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Phytochemicals and acute oral toxicity studies of the aqueous extract of *Vernonia amygdalina* from state of Malaysia

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Abstract
Vernonia amygdalina (VA) is a member of Asteracea family that use as traditional folk medicine in treating various infection and diseases. This study aim to determine the phytochemical characterization of Malaysian local VA and determine the acute oral toxicity. Phytochemical investigation was done by standard procedures. The crude extract of VA was observed to contained flavonoids, terpenoids, saponin and tannin. FTIR spectroscopy was done against VA aqueous extract revealed a presence of high content of flavonoids and terpenoids, phenols, methoxy compounds, ester carbonyl and amide. Sighting study for acute toxicity was conducted as per Organization of Economic Cooperation and Development (OECD) 425 guidelines which divided into sighting study and main study. One female Sprague Dawley rat was given single oral dose of VA aqueous extract at progressively dose of 175, 550, 2000 and 5000 mg/kg of body weight (BW), dissolved in 1ml distilled water and observed daily for 14 days. VA aqueous extract of 5000 mg/kg was used in main study as it exhibited no toxicity signs and mortality. All the treated rats survived and no toxicity signs were observed. No differences in body weights and organs weights. Liver function tests showed the levels of aspartate aminotransferase (AST) and globulin slightly increased but the levels of alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), albumin, total protein and total bilirubin were in normal range. Despite that, no elevation in the level of Alkaline phosphatase (ALP). Therefore, it can be concluded that the toxicity of VA is greater than 5000mg/kg BW as proved by AOT425 Statistical Programme.

Keywords: *Vernonia amygdalina*, phytochemical test, FTIR spectroscopy, acute toxicity

1. Introduction
VA is a shrub that can reach 23 feet in height when fully grown (Echem and Kabari, 2012) [5]. This plant commonly called as 'bitter leaf' with medium sized shrub and having petiolate green leaf of about 6 mm in diameter (Akah *et al*., 2009) [1]. It grows throughout the African tropics and in South Africa, and can be found in Kwazulu Natal, Mpumalanga, Eastern Cape and Northern Provinces (Erasto *et al*., 2005) [7]. VA is also a common homestead farming vegetables and fodder tree (Ndaeyo, 2007) [16], which used as an ingredient in preparing dishes by removal of its bitter taste through soaking in water with several changes or by boiling (Onabanjo and Oguntona, 2003) [19]. The macerated leaves of VA was reported to be consumed as vegetables and condiments (Arhoghro *et al*., 2009) [2]. Generally, VA has been used to cured fever, general rash, pain (headache, back pain), cough and stomach ache (Langlois-Klassen *et al*., 2007) [10]. VA was also reported to be the treatment against malaria, diarrhea, gastroenteritits, hepatitis, dysentry and diabetes mellitus (Gbolade, 2009; Mesfin *et al*., 2009; Vlietinck *et al*. 1995) [8, 14, 25]. In Malaysia, pulverized fresh leaves of VA are soaked in water and orally taken for the treatment of diabetes mellitus by local people (Erasto *et al*., 2005) [7].
The medicinal value of herbal plants lies in the phytochemical compounds that produce a definite physiological action on the human body (Mithraja *et al*., 2011) [13]. However, samples of medicinal plant from different plant varities and geographical origin have different complex chemical mixture (Sim *et al*., 2004) [22]. Therefore, the phytochemicals compound which produce the pharmalogical activities and act as a plant markers should be identified. Phytochemical defined as bioactive non-nutrient plant compounds that can be found in fruits, vegetables, grains and other plant foods that have been associated in reducing the risk of major chronic disease (Liu, 2004) [10].
Despite the long traditional use of VA, there has no indication of the toxicity of this medicinal plant. The LD_{50} of VA has been reported to be 1265.22 ± 56 mg/kg (Nwanjo, 2005) [17].

The leaf extract of VA also been reported of not having hepatotoxic in rats and the acute toxicity test gave an LD_{50} of 500 mg/kg (Ojiako and Nwanjo, 2006) [18]. As reported by Cosentino *et al*., 1999 and Hossain *et al*., 2013 [4, 9] geographical origin, locality, climate conditions and the time of harvesting could influnce the biological activities of the plant extract. Therefore, this study aims to determine the contain of phytoconstituent and measure the potential toxicity of Malaysian local VA aqueous extract as claim by local people having medicinal value.

Standard phytochemical screening and Attenuated total reflection - Fourier transform infrared spectroscopy (ATR-FTIR) have been applied in this study to characterize the phytochemical of VA. ATR-FTIR is relatively inexpensive, fast and easy technique, and only a small amount of samples needed to be scanned. It is also capables of providing wide informations, such as the peak positions, intensities, widths and shapes of the spectrum (Smith, 1999) [23]. In addition, ATR-FTIR is capable of greater speed and greater sensitivity than a dispersion instrument (Pavia *et al*., 2009) [20]. Acute oral toxicity study was conducted in in vivo model by using female Sprague Dawley (SD) rats, according to the Organization for Economic Cooperation and Development (OECD) guidelines for the testing of chemicals 420 adopted on 17th December 2001, in Fixed Dose Procedure with some modifications.

2. Materials and Methods

Plant material

The fresh leaves of VA were collected from Bachok, Kota Bahru, Kelantan, Malaysia. The identification of the leaves was performed by Natural Medicinal Products Centre, Kulliyah of Pharmacy, International Islamic University Malaysia (IIUM). The plant name also checked with www.theplantlist.org

Preparation of VA aqueous extract

The leaves were cleaned with towel to remove any debris and dust, then dried in an oven at 55 °C ± 1 °C for 72 hours prior crushing into coarse powder. The 25 g of VA coarse powder were extracted in 250 ml of distilled water at 60 °C ± 1 °C for 72 hours. The extract was filtered and further dried in oven incubator at 37 °C. The solid residue was referred as aqueous extract and the percentage of yield was calculated.

Phytochemical analysis

Phytochemical analysis for terpenoids, alkaloids, saponin, tannin, anthraquinone and flavonoids were conducted based on the standard procedures described by Trease and Evans, 1989 [22]. ATR-FTIR analysis was carried out using Bruker (Tensor 27) Spectophotometer. Briefly, the crystal spot was cleaned with a swab of acetone and the background measurement was collected. The sample of VA aqueous extract was then placed directly on the spot. Optical User Software (OPUS) version 7.0 was used for spectral data collection and analysis.

Acute oral toxicity study

Nulliparous and non-pregnant female SD rats *(Rattus norvegicus)* were obtained from Animal Research and Service Center (ARASC), Universiti Sains Malaysia (USM), Kubang Kerian, Kelantan. The rats weighing from 250-300 g and 8-12 weeeks old were chosen as experimental animals. They were kept in individual cages and have free access to water and standard pellet food (*ad libitum*). The rats were allowed to acclimatized for one week prior to the experiment. The Principles of Laboratory Animal Care (NIH, 1985) were followed during the experimentation period. Animal protocol was approved by the Animal Ethics Committe USM (AECUSM) under USM/Animal Ethics Approval/2012/(82)(418). The acute oral toxicity study was carried out according to OECD guidelines for the testing of chemicals 420.

Sighting study and main study

Four female SD rat were given single oral of VA aqueous extract at progessive dose of 175 mg/kg, 550 mg/kg, 2000 mg/kg and 5000 mg/kg of BW, respectively. The VA was freshly prepared based on curent BW of the rats. The extract was dissolved in distilled water prior to administer as the volume dosing. For main study, five female SD rats (four new rats and one from the sighting study which is the one received the highest dose that did not caused death) were administered with 5000 mg/kg of VA aqueous extract (the highest dose that found did not caused death). Both of the studies involved of observation of the rats at 0.5, 1, 2, 3 and 4 hours after administration of VA aqueous extract and followed by observation twice daily for 14 days for any signs of toxicity and mortality. Signs of clinical toxicity includes changes in the skin, fur, eyes and mucous membrane, behavior pattern, tremors, salivation and diarrhea. All of the observations were recorded in Observation Sheet Form.

Collection of blood and biochemical analysis

All rats were fasten for 16-18 hours before the necropsy. The rats were euthanized by intraperitoneal injection of 200 mg/kg BW sodium pentobarbital. Blood were collected by cardiac puncture and transferred into blood plain vacuum tube, and then centrifuged at 4500 rpm for 10 min. The serum was evaluated for liver function test (LFT). All organ includes lung, heart, liver, kidney, spleen, stomach and gastrointestinal (GI) tract were isolated and weighted (absolute organ weight). Any abnormality to the organs were documented. The organs were analysed in relative to the BW (ROW) of rats using the formula : ROW (g/100g) BW = Organ weight (g) / BW at day 15 (g) x 100.

3. Results

Identification of VA plant

The authentication of VA with voucher spesimen number PIIUM 0233 showed that VA belongs to Asteraceae family. The local name has been identified as ‘daun Bismillah’ and ‘Pokok Africa Selatan’ with its English common name as ‘Bitter leaf’.

Yield of VA extraction

The crushed powder of 25 g of VA yielded 8.28 g of crude extract which gave the total yield percentage of 33.1%.

Phytochemical analysis

Four major compounds were identified in Malaysian local VA extract by standard procedure of phytochemical analysis. They are terpenoids, saponin, tannin and flavonoids (Table 1). The FTIR spectrum in Figure 1 shows a broad band around 3373-3346 cm^{-1} assigned to O-H stretching vibrations of water molecules (Peak 1). This may also indicated the presence of phenol and flavonoid. The sharp peak observed at 2925-2854 cm^{-1} (Peak 2) was attributed to C-H stretching vibrations of methoxy compounds. The presence of sharp peak at 1650-1631 cm^{-1} (Peak 3) assigned to C=O stretching vibrations in carbonyl groups which characterized the high content of terpenoid. The sharp peak at 1390-1326 cm^{-1} (Peak

4) attributable to nitro compounds. Another sharp peak in between 1274-1036 cm^{-1} (Peak 4) was due to primary amines stretching.

Table 1: Qualitative phytochemical analysis by standard procedure of VA aqueous extract

+ = present - = absent

Phytochemical	Remark
Terpenoids	+
Alkaloids	-
Saponin	+
Tannin	+
Anthraquinone	-
Flavonoids	+

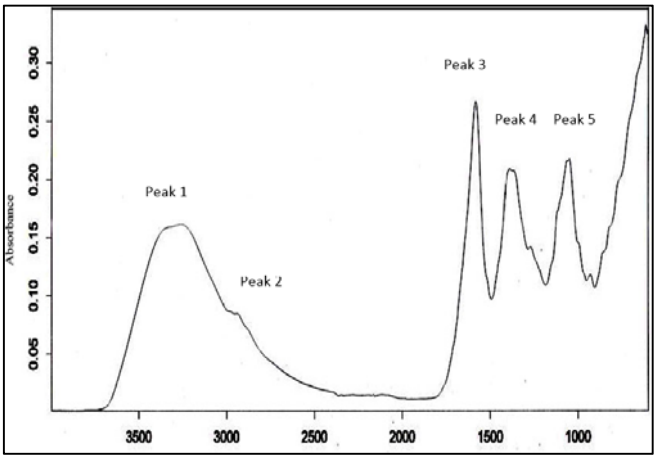

Fig 1: The ATR-FTIR spectrum of *V. amygdalina* aqueous extract

Acute oral toxicity study

Mortality, physical and behavioral observation

Throughout sighting study period, there was no mortality recorded and no physical and behavioral changes. Therefore, the highest dose of 5000 mg/kg of VA aqueous extract was used in the main study. As in the main study, no physical and behavioral changes observed throught out the 14 days of observation. Additionally, no mortality documented.

Body weight (BW) and relative organ weight (ROW)

There were no significant changes in BW and ROW observed for both sighting study and main study. The organs of the rats in both studies did not show any signs of abnormalities or gross lesions.

Liver function test (LFT)

In the sighting study, the level of total protein, ALT, GGT and total bilirubin were in between the normal range of rats. However, albumin levels were observed below the normal range (40 to 48 g/L) in the rats given 175 and 2000 mg/kg BW of VA aqueous extract. The level of ALP in rats given 175, 550 and 5000 mg/kg BW of VA aqueous extract detected below the normal range (132 to 312 U/L). In addition, globulin levels are above the normal range (12 to 20 g/L) in all the rats in sighting study. Rat given 5000 mg/kg BW of VA aqueous extract in the sighting study produced the level of AST above the normal range (77 to 157 U/L). However, there were no significant differences in the level of biochemical parameters observed between each rat (Table 2). Results from the main study show the level of total protein, albumin, ALT, GGT and total bilirubin are in between the normal range of rats. Globulin levels also above the normal range (12 to 20 g/L) in all the rats. Despite that, the level of AST were expressed above the normal range (77 to 157 U/L) in three out of five rats and all the rats were detected with ALP levels below the normal range (132 to 312 U/L). Therefore, no significant differences in the level of biochemical parameters were observed between each rat (Table 3).

Table 2: The liver function test results for the rats in sighting study given single orally *V. amygdalina* aqueous extract in different concentration

Group	1	2	3	4
Number of rat	1	1	1	1
Dose (mg/kg BW) Biochemical parameters:	175	550	2000	5000
Total protein (g/L)	63.0	68.0	70.0	73.0
Albumin (g/L)	35.0	41.0	38.0	41.0
Globulin (g/L)	28.0	27.0	32.0	32.0
Albumin/Globulin ratio	1.2	1.5	1.2	1.3
AST (U/L)	101.0	138.0	84.0	327.0
ALT (U/L)	34.0	38.0	37.0	54.0
AST/ALT ratio	3.0	3.6	2.3	6.1
ALP (U/L)	123.0	99.0	166.0	60.0
GGT (U/L)	<3.0	<3.0	<3.0	<3.0
Total bilirubin (umol/L)	<2.0	<2.0	<2.0	<2.0

Table 3: The liver function test results for the rats in main study given single orally 5000 mg/kg BW of *V. amygdalina* aqueous extract (n =5). *Rat Number 3 has no result due to small amount of blood collected making it insufficient to run liver function tests

Rat	1	2	3	4	5	Mean±SEM
Dose (mg/kg BW) Biochemical parameters:	5000	5000	5000	5000	5000	
Total protein (g/L)	73.0	81.0	-	69.0	69.0	73.0 ± 2.4
Albumin (g/L)	41.0	46.0	-	39.0	40.0	41.5 ± 1.3
Globulin (g/L)	32.0	35.0	-	30.0	29.0	31.5 ± 1.1
Albumin/Globulin ratio	1.3	1.3	-	1.3	1.4	1.3 ± 0.02
AST (U/L)	327.0	346.0	-	156.0	172.0	250.3± 43.3
ALT (U/L)	54.0	149.0	-	52.0	52.0	76.8 ± 20.9
AST/ALT ratio	6.1	2.3	-	3.0	3.3	3.7 ± 0.7
ALP (U/L)	60.0	45.0	-	122.0	84.0	77.8 ± 14.5
GGT (U/L)	<3.0	<3.0	-	<3.0	<3.0	-
Total bilirubin (umol/L)	<2.0	<2.0	-	<2.0	<2.0	-

4. Discussion

In order to confirm the traditional claim of VA in traditional practice, aqueous extract of Malaysian local VA leaves were used in order to mimic as close as possible. Most of the traditional healers used water to make herbal decoctions juices. In addition, the aqueous extract was chosen as water is an universal solvent, non-toxic and does not interfere with the end product of extraction. This study revealed that the

aqueous extract of Malaysian local VA leaves contained flavonoids, tannin, saponin and terpenoids. ATR-FTIR spectrum also demostrated the present of phenol, methoxy compounds, and high content of flavonoid and terpenoid in Malaysian local VA extract. Previous research on VA has found polyphenols, glycosides, steroids, carbohydrates, alkaloids, saponin, tannin, flavonoids and glycosides in the aqueous extract of VA leaves (Atangwho *et al*., 2007; Nwanjo, 2005) [3, 17]. Thus, the main differ between Malaysian local VA and any other reported VA are in the contain of terpenoids. Terpenoids is one of the plant antioxidants substances besides ascorbic acid and tocopherols which perform an important function in human and plant itself. During the assessing of plant antioxidant activity, an important point to consider is the interaction with other antioxidants. Flavonoids also act as antioxidant (Akah *et al*., 2009) [1]. The combinations of hydrophilic and lipophilic antioxidants may exert synergistic effects which could significantly increase the potential of that biological property.

The acute oral toxicity test was carried out according to the OECD 420 guidelines. The purposes of the procedures in this guideline were to minimize the number of animals and causes less suffering than the traditional methods. Consideration for selection of this guidelines is moderate toxic dose was used and avoiding the use of death as an end point. The acute oral toxicity test was divided into two sections, sighting study and the main study. The purpose of the sighting study is to allow selection of the appropriate starting dose for the main study and minimizing the number of animals. As for the main study, only moderate toxic dose was used and doses which are expected to be lethal should be avoided. Adult female SD rats were used as animal model as the guidelines reported that there are differences in sensitivity between sexes, with females are slightly more sensitive. For both of the studies, no toxicity signs were observed and no mortality documented. There were also no significant changes in body weight, ROW and the organ weight. Previous study on VA extract also showed that there are no significant changes in the body weight, wet organ weight and abnormalities in the vital organs (Akah *et al*., 2009; Ekpo *et al*., 2007) [1, 6].

Aminotransferase which includes AST and ALT is usually used to detect liver damage. The enzymes in the cells will be leak out into the blood during liver damaged or destroyed. These liver enzymes can be measured by blood test or called liver function test (LFT). GGT, albumin and bilirubin also part of measured parameters in LFT. Based on this present study, there were no increased in the level of ALT but a slightly increased in the level of AST. ALT found predominantly in the liver, thus make it liver specific enzyme than is AST. Besides the liver, the AST enzyme is also found in muscles and many other tissues. In clinical laboratory, sometimes the ratio of AST/ALT was used to distinguish between different causes of liver damage and to distinguish liver injury from damage to heart or muscle. Thus, AST level test often confirmed with other liver tests such as total protein, bilirubin and the important liver marker ALT and GGT.

The level of total protein, albumin and total bilirubin were observed in between the normal range of rats. Albumin is an essential protein that circulates in blood and helps to keep our body fluid pressure in a stable state. Albumin also acts as a transport protein in order to carries many substances in the body. Basically, people with severe chronic liver disease will have low levels of Albumin. However, albumin level also may fall in a variety of medical conditions such as in person with impaired protein metabolism (Mauro *et al*., 2006) [13]. In addition, the albumin level itself could not be a liver diagnosis since a low level of albumin is often temporary. Bilirubin is a waste product from the breakdown of hemoglobin and unsoluble in water. In an order to excrete this bypassed product and make it soluble, the liver has to process the bilirubin so that it can be excreted in urine and stool. Some of them dissolved in bile and flow through the liver's bile ducts. Therefore, the level of bilirubin also used as a liver disease indicator because any damage of liver will cause the accumulation of bilirubin in the body. The elevation of bilirubin could be seen in people with impaired bile flow. This condition can occur in severe liver disease, gall bladder disease or other bile system conditions. Therefore, bilirubin can be a useful liver function test in people with a known bile flow problem (Rosalki and Mcintyre, 1999) [21].

Since the level of AST increased and no elevation of ALT level detectecd in this present study, GGT enzyme was measured to confirm if there was a liver damage. The result found no increased in GGT level. The level was measured in between the normal range. Liver dysfunction or damage is most likely present if the levels of ALT and AST are found together in elevated amounts in the blood. If both the levels of ALP and GGT are increased, the persons likely have a problem with bile flow which can be due to a problem in the liver or the gall bladder (Martin *et al*., 1976) [12]. With the normal results of ALP, GGT, total protein, albumin and bilirubin, proved that Malaysian local VA with concentration of 5000 mg/kg BW has no toxicity towards the rats. Sometimes, mild to moderate increases of AST not an indicator for liver damage but it may due to vigorous exercise. However, the study done by Ojiako and Nwanjo (2006) [18] reported that VA may be toxic to the body if consumed in very large quatities, but the potential danger is not higher than other common vegetables that are routinely consumed in larger quantities. No toxicity presented in Malaysian local VA might be due to the phytochemicals constituent present in the plant leaves which differed in the contain of terpenoids when compared to VA in other previous studies. In this Malaysian local VA extract, the presence of flavonoids as a potent antioxidant together with terpenoids may contribute to the lack of direct organ toxicity by free radical scavenging activities. Hence, it can be concluded that the local equeous extract of VA from Malaysia is safe to be consumed as vegetables or herb since the toxicity may greater than 5000 mg/kg of BW.

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