negative side effects on the blood parameters.

Based on these preliminary findings the initial hypothesis that the 'dukung anak' plant extracts were able to prevent significance changes on MPV and hence preventing platelets activation with no side effect on blood were substantiated. However this result should be confirmed with more extensive studies on animals (*in vivo* studies) based on platelet activities using a specific instrument such as platelet function analyzer or platelet aggregometer. For control, all the three doses of water extracts of 'dukung anak' plant (0.001mg, 0.01mg, 0.1mg), 0.001mg and 0.01mg ethanol extracts of 'dukung anak' plant for every 1 milliliter of blood following treatment were shown to have some significance changes on MPV.

ABSTRAK

Tujuan kajian ini adalah untuk mengkaji kesan pokok dukung anak (*Phyllanthus niruri* Linn) ke atas darah manusia terutama ke atas isipadu platelet min ('mean platelet volume'- MPV) melalui ujian diluar badan (*in vitro*). Kesan ekstrak pokok ini dikaji berdasarkan kepada pemerhatian dan analisis terhadap perubahan nilai MPV dan parameter darah yang lain seperti haemoglobin dalam masa dua jam selepas darah dirawat dengan ekstrak pokok dukung anak. Analisis parameter darah dibuat menggunakan penganalisis hematologi automatik (Abbott Cell Dyn. Model: 4000). Tiga dos ekstrak air suling dan ekstrak etanol pokok dukung anak digunakan untuk menentukan kesannya keatas MPV bagi mengetahui keupayaan ekstrak untuk menghalang agregasi platelet.

Pokok dukung anak diperolehi dari sekitar kawasan Pasir Tumboh di Kelantan. Kemudian keseluruhan bahagian pokok dukung anak diekstrak dengan larutan organik dan larutan bukan organik. Larutan organik yang digunakan adalah etanol, manakala larutan bukan organik adalah air suling. Ekstrak dikeringkan menjadi serbuk.

Daripada serbuk ekstrak yang diperolehi, pengiraan tiga dos untuk setiap jenis ekstrak dibuat berdasarkan berat serbuk ekstrak, isipadu pelarut dan isipadu darah yang digunakan untuk ujian. Ekstrak kemudian dilarutkan dalam larutan saline berfosfat (PBS) pada pH 7.3 untuk mendapatkan larutan ekstrak. Larutan ekstrak yang diperolehi kemudian diuji ke atas darah manusia yang diperolehi daripada lima penderma sihat yang berlainan. Kesan ekstrak keatas darah dikaji pada 0 minit hingga 120 minit selepas rawatan, dengan

selangan selama setiap setengah jam iaitu pada 30 minit, 60 minit, 90 minit dan akhirnya selepas 120 minit.

Pada keseluruhan penyelidikan, dos ekstrak yang digunakan adalah 0.001mg, 0.01mg dan 0.1mg ekstrak untuk setiap 1mililiter (ml) darah dan ujian dilakukan dalam masa 2 jam selepas rawatan dengan ekstrak. Data yang diperolehi kemudian dianalisis dengan program SPSS versi 11.5 untuk Windows.

Berdasarkan kepada keputusan untuk ujian, ini menunjukkan bahawa 0.1mg ekstrak etanol pokok dukung anak dalam 1ml darah tidak memberikan sebarang perubahan yang ketara kepada MPV. Keputusan ujian juga menunjukkan bahawa ekstrak etanol dan air suling pokok dukung anak tidak menyebabkan perubahan kepada kandungan haemoglobin. Ini bermakna bahawa pokok dukung anak tidak menyebabkan kesan sampingan ke atas parameter darah.

Ini bermakna hipotesis yang mengatakan ekstrak etanol pokok dukung anak berupaya mengurangkan perubahan MPV yang ketara yang seterusnya menghalang aktiviti platelet dan tidak menyebabkan kesan sampingan ke atas parameter darah telah dapat dibuktikan. Namun, pengesahan dengan kaedah yang lebih tepat perlu dilakukan iaitu dengan membuat kajian menggunakan haiwan (*in vitro*) berdasarkan kepada aktiviti platelet dengan menggunakan peralatan yang khusus seperti penganalisis fungsi platelet atau aggregometer platelet. Bagi kawalan, ketiga-tiga dos ekstrak air suling pokok dukung anak (0.001mg, 0.01mg, 0.1mg), 0.001mg dan 0.01mg ekstrak etanol pokok dukung anak untuk setiap 1 mililiter darah, didapati bahawa terdapat perubahan yang ketara pada MPV selepas rawatan.

1.0 INTRODUCTION

Platelets are directly involved, by their aggregation, in many physiological events such as hemostasis. Their hyperactivities contribute in the development and progression of many cardiovascular diseases like arterial hypertension, atherosclerosis and thrombosis. Indeed, it has been reported that patients with hypertension or coronary heart disease tend to have increase platelet reactivity (Mekhfi *et al.*, 2004).

Activation of platelets plays a key role in hemostasis and circulation. Following activation, platelets provide a catalytic membrane surface for thrombin generation, which accelerates the formation of fibrin necessary to stabilize thrombi. These thrombi are the source of thromboembolic complications of atherosclerosis, heart attacks, strokes and peripheral vascular disease (Ruggeri, 1997). Therefore, many investigations were carried out in order to prevent this abnormal hyperactivity of platelets reported in cardiovascular disorders using different mode of therapies, including the use of medicinal plants. It has been shown that some plants, such as garlic (Rahman and Billington, 2000), ginko biloba (Steriti, 1998) and tomato (Dutta-Roy *et al.*, 2001) may be benefial in protecting against cardiovascular disease as a result of their ability to inhibit platelet aggregation.

Phyllanthus niruri Linn or in Malaysia it is locally known as 'dukung anak' plant, is a medicinal plant that are widely used in the folk medicinal practice to threat diseases like hepatotoxicity and hypertension. In the literature searched, it was reported that 'dukung anak' plants contain a flavonoids like quercetin. Several in vitro studies have

showed that quercetin is able to inhibit platelet aggregation (Xie *et al.*, 1996., Guglielmone *et al.*, 2004).

Nevertheless, there was no information about the eventual influence of this 'dukung anak' plant on blood parameters particularly on the platelet activities. Thus, the present study was undertaken with the aim to examine the *in vitro* effect of water and ethanol extracts of 'dukung anak' plant on blood parameters, particularly on mean platelet volume (MPV) which may serve as an indicator of platelet activation. The observations were based on blood parameters using the automated haematology analyzer.

2.0 LITERATURE REVIEW

2.1 'Dukung anak' (*Phyllanthus niruri* Linn) plant

2.1.1 The plant

Phyllanthus niruri Linn or locally known as 'dukung anak' plant is a herbal plant that also called as 'amin buah' or 'keman jolok' (Zakaria and Mohd, 1999). 'Dukung anak' plant is a small herb growing up to 50-70 centimeters height and is indigenous to the rainforest of tropical and subtropical area (Jaganath and Teik, 2000). The flower and fruit along the undersurface of the branch of the leaves characterize this weedy herb. They grow and spread freely on an open, abandoned wasteland and by the roadside or riverside. (Zakaria and Mohd, 1994., Jaganath and Teik, 2000., Goh *et al.*, 1995). 'Dukung anak' plant belongs to the Eupharbiaceae family, which groups over 6500 species and 300 genera (Rain, 2004).

Figure 1: A picture of 'dukung anak' plant. (Image from:www.secure-shopping-cart.com/niam/cart/cart84.html. Accessed on 17 December 2004).

Figure 2: A picture of 'dukung anak' plants used in this research.

Kingdom – Plantae

Subkingdom –Tracheobionta (vascular plant)

Division –Magnoliophyta (Flowering plant)

Class –Magnoliopsida (Dicotyledonts)

Subclass –Rosidae

Order –Euphorbiales

Family –Euphorbiaceae

Genus –*Phyllanthus* Linn

Species –*Phyllanthus niruri* Linn

Subspecies –*Phyllanthus niruri* Linn *ssp*

('Dukung anak'plant)

Figure 3: Taxonomy of 'dukung anak' plant.

(From: http://www.itis.usda.gov/servlet/singleRpt/singleRpt?

Accessed on 17 December 2004).

2.1.2 Plant description

Seeds propagate 'dukung anak' plants. The seeds can be collected from matured plants that are about 2-3 months of age. Normally, a lot of seedling can be seen growing around mothers plants which can act as a good source of planting material (Jaganath and Teik, 2000). The plant is bitter in taste, the leaves are small, green in color and short petiole. The flowers are unisexual, and are borne solitary on the underside of the leaves. There are two types of flowers; the male and the female, both of which are very minute (Ross, 1999., Jaganath and Teik, 2000).

2.1.3 Chemical composition

'Dukung anak' plant contains bitter compound, phyllantin and hypophyllanthin (Zakaria and Mohd, 1994., Li,2002). Chemical compositions in this plant with commercial potential are geraniin, niruriside, phyllanthin, repandusinic and rutin. Niruriside from methanol extract of 'dukung anak' plant are found to act as HIV REV/RRE binding inhibitor (Jaganath and Teik, 2000). Other compounds in this plant are tannin, saponine and calcium oxalate (Zakaria and Mohd, 1999). This plant also contains terpenes and flavanoids like quercetin that can give effect on blood coagulation, quercitrin, isoquercetrin, astragalin, rutine and physetinglucoside. Lipids, benzenoids, alkaloids, steroids, alcanes and vitamin C are also compounds found in this plant (Rain, 2004., Jaganath and Teik, 2000., Goh *et al.*, 1995). Among the compounds that can be isolated from 'dukung anak' plant are phyltetralin, nirtetralin, niranthin and phyllanthin (Zakaria and Mohd, 1994., Li, 2002).

2.1.4. Traditional medicine uses

'Dukung anak' plants have good medicinal properties and are prominent ingredients in traditional medicine. All parts of the plant have medicinal value but not much research especially in Malaysia has been done on this aspect. The plant is traditionally believed to have antidysenteric, purgative, antihepatotoxic, antihypertensive and diuretic properties. It is used for treating jaundice, dropsy and genito-urinary infectious, emmenagogue, febrifuge, stomachache and swelling. In Malaysia, it has been used as an emmenagogue, diuretic, as a tonic and used after a miscarriage or childbirth (Jaganath and Teik, 2000., Ross, 1999).

The extract is effective in healing wounds and scurf (Zakaria and Mohd, 1994., Jaganath and Teik, 2000). Poultice of leaves is used for treating cuts, superficial wounds, ulcers and sores (Jaganath and Teik, 2000., Rain, 2004). It is also used for treating diarrhea, kidney trouble, gonorrhea, syphilis and for purifying the blood (Zakaria and Mohd, 1994., Jaganath and Teik, 2000., Rain, 2004). It is has been reported that hepatitis was cured when goat's milk boiled with the plant was consumed for a period of 1-2 months (Zakaria and Mohd, 1994). Hot water extract of dried entire plant is administered orally for diabetes, as a diuretic, for gonorrhea and genital tract infections, for jaundice, for leucorrhea and for asthma in Ayurvedic medicine (Ross, 1999). There have been no side effects or toxicity reported in any of the clinical studies done. Aqueous extract of 'dukung anak' plant is reported to be nontoxic to mice (Jaganath and Teik, 2000., Ross, 1999).

2.1.5. Pharmacological activities and clinical trials

'Dukung anak' plant has gained world attention in the late 1980's due to the plants antiviral activity against Hepatitis B and was found to contain the same beneficial phytochemical as in green tea, which helps to protect cells from stress and pollutants in the environment (Jaganath and Teik, 2000).

Beneficial effects for liver especially as treatment for hepatotoxic damage have been proven in scientific condition by Rohani (1998) and Sharidan (1998). The most recent research on 'dukung anak' revealed that its antiviral activity extends to human immunodeficiency virus (HIV). Ogata *et al.* (1992) discovered that an aqueous extract of *Phyllanthus niruri* (Euphorbiaceae) inhibit human immunodeficiency virus Type 1 reverse transcriptase (HIV-1-RT) and Naik *et al.* (2003) found that alkaloidal extract of *Phyllanthus niruri* showed suppressing activity on strains of HIV-1 cells cultured on MT-4 cell.

Khanna *et al.* (2000) reported that *Phyllanthus niruri* can cause a decrease in serum lipids level and also acts as an antioxidant. Clinical observation revealed that 'dukung anak' plant has a potential use as agent for diuretic, hypotensive and hypoglycemic drug on humans with no harmful side effect (Jaganath and Teik, 2000).

Besides those mentioned above, there were also researches on the effect of 'dukung anak' plant on analgesic activity, antibacterial activity, antifungal activity, antihyperglycemic activity, antitumour and antihyperlipemic activity (Ross, 1999).

2.2. Effect of 'dukung anak' plant on platelets

'Dukung anak' plants are use in traditional medicine to purifying the blood. Several compound including alkaloids, flavanoids, lignans, phenols and terpenes were isolated from these plant (Calixto *et al.*, 1998). 'Dukung anak' plants contain quercetin that is a flavanoids that can inhibit the aggregation of platelet and act as antithrombotic agent (Otto *et al.*, 1999., Xie *et al.*, 1996., Steriti, 1998).

In vitro studies have consistently shown that flavanoids rich foods and flavanoid extracts inhibit platelet aggregation (Duffy *et al.*, 2001). Several recent reports have suggested that increase dietary flavanoids consumption may inhibit platelet activation and function in human (Duffy *et al.*, 2001).

'Dukung anak' plant also contain vitamin C. Vitamin C can cause a delay of time in thrombus formation and also decrease platelet aggregation and activation (Mehta *et al.*, 1999., Wilkinson *et al.*, 1999). Vitamin C may be considered as an important compound in the prevention of chronic thromboatherosclerotic disease of the arteries (Bordia and Verma, 1985., Wilkinson *et al.*, 1999).

2.3 Mean platelet volume (MPV)

Platelet size measured as mean platelet volume (MPV) is a marker of platelet function and positively associated with indicators of platelet activity, including aggregation (Bath *et al.*, 2004). MPV is increased in patients at high risk for athero-thrombotic diseases. Thus, an elevated MPV may be considered as an important risk marker for platelet activation (Park *et al.*, 2002). Therefore, MPV can be a good parameter to study the activities of platelet in the blood following the addition of 'dukung anak' plant extracts.

3.0 AIMS AND OBJECTIVES

The aim of this study is to assess the effect of 'dukung anak' plant on human blood with the following objectives: -

i) To determine the effect and permeability of water and ethanol extracts of 'dukung anak' plant on blood especially platelets by focusing on mean platelet volume (MPV) and other blood parameters.

ii) To make a comparison effect of water and ethanol extracts of 'dukung anak' plant with different doses on the mean platelet volume (MPV).

iii) To determine the potential use of this plant as an anti platelets aggregating agent based on the study of platelet activities.

4.0 MATERIALS AND METHODS

4.1 Equipment and instruments

The equipments that have been used in this research project are as in the list below. The equipments and instruments can be found in the Pharmacology Laboratory (PPSP) and in the Laboratory Facility Unit (UKM-Unit Kemudahan Makmal), PPSK.

1. Hot air oven (Memmert) [Figure 7]
2. Grinder [Figure 8]
3. Weighing scale (Shimadzu. Model: BL-22004) [Figure 9]
4. Refrigerator (Panasonic. Model: NR-B30TA) [Figure 10]
5. Freeze dryer (Heto Power Dry. Model: LL3000) [Figure 11]
6. Water bath. (Memmert) [Figure 12]
7. Soxlet extractor apparatus [Figure 13]
8. Rotary evaporator with chiller cooling system (Buchi. Model: R114) [Figure 14]
9. Haematology analyzer (Abbott Cell Dyn. Model: 4000) [Figure 15]

4.2 Materials

4.2.1 'Dukung anak' Plant

'Dukung anak' plants (Figure 2) were harvested freshly from Pasir Tumboh, Kota Bharu area. All parts of the plant were used in this study. The plants were freshly collected, washed and dried in the hot air oven. The plants were then grinded into a powder form.

The extraction processes were performed with an organic based solvent (ethanol extraction) and with the non-organic based solvent (water extraction).

4.2.2 Phosphate buffered saline (PBS) - pH 7.3 (Green *et al.*, 1986).

- **Materials:**

  Solution A: 0.1 M KH_2PO_4

  13.6g of KH_2PO_4 was dissolved in an approximately 600mL of normal saline and made up to 1 liter with normal saline.

  Solution B: 0.1M Na_2PO_4

  14.2 g of Na_2PO_4 was dissolved in an approximately 600 mL of normal saline and made up to 1 liter with normal saline.

  (Both solutions were stored at 4^0C until they are used).

- **Method:**

  100 mL of a mixtures of solution A and B that consist of 23.6 mL of solution A and 76.4 mL of solution B were added to 900 mL of normal saline. The pH of this solution was checked and adjusted to 7.3 with the addition of a few drops of 0.1M HCL or 0.1M NaOH.

4.2.3 Human blood for test

The study was performed on 5 venous blood samples taken via venipuncture from healthy young volunteers (aged 19-23) following their inform consents [Figure 16]. Blood specimens collected with minimal venous occlusion were placed into tubes containing EDTA and aliquot into seven tubes.

4.2.4 Dose and duration of observation

For the entire test that was conducted, the doses of water and ethanol extract of 'dukung anak' were set at 0.1 mg, 0.01mg and 0.001mg per 1ml of blood. The test duration for the blood changes were observed at every half an hour intervals for a total of two hours duration. The doses were chosen based on the previous study of the 'dukung anak' plant extract effect on the hepatotoxicity activity (Ross, 1999).

4.3 Methods

Two types of 'dukung anak' plant extract, the water and ethanol extracts were used. Both of the extracts then were dissolved in PBS with three different concentrations. The extract solution in PBS was added to 1 ml fresh human blood in separate tubes. After every half an hour the blood that have been treated with the two different extracts at three different concentrations were analyzed with automated haematology analyzer. The complete research procedures were summarized in Figure 4.

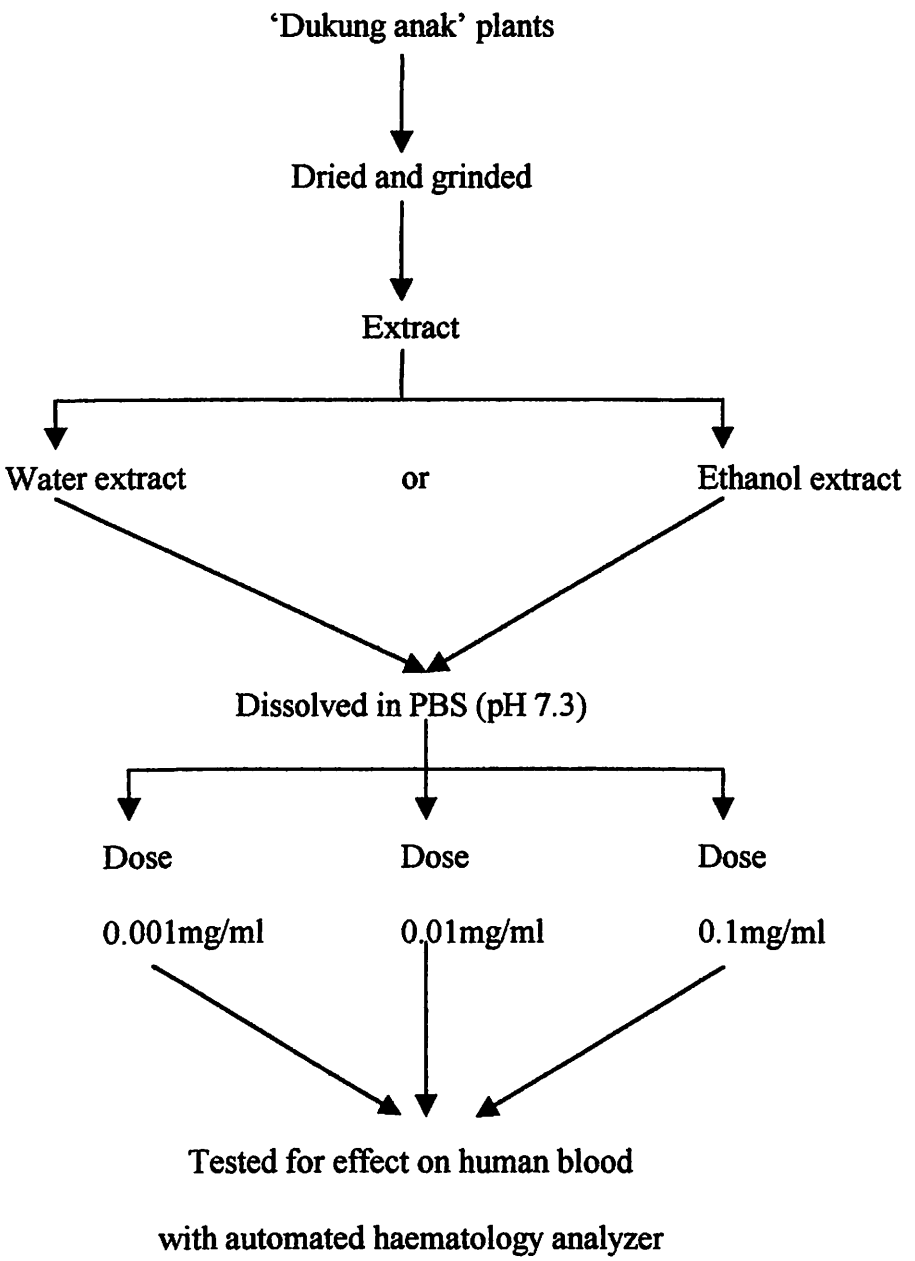

Figure 4: Summary of research procedure.

4.3.1 Processing 'dukung anak' plant for extraction

- **Materials**

1. Macer/grinder machine.
2. Distilled water.
3. Hot air oven.

- **Procedure**

Fresh plants of 'dukung anak' were cleaned from any dirty material by washing with tap water. The plants then were rinsed with distilled water. Following the washing and cleaning steps, the plants were then placed in a hot air oven for 3 days to dry at the temperature of 40°C or until no weight changes of the plants were observed. The dried plants were then grinded with a macer machine to increase the surface area for easy extraction. The milled plants were extracted with two types of solvent, water and ethanol in order to obtained two types of extract, the water and ethanol extracts.

4.3.2 Extraction process

Two types of extraction were done which are organic extract and non-organic extract. For the organic extract, ethanol was used as the extract solvent and for the non-organic extract, distilled water was used.

4.3.2.1 Preparation of ethanol extracts

The ethanol extract of 'dukung anak' was prepared with the use of denatured 95% ethanol.

- **Materials:**
  1. Water bath.
  2. Petri dish.
  3. Volumetric flask.
  4. Separating funnel.
  5. Gauze.
  6. Filter paper (Whatman 110mm diameter).
  7. Rotary evaporator with chiller cooling system (Buchi. Model: R114).
  8. Scapel.
  9. Hot air oven.

- **Procedure**

Milled 'dukung anak' plant (about 200g) was placed into volumetric flask. Then 500ml of 95% denatured ethanol was placed in the volumetric flask that contains milled 'dukung anak' plants. The milled 'dukung anak' plants and ethanol were mixed and then soak in waterbath at 60^0C for overnight. The mixture then filtered with gauze and Whatman filter paper. This first filtrate product was saved. The procedure was repeated by adding another 500ml of ethanol into the milled 'dukung anak' plants from the first step procedure to obtain the second and third filtration products. Products from all three

filtration processes were mixed and then evaporated using rotary evaporator (temperature 40^0C) until the solution become concentrated. The concentrated extract then was placed in petri dish and then was placed in an oven at 50^0C until the extract become fully dried (approximately overnight). The dried extract was then dug out from petri dish with scapel blades. The powdered ethanol extract obtained was placed in a bottle and kept in refrigerator (4^0C) to restore its composition until it was ready for use in the test.

4.3.2.2 Preparation of water extracts

A water extract procedure of 'dukung anak' plants was done using Soxlet apparatus extractor.

- **Material**

1. Soxlet apparatus extractor.
2. Rotary evaporator with chiller cooling system (Buchi. Model: R114).
3. Freeze dryer machine (Heto Power Dry. Model: LL 3000).
4. Mc Cartney bottle.
5. Gauze and filter paper (Whatman 110 diameter).
6. Separating funnel.
7. Volumetric flask.
8. Distilled Water.

- **Procedure**

Approximately 220g of milled 'dukung anak' plants was placed in thimbles (Soxlet apparatus). Two liters of distilled water was placed in a round bottom flask (Soxlet apparatus). Soxlet apparatus was set and switch on to start the extraction process. After 4 days, Soxlet apparatus was switched off. The water extract available then was filtered with gauze and Whatman filter paper. The filtrate then was kept in a cold room. This water extract then was concentrated with rotary evaporator at 80°C. The concentrated water extract then placed in Mc Cartney bottle and freeze in the freezer until the solution becomes frozen. This process took approximately overnight. The frozen extract was then subjected to freeze drying technique at the temperature of 55°C in vacuum chamber. The powder that obtained was placed in a bottle and kept in desiccators and placed in the refrigerator (temperature 5°C) until ready to be used in the test.

- **Principle of Soxhlet apparatus procedure**

The principle of Soxlet apparatus is use a reaction distilled water solvent to keep the materials dissolved at a constant temperature by boiling a solvent (distilled water), condensing it and returning it to the vessel. The water dissolved materials in 'dukung anak' plant were extracted by repeated washing with distilled water under reflux in special glassware.

The flask was heated and the solvent (distilled water) evaporated and moved up into the condenser where it was converted into a liquid that trickled into the extraction chamber (thimble) containing the sample and after up to certain levels it overflowed and

trickled back down into the boiling flask. At the end of extraction process the distilled water was distilled off and the extracted substance remained in the evaporating flask.

- **Principle of freeze-drying**

The principle of freeze-drying consists of the removal of the ice contained in a frozen product by sublimation (vaporization of ice without going through the liquid stage). Following that, only the extract without solution (distilled water) is left in the bottle. In this process, pressures from cold air were used, which resulted in drying of sample, by sucking out the water from them.

4.3.3 Dose Preparation

- **Materials**

1. PBS.
2. Dimethyl sulphoxime (DMSO).
3. Spatula.
4. Digital balance.
5. Extract's powder.
6. Beaker (1-10ml).
7. Bottle (1-20ml).

- **Methods**

From the extracts that were obtained, the extracts solution with a concentration 0.1mg/ml, 1.0mg/ml and 10.0mg/ml were prepared. The water extract solution with a concentration 10.0mg/ml was prepared by dissolving 100mg of extract in 10ml of PBS. For the ethanol extract solution, 100mg of extract was dissolved in PBS and a few drop of DMSO were added to facilitate in dissolving the insoluble compounds in PBS. The final volume of solution obtained was 10ml. For extracts solution with concentration of 1.0mg/ml, 10mg of extract was dissolved in 10ml of PBS and a few drop of DMSO were added to the ethanol extract solution. Approximately 1ml of this solution with concentration 1.0mg/ml was the used for the preparation of extract solution with the concentration of 0.1mg/ml. The solution with concentration of 0.1mg/ml was prepared by adding 1ml of 1.0mg/ml solution in PBS and made up to the final volume of 10ml.

Based on the calculation, the doses of the extract that was added into 1ml of blood were at 0.001mg, 0.01mg and 0.1mg, respectively.

On completion of the dosage preparation, the prepared solution was stored in a refrigerator before being tested on blood.

4.3.4. Haematological study design

- **Materials**

1. Gauze.
2. Haematology analyzer (Abbott Cell Dyn. Model: 4000).
3. EDTA tube.
4. Plain test tube.

- **Method**

10ml of human blood specimen was taken freshly from a donor and placed in an EDTA tube. Approximately 1 ml each of the blood specimen then was placed into separate plain test tubes. Seven plain tubes were used, which in each tube contains 1ml of blood. The first tube then was added with 0.01ml of PBS only. This tube served as control for the test. Into the other six tubes that contain 1ml of blood, 0.01ml of the water and ethanol extracts at 0.001mg, 0.01mg and 0.1mg dose were added.

All the tubes were gently mixed and then run and analyzed using an automated haematology analyzer [Figure 15] to obtain the result of haematology parameters particularly the mean platelet volume (MPV). The blood specimens were further incubated at room temperature and the same procedures were repeated at every half an hour intervals for a total time of two hours duration.

These test procedures were done on five blood specimens from different donors. The outline of the study design used in this research was shown in Figure 5.

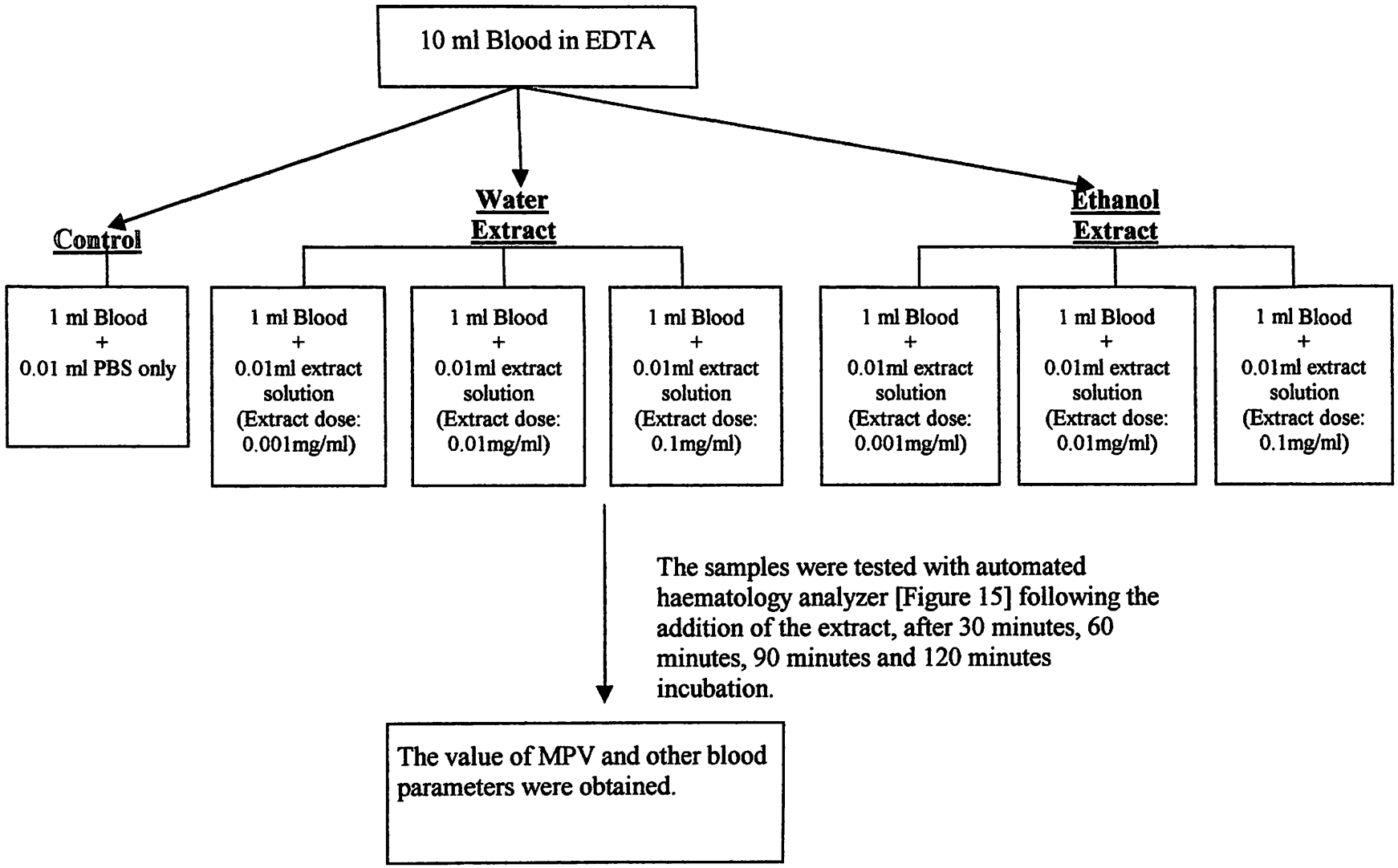

Figure 5: An outline of the study design.

4.3.4.1 Measurement of haematology parameters

Abbott Cell Dyn model 4000 of haematology analyzer was used to determine the changes of blood parameters after following treatment with 'dukung anak' plant extracts. Before proceeding with the test, the calibration of haematology analyzer was first done to ensure that the instrument was fully functioning and the results obtained were reliable. The blood parameters that can be determined by haematology analyzer are:

1. Total red blood cell (RBC)
2. Haemoglobin (HGB)
3. Hematocrit (HCT)
4. Mean cell volume (MCV)
5. Mean cell haemoglobin (MCH)
6. Mean cell haem. conc. (MCHC)
7. Red cell distribution width (RDW)
8. Reticulocyte counts (RETC)
9. Immature reticulocytes fraction (IRF)
10. Neucleated RBC (NRBC)
11. Total platelets count (PLT)
12. Mean platelet volume (MPV)
13. Platelet Distribution width (PDW)
14. Plateletcrit (PCT)
15. Total white clood cell (WBC)
16. Neutrophils (NEU)

17. Lymphocytes (LYM)
18. Monocytes (MONO)
19. Eosinophil (EOS)
20. Basophil (BASO)

4.3.5 Statistical analysis

The data obtained were subjected to repeated measure ANOVA (RM ANOVA) using SPSS 11.5 for Windows, with post hoc multiple comparisons for between groups treatment effect analysis and paired sample T-test for within group's treatment analysis based on time of the test. Statistical significance was accepted at $p < 0.05$ for post hoc multiple comparison analysis and $p < 0.005$ for paired sample T-test analysis.