Toxicity Evaluation of *Manihot esculenta* and *Anacardium occidentale* Based on Two Solvent Extraction Techniques

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ABSTRACT: *Manihot esculenta* and *Anacardium occidentale* leaves are famous traditional vegetables which are extensively cultivated by Malaysian society. These leaves possess an impressive range of medicinal uses with high nutritional values. However, there is limited scientific data available regarding safety aspects for them. Thus, this study was carried out to evaluate their respective toxicity levels using Brine Shrimp Lethality Test (BSLT) and phytochemical screening based on different solvent extractions. BSLT assay is a convenient system for monitoring biological activities of various plant species and very useful for preliminary assessment of toxicity of plant extracts in terms of its rapidness, simplicity and low requirements. The result showed that no toxicity was observed in the extracts. The median lethal concentration (LC_{50}) of the test samples was obtained with a low range of value at less than 1000 ppm. However, the methanolic extract of *Anacardium occidentale* can be toxic if the concentration was increased up to 2370 ppm. Tannin and alkaloids were seen to be present in all extracts except terpenoids and saponin. In conclusion, the extract with different solvent extraction exhibited different results due to their different polarities and chemical properties. Therefore, further investigation was recommended to check the chemical profile in both leaves.

Keywords: *Manihot esculenta, Anacardium occidental*e, solvent extraction, toxicity, brine shrimp lethality test

Introduction

Cassava or tapioca can also be known as *Manihot esculenta* from *Euphorbiaceae* family. There are 6300 species in the same family as cassava (Soto *et al.*, 2015). It is commonly cultivated in tropical and subtropical region around the world (Bahekar & Kale, 2015). Cassava is originated from South America (Hue *et al.*, 2010) and currently there are more than 100 countries cultivate this type of plant (Soto *et al.*, 2015). It was introduced into India at 19^{th} century (Bahekar & Kale, 2015) and known as an all-season food crop in Africa, Asia and Latin America (Tsumbu, *et al.*, 2011). Cassava is an important crop because it can be the source of calories for tropical countries and thus secure the food needs (Soto *et al.*, 2015). Cassava can grow easily because it can tolerate drought and adapt to the climate exchange (Soto *et al.*, 2015).

Cassava consists of several parts, including tuber and leaves (Bahekar & Kale, 2015). Both of these parts are important because they can be used as food and feeds for human and animals (Soto *et al.*, 2015). Leaves of cassava plants are also the byproduct of the plant (Latif & Muller, 2015). The demand for cassava studies increase since the beginning of research on the biofuel, due to the potential of cassava to be converted as biofuel (Soto *et al.*, 2015). Tubers are consumed by human for their starch contents which can be substituted the corn and rice in daily meal. On the other hand, the leaves are also important to human because they can be consumed as herbs. This was proven by sub-Saharan Africans who live in Western Europe and consume cassava leaves as vegetables in their meals (Tsumbu *et al*., 2011). This type of leaves had also been used in folk medicines for a long time. Cassava leaves were also known to be rich in protein, minerals, vitamin B1, vitamin B2, vitamin C and carotenes (Fasuyi, 2005). They have been used to treat rheumatism, fever, headaches and loss of appetite generally (Bahekar & Kale, 2015). Somehow, in Nigeria, the folks used the cassava leaves to treat ringworm disease, tumor, conjunctivitis, sore and abscesses (Bahekar & Kale, 2015).

Cassava leaves contain high level of protein (Ngudi *et al.*, 2003). However, researchers believed that consuming cassava can cause cyanide poisoning (Bradbury & Denton, 2014). This is because it contains high amount of cyanide glucosidase, linamarin and small amount of lotaustralin (Bradbury & Denton, 2014). The cyanide content in leaves are six times higher than the roots (Fasuyi, 2005). Due to this issue, the cassava is only being used particularly by

Congolese population in Central Africa, Liberia, Sierra Leone and Guinea but not in South Pacific (Bradbury & Denton, 2014). However, the cyanogenic material in the cassava especially in cassava leaves can be detoxified by combination of several methods such as boiling and pounding before being consumed (Ngudi *et al.*, 2003).

Cashew plant (*Anacardium occidentale*) belongs to *Anacardiaceae* family together with other 75 genera and 700 species including mango and pistachio (Chabi Sika *et al.*, 2013). Cashew plant is originally a native to Brazil (Konan & Bacchi, 2007). It is cultivated in tropical countries and able to grow on the harsh environment conditions, such as waste land and degraded land (Konan *et al.*, 2007). Chabi Sika *et al.* (2013) stated that the cultivation of cashew can resolve problems regarding to forest planning in Africa as the cashew is also cultivated for its nuts and apple. This plant is known as a multipurpose tree which can grow up to 15m high and it has a thick and tortuous trunk with branches (Edet *et al.*, 2013).

Cashew plants are rich in proteins, steric acids, riboflavin, thiamin, vitamin A, vitamin B, vitamin C, calcium, magnesium, zinc and sulfur. The bark and leaves of the plant can be used to treat hypertension, inflammatory disease, asthma, bronchitis and gastric (Edet *et al.*, 2013). Apart of that, cashew apples and nuts can be used to treat fever and sweetened bread in Brazil (Edet *et al.*, 2013). In Peru, the cashew leaves are served as a tea to treat diarrhea Edet *et al.*, 2013) and the leaf tea can also be prepared to treat mouth ulcers, tonsillitis, throats problem and washing wounds. Surprisingly, the plant also can be used to treat diabetes mellitus (Edet *et al.*, 2013). Despite of all the good health benefit of it, the leaves can also cause asthenia, anorexia, diarrhea and syncope if it being overly consumed (Tedong *et al*., 2007). Konan *et al.*, (2007) stated that the leaves contain two major chemicals called flavonoid and tannin. Both of the chemicals are said to be involved in the antihypertensive effect on the organism (Konan *et al.*, 2007). On the other hand, the leaves extract also contain anti-inflammatory and analgesic activity (Pawar *et al.*, 2000) and had been proved to inhibit 25% human rotavirus and 84.5% sirnian rotavirus (Konan *et al.*, 2007). Traditionally, the leaves of *Manihot esculenta* and *Anacardium occidentale* have been used as a folk medicine in treating the diseases. However, limited studies have been performed on these plants in Malaysia, especially in relation to their toxicity levels of consumption.

Material and methods

Plant materials

Leaves of *Manihot esculenta* were collected from a farm in Pasir Hor, Kota Bharu District, Kelantan while the leaves of *Anacardium occidentale* were purchased from market in Rural Transformation Center (RTC), Tunjung, Kota Bharu District, Kelantan. The cassava leaves were chopped into small pieces and grinded. Cashew leaves were separated from its stalk and dried in an incubator for several days. Distilled water and methanol 95% from QREC were used as a solvent extractor.

Extraction

For extraction purpose, 50 g of grinded leaves were soaked in 500 mL of methanol for 24 hours. The methanolic extract were filtered, concentrated in a rotary evaporator and dried in a fume hood before preserved for further use. However, in water extraction, the solution was concentrated using freeze dryer before kept in an air tight container and placed in 4˚C. The percent yield of the obtained extracts was calculated based on equation below:

$$Yield\ of\ Extract\ \left(\%\ \frac{w}{w}\right) = \frac{g\ of\ solid\ content}{g\ of\ raw\ material} \times 100\ \%$$

Phytochemical screening

Phytochemical screening was done to test the presence of flavonoid, tannin, saponin, terpenoid and alkaloid. The procedure was briefly described by Solihah *et al*. (2012). All the extracts were diluted with distilled water at 1:100 (w/v) ratio.

Test for flavonoid

1mL of the extract was added to 1 mL of 10% $FeCl_3$. Then, the mixture was shaken. Formation of a wooly brownish precipitate indicates the presence of flavonoid.

Test for tannin

1mL of the extract was added to 1mL of 3% $FeCl_3$. Formation of a greenish black precipitate indicates the presence of tannins.

Test for saponin

Approximately 0.2mL extract was mixed with 5mL distilled water. It was shaken vigorously for 5 minutes. The persistence of foams was the indicator for the presence of saponin.

Test for terpenoid

To test for terpenoid, 5mL extract was mixed in 2mL chloroform. Then, 3mL of concentrated sulphuric acid was carefully added into the mixture. A reddish brown colouration between upper and lower layer of the mixture was observed to determine the presence of terpenoid.

Test for akaloid

In testing for the presence of alkaloid, 1mL of extract was mixed with 1% HCl and stirred. The mixture was heated in steam bath at 60°C for 15 minutes and filtered. Then, 1mL of Dragendorff reagent was added to 1ml of extract. The formation of cloudy orange was formed indicating a positive reaction.

Brine Shrimp Lethality Test

In this test, 0.25 g of brine shrimp egg was hatched well in an aerated flask, filled with 250 mL of 3.6% sea water. This process was performed at room temperature for 48 hours. Light source is needed for the hatching process. After the hatching process, the shrimp was placed into petri dishes and 0.2g of the extract was diluted in 100mL distilled water to produce 1000 ppm stock solution. The respective methanolic and water extracts were tested each made in concentrations of 200, 400, 600, 800, and 1000 ppm. The serial dilutions were duplicated for each concentration. Then, 5 mL from each solution was put into petri dish and left for 24 hours at room temperature. Lastly, the petri dish was observed using magnifying glass and average of survived shrimp was calculated.

Result

In this study, Table 1 shows the result of the percent yield obtained for both methanolic and water extract of *Manihot esculenta* and *Anacardium occidentale* leaves.

Table 1: Percent yield of the extracts

Sample	**Yield percentage (%)**	
	Methanol	**Water**
***Manihot esculenta* leaves**	11.00	7.52
***Anacardium occidentale* leaves**	3.10	11.88

Qualitative phytochemical screening test

Components contained in the methanolic and water extracts of respective *Manihot esculenta* and *Anacardium occidentale* leaves were analysed on the basis of the group of secondary metabolic compounds by colour tests using several classes of reagents for flavonoid, tannin, saponin, terpenoid and alkaloid. The results of phytochemical screening of the extracts are presented in Table 2 and Table 3.

Table 2: Phytochemical tests for *Manihot esculenta* extract

Extract	**Flavonoid**	**Tannin**	**Saponin**	**Terpenoid**	**Alkaloid**
Methanol	-	+	+	-	+
Water	-	+	+	+	+

Key: (+) Present (-) Absent

Table 3: Phytochemical tests for *Anacardium occidentale* extract

Extract	**Flavonoid**	**Tannin**	**Saponin**	**Terpenoid**	**Alkaloid**
Methanol	-	+	-	+	+
Water	-	+	-	+	+

Key: (+) Present (-) Absent

Brine Shrimp Lethality Test (BSLT)

The level of toxicity (LC_{50}) and toxicity categories was determined according to Table 4 below:

Table 4: LC_{50} values and toxicity category

Categories	LC_{50} values
Super toxic	< 5 mg/kg
Very toxic	5 – 50 mg/kg
Toxic	50 – 500 mg/kg
Toxic medium	0.5 – 6 g/kg
Mild toxic	5 – 15 g/kg
Practically non-toxic	>15 g/kg

Observations were made after 48 hours to calculate the percentage of mortality shrimp larvae *A. saline*. Mortality data are used to calculate the value of Lethal Concentration 50 (LC_{50}). The graph is plotted with the percentage of mortality as y-axis against the log concentration as the x-axis. The LC_{50} value is the concentration of a substance which causes the death of 50% obtained using linear regression equation y = a + bx. A substance said to be active or toxic when LC_{50} values <1000 µg/mL to extract and <30 µg/mL for a compound. Figures 1, 2 and 3 show the experimental outcome for BSLT on each extract.

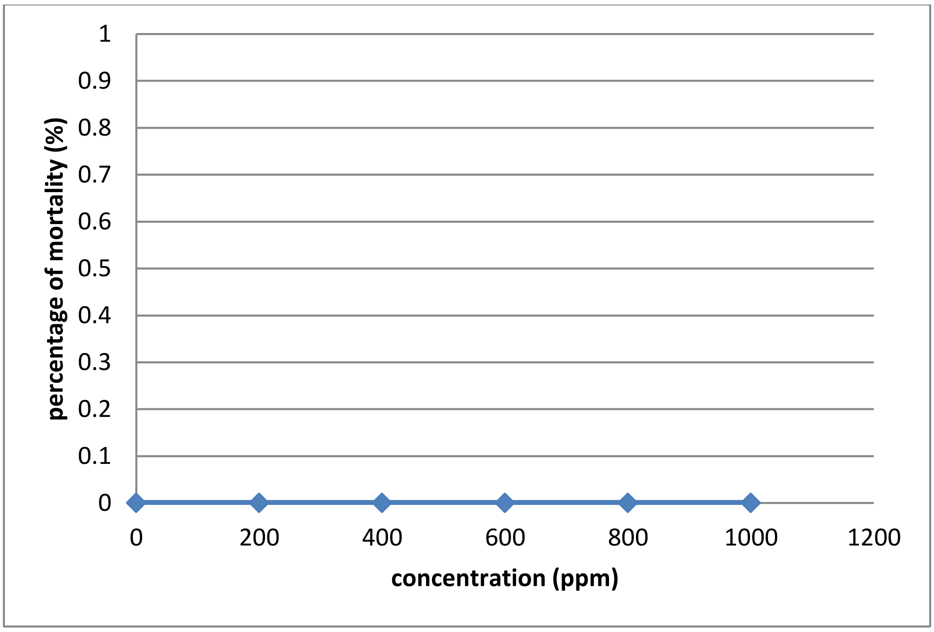

Figure 1: Graph of the percentage of mortality against log concentration for *Manihot esculenta* water extract

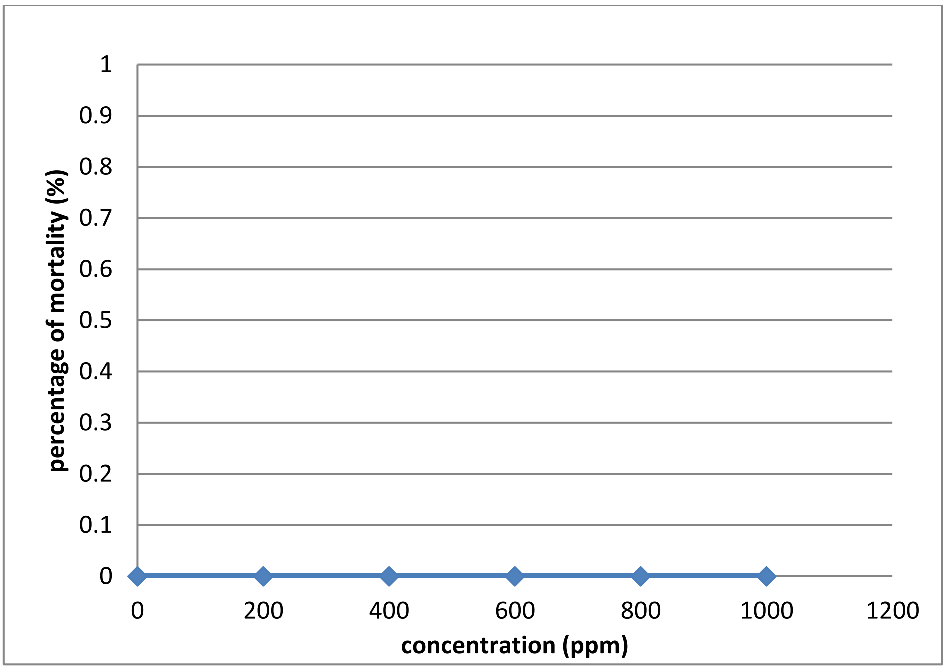

Figure 2: Graph of the percentage of mortality against log concentration for *Manihot esculenta* methanol extract

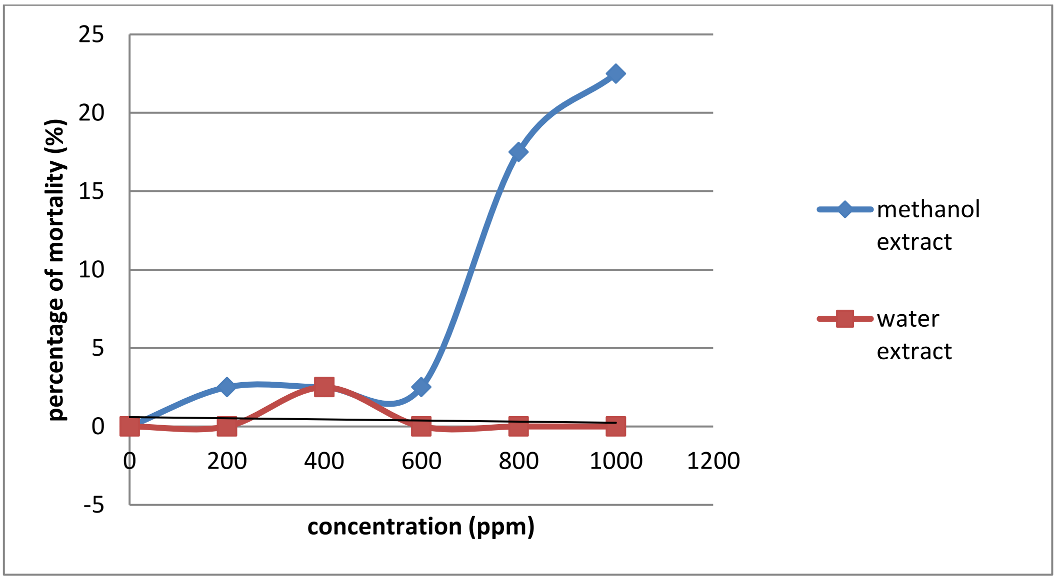

Figure 3: Graph of the percentage of mortality against log concentration for *Anacardium occidentale* extracts

Discussion

Extraction

Based on our extraction results, it was found that the methanolic extract of *Manihot esculenta* (11%) contained more yield than *Anacardium occidentale* (7.52%). Meanwhile, the water extract of *Anacardium occidentale* (11.88%) was reported with the highest yield, due to the different extraction methods and polarity of solvent. Among the different solvent extraction techniques, the successive freeze dried method was found to give higher recovery over other extraction methods. Since water extract was concentrated with freeze dryer, it could conserve more particles and the freeze dried products remained as a benchmark quality because of the structure preservation during the removal of water (Gutie´rrez *et al.,* 2008). The drying methods have different effects on the quality of dehydrated product.

The water extract of *Anacardium occidentale* was reported to be highest was because of the polarity of the solvent. In this study, the water extract may either contain more non-phenolic compounds or possess phenolic compounds that contain a smaller number of active groups than other solvents. Since the plants contain more polyphenols compound, the extracts tend to extracted more in water rather than methanol due to the polarity level. Although both water and methanol contain hydroxyl group that can form hydrogen bonding with the solute, water is more effective in extracting the solute because it has higher polarity and shorter chain (Pin *et al*., 2010). This characteristic of water improved its capability to extract the polar compounds. The difference in yields in this study suggested that polar compounds are easier to be extracted compared to non-polar compounds.

Phytochemical screening test

Phytochemical screening is performed on the naturally occurring compounds in medicinal plant which have defense mechanisms and protect users from various diseases (Wadood *et al*., 2013). Based on the results, phytochemical element which is contained in methanol extract of *Manihot esculenta* leaves is slightly different from those in the water extract of the same sample. Terpenoid was absent in methanol extract but appeared in the water extract. Terpenoid is important to exhibit various kinds of pharmacological activities such as anti-inflammatory, anticancer, antimalarial, inhibition of cholesterol synthesis, antiviral and antibacterial (Wadood *et al*., 2013). The elements of tannin, saponin and alkaloid were found in the extracts. Tannin is important in medical plants due to its stringent properties to hasten the healing of wounds and inflamed mucous membranes (Okwu & Josiah, 2006). Similar to tannin, saponin is also important in wound healing process. This is because it has the property of precipitating and coagulating red blood cell (Okwu & Josiah, 2006).

In *Anacardium occidentale* leaves, both extracts showed the presence of tannin, terpenoid and alkaloid. However, flavonoid and saponin were found in the leaves in previous study (Jaiswal e*t al*., 2012). The other findings also showed the presence of alkaloid, saponin and polyphenol in hexane extract of *Anacardium occidentale* (Tedong *et al*., 2006). Alkaloid seems to be the most potential compound in *Anacardium occidentale* leaves throughout all the findings. Alkaloid is important for its antibacterial and analgesic properties which can be used to treat headache and fever (Wadood *et al*., 2013). Different solvents were used in previous studies to detect the presence of this element. In addition, the changes of location and genetic variation due to cross pollination can also be the reason of different phytochemical screening results (Wadood *et al*., 2013).

Brine Shrimp Lethality Test (BSLT)

Toxicity tests on methanolic and water crude leaves extracts for both plants were conducted to determine the level of toxicity of these extracts against larvae shrimp. The test results showed that both leaves at different concentration levels shown no significant impact on mortality and larval toxicity. Based on BSLT, both samples were considered as not toxic. This is because all the results did not shown the LC50 value. The percentage of mortality for *Manihot esculenta* was continuously to be shown as zero with increase concentration. This phenomenon was found for both methanol and water extract, suggesting that the *Manihot esculenta* extracts were not toxic to brine shrimp.

However, in *Anacardium occidentale* water extract, there was a number of mortality being reported at the concentration of 400 ppm. Even though there was 2.5% of mortality, the extract was still considered as not toxic, as only 1 out of 10 brine shrimp was lethal in the petri dish and no lethality occurred in its duplicate petri dish. The condition was different for the methanol extract of *Anacardium occidentale* leaves in which the percentage of mortality increased as the concentration increased. The highest percentage of mortality was found in methanol extract of *Anacardium occidentale* leaves with 22.5% in 1000 ppm concentration. However, the data analysis was not adequate enough to conclude the methanol extract of *Anacardium occidental* as toxic. This is because it did not reach the LC_{50} value for standard brine shrimp lethality test. According to Nik Aina & Mohd Dasuki (2014), LC_{50} value for standard brine shrimp lethality test of less than 1000 ppm was considered as bioactive in toxicity evaluation of plant extracts (Meyer *et al*., 1982). Since the results showed LC_{50} is more than 1000 ppm, the expected value for the *Anacardium occidentale* methanol extract to reach its LC_{50} and become toxic was calculated and identified. *Anacardium occidentale* methanol extract can be toxic for brine shrimp test if the concentration was increased up to 2370 ppm. This was in agreement with the previous *in vivo* findings studies of *Anacardium occidentale*. There is no toxic effect in mice, which were treated with aqueous extract of *Anacardium occidentale* at doses up to 2g/kg by oral route (Paris *et al*., 1977). Tedong *et al*. (2007) also stated that there were no deaths or sign of toxicity observed after oral administration of *Anacardium occidentale* hexane extract up to 14 g/kg. In addition, the 100% mortality percentage was reported at a dose of 26 g/kg (Tedong *et al*., 2007).

Conclusion

In conclusion, different method drying and solvent extraction produced different percent yield of extract. The highest yield of extract was reported for water extract of *Anacardium occidentale* leaves (11.88%), followed by methanol extract of *Manihot esculenta* leaves (11%), water extract of *Anacardium occidentale* leaves (7.52%) and methanol extract of *Anacardium occidentale* leaves (3.1%). The difference values occurred due to the polarity of the solvent where water is more polar than methanol. In phytochemical screening, the different elements found in the samples are important in pharmacological studies. Each phytochemical element showed different effect on human in treating diseases. The phytochemical test was proved that the *Manihot esculenta* leaves and *Anacardium occidentale* leaves can be used as folk medicines since they contain the elements which can protect users from diseases. The findings indicated that both leaves were potential sources of highly nutritious ingredients and phytomedicine. The study on both samples was further carried out by testing with BSLT assay. The analysis of both *Manihot esculenta* and *Anacardium occidentale* samples in methanol and water extracts were proved to be considered as non-toxic even at the concentration of 1000 ppm. However, the methanol extract of *Anacardium occidentale* was expected to be toxic if the concentration up to 2370 ppm. Therefore, further studies need to be performed to prove the toxicity of the expected concentration in future.

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