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The *in vitro* Antibacterial Activity and Brine Shrimp Toxicity of *Manihot esculenta* var. Sri Pontian (Euphorbiacea) Extracts

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Abstract: To determine on the antibacterial activity of the leaves and acute toxicity level of the leaves, tender leaves and rhizomes of *Manihot esculenta* var. Sri Pontian extracts. The chloroform (CME1) and ethanol (EME1) leaves extracts of *M. esculenta* (25, 50 and 100% concentrations) were tested against a selected groups of Gram positive and Gram negative bacteria using the disc diffusion method. On the other hand, the chloroform and ethanol leaves (CME2 and EME2), as well as the tender young leaves (CME3 and EME3) and rhizomes (CME4 and EME4), extracts of *M. esculenta*, (concentration ranging from 200 to 2600 ppm) were tested for their chronic toxicity level using the brine shrimp bioassay. CME1 was found to give positive antibacterial activity against *L. monocytogenes, Vibrio cholerae*, *Shigella flexneri* and *Salmonella typhi* while EME1 was effective against *P. aeroginosa, C. diphtheria* and *V. cholerae*. The results also showed that among the chloroform extracts, CME4 (LC_{50}) = 413.9±51.6) possessed significantly ($p<0.05$) high toxicity followed by CME3 (LC_{50} = 496.2±33.1) and CME2 (LC_{50} = 532.9±22.9) while among the ethanol extracts, EME3 (LC_{50} = 344.7±33.9) was significantly ($p<0.05$) more toxic followed by EME2 (LC_{50} = 534.3±81.5) and EME4 (LC_{50} = 609.6±74.8). Overall, EME3 and CME4 were highly toxic than their counterpart (CME3 and EME4), respectively, while CME2 and EME2 did no show any discrepancy in their LD_{50} value. *M. esculenta* possess an antibacterial property and low toxicity level.

Key words: *Manihot esculanta* Crantz, antibacterial activity, acute toxicity, median lethal dose (LD_{50}), chloroform extract, ethanol extract, disc diffusion method

INTRODUCTION

Manihot esculenta, commonly known as tapioca or cassava and native to South America (Olsen and Schaal, 1999) and widely distributed in the Southeast Asia region, is a plant belonging to the family Euphorbiacea. It is a tropical perennial shrub and is grown for its enlarged starch-filled tuberous roots (El-Sharkawy, 2004). It is classified under roots and tubers, which is the class of foods that basically provide energy in the human diet in the form of carbohydrates (Alfonso *et al*., 2004). Other than that, the young leaves are used as vegetable (Lancaster and Brooks, 1983) and contain a high amount of protein (Ravindran, 1993), as well as vitamin A and C.

There is a bitter, poisonous and a sweet, nonpoisonous-variety of *M. esculenta* (Essers, 1995). The poisoning effect of *M. esculenta* is attributed to the presence of hydrocyamic acid (HCN), a type of glycosides, found in all parts of the bitter, poisonous variety, but presence only in the skin of sweet, nonpoisonous variety of *M. esculenta* (Vetter, 2000). These glycosides can be removed or destroyed by peeling the skin of the sweet variety roots and boiling the bitter variety in water, respectively (Balagopalan and Rajalakshmy, 1998). The peeled roots of the sweet variety are usually eaten cooked or baked. The bitter ones are grated, mixed with water and pressed to extract the juice. The paste of cassava is baked into pancake-like bread while the extracted juice is fermented into strong liquor called kasiri. The juice can also be concentrated and sweetened until it becomes dark viscous syrup called kasripo (casareep). This syrup has antiseptic properties and is used for flavoring.

Traditionally, the medicinal folklore used of *M. esculenta* is attributed to its roots/rhizomes. In Suriname's traditional medicine, the Amerindians use the

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brown juice, obtained during processing, for burns. Bark decoction of the tuber of *M. esculenta* when drunk is said to be antihelmintic. According to Hartwell (1967-1971), *M. esculenta* is used in folk remedies for cancerous affections, condylomata, excrescences of the eye and tumors. Other than being reported to be antiseptic, cyanogenetic, demulcent and diuretic, *M. esculenta* is also a folk remedy for abscesses, boils, conjunctivitis, diarrhea, dysentery, flu, hernia, inflammation, marasmus, prostatitis, snakebite, sore, spasm and swellings (Duke and Wain, 1981). Furthermore, *M. esculenta* is indigenously known, to most Ugandans, to have more value as medicine for various diseases such as asthma, coughs, wounds, tuberculosis (TB), measles, backache, chest pain, skin rash, sweating, stomachache, headache and medicinal anesthesia. However, there are no scientifically proven studies reported on the medicinal properties of this plant.

Based on the folklore medicinal believes mentioned above and the fact that there is no well-documented medicinal uses for *M. esculenta*, the objectives of the current study are to determine for the presence of antibacterial activity in the leaves and to elucidate on the chronic toxicity level in the leaves, tender young leaves and roots/rhizomes of chloroform and ethanol extracts of *M. esculenta*.

MATERIALS AND METHODS

Materials: *M. esculenta* var. Sri Pontian leaves, tender young leaves and roots were supplied by Malaysian Agriculture Research and Development Institute Serdang, Selangor, Malaysia and a voucher specimen was deposited at the Herbarium of MARDI, Serdang, Selangor, Malaysia.

Microorganisms tested in this study were *Streptococcus pneumoniae*, *Bacillus cereus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Corneybacterium diphtheria*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Shigella flexneri*, *Proteus vulgaris*, *Vibrio cholerae*, *Escherichia coli*, *Vibrio parahemolyticus* and *Salmonella typhi*.

Preparation of *Manihot esculanta* extracts: Fresh leaves of *M. esculenta* were oven-dried for 24 h at 40°C according to the methods described by Somchit *et al.* (2003) but with slight modifications. It was then ground into small pieces under sterilized condition and extracted separately with chloroform and ethanol in the ratio of 1:20 (w/v) for 24 h by using Soxhlet apparatus. The aqueous extract obtained was kept at -80°C for 48 h and then freeze-dried for 72 h while the resultant extraction of chloroform and ethanol was completely evaporated by using rotary evaporator machine (Somchit *et al.*, 2003). The obtained dried crude extracts of chloroform (CEME) and ethanol (EEME), dissolved in dimethyl sulfoxide (DMSO) in the ratio of 1:10 (w/v) and considered as stock solutions with 100% concentration/strength, were then diluted using DMSO to the concentrations of 25 and 50% and used in the antibacterial studies. The same dried crude extracts of CEME and EEME, prepared on another occasion, were diluted using DMSO to the concentrations ranging from 200 to 2600 ppm and used in the toxicity studies.

Antibacterial study: The above-mentioned bacteria were incubated at 37°C±0.5 for 24 h after injection into nutrient broth. Mueller Hinton Agar (MHA) (Oxoid, UK), sterilized in a flask and cooled to 40-50°C, was poured (15 mL) into sterilized Petri dishes (diameter of 9 cm) and allowed to harden under room temperature. This is followed by homogenous distribution of 0.1 mL bacteria cultures (10^6 bacteria per mL) onto medium in Petri dishes.

Twenty microliter of the respective extract were then loaded into empty sterilized blank discs (6 mm diameter, Oxoid, UK) and dried under room temperature. Dried discs loaded with extracts were then positioned on the solid agar medium by pressing slightly (Sundar, 1996). Petri dishes were placed in incubator according to their respective growth temperature and condition for 18 to 24 h. At the end of the period, inhibition zones formed was measured in mm. The study was performed in triplicate and the formation of the inhibition zones were compared with those of antibiotic discs. In addition, commercial antibiotic discs (Chloramphenicol; 30 μg μL^{-1}) were used for comparison.

Chronic toxicity study: The chronic toxicity level of leaves, tender young leaves and rhizomes of *M. esculenta* were determined using the brine shrimps (*Artemia salina*) bioassay described previously by Lieberman (1999). Briefly, brine shrimps were used at hatching stage (nauplii), after 46 h incubation of its eggs in a petri dish filled with sea salt water. Ten naupliis were placed in each vial containing the CEME or EEME extracts, at their respective concentration (ranging from 200 to 2600 ppm), an observed for 24 h. The brine shrimp are best seen against a dark background with good light from the side of the tube. Normally, most of the shrimp swim vigorously with their swimming rate, at room temperature, of several beats per second and show a phototaxis activity. Shrimp that lie motionless at the bottom of the tube are dead. The most common sub-lethal effect is impairment of swimming rate. For each tube, the fraction of shrimp that are dead was counted. LD_{50} values were obtained from the data according to the Reed-Muench method (Reed and

Muench, 1938) by plotting data of the percentage of mortality rate against the respective dosage, plotted to a logarithmic scale, in each test tube. Each experiment was carried out with three replications, two sets of controls and was repeated five times. Statistical analysis was by Sigmastat 2.0 (Jandel Scientific Software, San Rafael, CA, USA; copyright 1995).

RESULTS

As can be seen from the Table 1, the CEMC was less effective when compared to AEMC and MEMC, in term of number of bacteria inhibited. At the concentration ranging from 10,000 to 50,000 ppm, the AEMC was effective against *S. aureus* and *K. rhizophila* while the CEMC was effective against the two bacteria, as well as *B.cereus*, *P. vulgaris*, *S. flexneri* and *A. hydrophila*. At the concentration ranging from 40,000 to 100,000 ppm, the AEMC was effective against *C. diphtheriae*, *P. vulgaris*, *S. epidermidis* and *A. hydrophila*; the MEMC was effective against *C. diphtheriae* and *S. typhi*; and the CEMC was only effective against *S. aureus*. Interestingly, the MEMC was also found to be effective against *E. coli*, which is observed at the concentration of 70,000 ppm and above.

Our further studies to evaluate the level of chronic toxicity of both extracts of the leaves, young tender leaves and rhizomes of *M. esculenta* have demonstrated that all parts of *M. esculenta* possessed certain level of toxicity, which is considered very high when assessed using the brine shrimp bioassay (Table 2-4). This is indicated by the low level of concentration of each part of the plant that is needed to cause toxicity to half of the population of *Artemia salina*. In our studies, the chloroform and ethanol extracts of *M. esculenta* leaves (CME2 and EME2), as well as the tender leaves (CME3 and EME3) and rhizomes (CME4 and EME4), prepared in the concentration ranging from 200 to 2600 ppm, were found to possess different level of median lethal concentration (LC_{50}) as described below. The results demonstrated that among the chloroform extracts, CME4 (median lethal dose (LC_{50}) = 413.9±51.6) possessed significantly ($p<0.05$) higher toxicity level followed by CME3 (LC_{50} = 496.2±33.1) and CME2 (LC_{50} = 532.9±22.9) while among the ethanol extracts, EME3 (LC_{50} = 344.7± 33.9) was significantly ($p<0.05$) more toxic followed by EME2 (LC_{50} = 534.3±81.5) and EME4 (LC_{50} = 609.6±74.8). Overall, EME3 and CME4 were highly toxic than their counterpart (CME3 and EME4), respectively, while CME2 and EME2 did no show any discrepancy in their LC_{50} value.

Table 1: The Antibacterial Activity of Aqueous, Methanol and Chloroform Extracts of *Muntingia calabura* Determined by Disc Diffusion Method

	Concentration (%)					
	CME			EME		
Bacteria	25	50	100	25	50	100
C. diphtheriae	-	-	-	9.0	10.0	13.0
S. aureus	-	-	-	-	-	-
B. cereus	-	-	-	-	-	-
P. vulgaris	-	-	-	-	-	-
L. monocytogenes	7.5	8.0	9.0	-	-	-
S. pneumoniae	-	-	-	-	-	-
E. faecalis	-	-	-	-	-	-
S. flexneri	-	-	-	-	-	-
E. coli	-	-	-	-	-	-
P. aerginosa	7.0	10.0	12.0	10.0	16.0	19.0
V. parahemolyticus	-	-	-	-	-	-
V. cholerae	-	7.0	7.0	-	7.0	9.0
S. typhi	-	7.5	9.0	-	-	-
K. pneumoniae	-	-	-	-	-	-

IZ = Inhibition zone (mm), Except for *P. vulgaris* (IZ = 9 mm), Chloramphenicol gave inhibition zone of ≤20 mm against all bacteria

Table 2: Acute LD_{50} of ethanol and chloroform extracts of *M. esculenta* leaves after 24 h exposure to *Artemia salina* assessed using the Reed-Muench method

Solvent	Mean LD50
EME2	534.3±81.5
CME2	532.9±22.9

Table 3: Acute LD_{50} of ethanol and chloroform extracts of *M. esculenta* young tender leaves after 24 h exposure to Artemia salina assessed using the Reed-Muench method

Solvent	Mean LD50
EME3	344.7±33.9[a]
CME3	496.2±33.1[b]

[a,b]Data with different asterisks differed significantly ($p<0.05$)

Table 4: Acute LD_{50} of ethanol and chloroform extracts of *M. esculenta* rhizomes after 24 h exposure to Artemia salina assessed using the Reed-Muench method

Solvent	Mean LD50
EME4	609.6±74.8[a]
CME4	413.9±51.5[b]

[a,b]Data with different asterisks differed significantly ($p<0.05$)

DISCUSSION

M. esculenta var. Sri Pontian, locally known to the Malays as 'ubi kayu', is grown basically as an energy provider for human in the form of carbohydrates due to its enlarged starch-filled tuberous roots (El-Sharkawy, 2004). Other than that, its leaves and young tender leaves are used as vegetable in many part of the world (El-Sharkawy, 2004), including Malaysia. In Malaysia, particularly, *M. esculenta* is traditionally used in folklore medicine to treat various ailments such as headache, colds and fever and to treats gastric constipation.

However, there are many studies reported on its toxic effect, which is greatly attributed to the presence of free and bound cyanogenic glycosides, linamarin and lotaustralin (Frakes *et al*., 1985; Elias *et al*., 1997; Nambisan, 1999; Andersen *et al*., 2000). These

compounds are converted to HCN in the presence of linamarase, a naturally occurring enzyme in *M. esculenta* (Nambisan, 1999). Linamarase acts on the glycosides when the cells are ruptured. All plant parts contain cyanogenic glycosides with the leaves having the highest concentrations. In the roots, the peel has a higher concentration than the interior. In cases of human malnutrition, where the diet lacks protein and iodine, under processed roots of high HCN cultivars may result in serious health problems (Tylleskar *et al*., 1992). According to World Health Organization (1993), the toxicity of cyanogenic plants depends primarily on the potential concentration of HCN that may be released upon consumption. If the cyanogenic plant is inadequately detoxified during processing or preparation of the food, the potential HCN concentration, which may be released, can still be high. Upon consumption of a cyanogenic plants, β-glycosidase will be released during digestion and remain active until deactivated by the low pH of the stomach (Thayer and Conn, 1981; Poulton, 1990). This enzyme will hydrolyze the cynogenic glycosides and release, at least, the potential HCN content of the plant (Thayer and Conn, 1981; Poulton, 1990) Cyanide exerts its toxic effects by binding to the ferric ion of cytochrome oxidase, an enzyme that accounts for about 90% of the total oxygen uptake in most cells via the electron transport chain (Friedman, 1980). Inhibition of cytochrome oxidase thus virtually completely disrupts cellular oxygen utilization resulting in cytotoxic hypoxia, disfunctioning and death (Friedman, 1980, Abu-Bakare *et al*., 1986; Gruhnert *et al*., 1994). This situation leads to the exposure of humans with high diets of cassava and cassava products and of human engaged in the cassava product industries to varying levels of cyanide poisoning resulting in chronic and acute cyanide toxicity (Tylleskar *et al*., 1992; Abu-Bakare *et al*., 1986; Akanji *et al*., 1990; Osuntokun, 1981; Bennet *et al*., 1987; Howlett *et al*., 1990).

The present study has demonstrated the potential used of *M. esculenta* as an antibacterial agent against the infection of some of the bacteria used. The chloroform and ethanol extracts of *M. esculenta* leaves were found to be effective in the treatment against gram positive (*C. diphtheria*, *L. monocytogenes*, *P. aeroginosa*) and gram negative (*Vibrio cholerae*, *Shigella flexneri* and *Salmonella typhi*) bacteria. Present study also demonstrated that both extracts were only effective against six out of fourteen bacteria used, which might help explained the fact that the Malays folklore medicinal used of *M. esculenta* did not include its used in treatment against bacteria infection. However, this does not exclude the fact that *M. esculenta* did possess antimicrobial activity. Although isolation and identification of the bioactive compounds responsible for the observed activity is not the main objective of this study, the activity might be attributed to the presence of bioactive compounds, such as tannins and flavonoids (Diaz *et al*., 1988), glycosides (Chukwurah and Ajali, 2000) and saponins (Pretorius *et al*., 2003). The presence of tannins, flavonoids and glycosides (Ogunleye and Ibitoye, 2003), which have been known to occur abundantly in leaves of all plants, was expected to play major role in the observed antibacterial activity of *M. esculenta* extracts.

Further study using the brine shrimps bioassay has demonstrated that the leaves, young tender leaves and rhizomes of *M. esculenta* possessed chronic toxicity effect that is different among them and did not depend on the type of solvents used in their extraction. The chloroform and ethanol extracts of *M. esculenta* leaves did not show any discrepancies in their LC_{50}, while the ethanol extract of its tender young leaves and chloroform extract of its rhizomes exhibited high toxicity when compared to their counterparts, respectively. When compared among the chloroform extracts, the rhizomes was significantly more toxic followed by the tender young leaves and the leaves, while comparison among the ethanol extracts showed in sequence that the tender young leaves was much more toxic than the leaves and the rhizomes.

Based on the results obtained, it is plausible to suggest that *M. esculenta* leaves do possess certain level of antimicrobial activity, depending on the type of bacteria used. Furthermore, its leaves, tender young leaves and rhizomes do possess different level of toxicity, which is due to the presence of various types of glycosides that are converted to HCN. The toxicity level of each part of *M. esculenta* tested is considered higher since they gave the LD_{50} concentration below 1000 ppm, respectively.

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