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Heavy metal and microbiological profiles of defatted pili (*Canarium ovatum*, Engl.) pulp meal residue and acute oral toxicity of its ethanolic extract in mice

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Abstract

Canarium ovatum, Engl. (Burseraceae) or Pili is a valued indigenous fruit tree crop in the Philippines. The defatted pili pulp meal, a mixture of fruit peel and pulp that remains after pili pulp oil extraction, may be considered a functional ingredient because of its high dietary fiber and phytonutrient content. This study provides initial information on the microbiological and heavy metal contents of defatted pili pulp meal residue and preliminary toxicity report of its ethanolic extract. Ground lyophilized samples were subjected to heavy metal and microbiological analyses. An ethanolic extract of the plant material was prepared for phytochemical screening and acute oral toxicity testing. Results showed that both heavy metals and microbiological profiles of defatted pili meal residue pass the criteria for botanical ingredients set by relevant regulatory agencies. Phytochemical screening showed the presence of important bioactive compounds namely, flavonoids, tannins, anthraquinones, indoles, alkaloids, sterols, and terpenes. In acute oral toxicity, no mortalities were recorded over a 14-day experimental period. Neither significant gross and histopathological findings in liver and kidneys were detected. The LD50 of ethanolic extract in mice was estimated to be greater than 5 g/kg body weight. In conclusion, the pili pulp mixture may not present any potential public health risk when used as a component for food and drug products.

Introduction

Canarium ovatum, Engl. (Burseraceae) is a valued indigenous fruit tree crop in the Philippines. It is a deciduous tree measuring about 20-25 meters in height and 40-50 centimeters in diameter. Locally known as pili, this plant is abundantly found in the Bicol region of Southern Luzon, particularly in the provinces of Sorsogon, Albay and Camarines Sur (Coronel *et al*., 1983). It thrives in the primary and secondary forests of low to medium elevations (Orwa *et al*., 2009).

Pili is cultivated chiefly for its kernels. The Philippines holds monopoly over processed pili products in the global market (Coronel *et al*., 1983). Aside from pili nut confections, pili pulp oil is one of the highly acknowledged pili commodities. This oil is utilized for culinary and cosmetic products. Rural folks have attested to the curative properties of pili oil against skin diseases. It is also utilized for the treatment of worms in livestock such as pigs and chicken (Tacio, 2010).

Oil is obtained by manual extraction using a mechanical pulp press. Defatted pili pulp meal residue is the solid waste of such processing. Remnants consist of a mixture of fruit peels and fibrous pulp. Typically, this by-product is turned as livestock feed and compost. Recent experiments carried out in our laboratory have shown that this agro-industrial waste is a good source of dietary fiber and phytonutrients. As a potential functional ingredient for the food and pharmaceutical industries, the safety of this food-derived material needs to be evaluated. No toxicological studies have been done to determine any possible adverse health effects. Heavy metal accumulation in the edible and non-edible parts of pili fruit is likewise unknown. Because defatted pili pulp meal residue has not yet found its way into the human food chain, microbial quality is ignored. The present study provides initial information on the microbiological and heavy metal contents of defatted pili pulp meal residue and preliminary toxicity report of its ethanolic extract.

Materials and Methods

Plant material

Defatted pili pulp meal residue was collected

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from a pili pulp oil manufacturer in Sorsogon City, Philippines. Fruit material was lyophilized and pulverized into fine particles using a household grinder. Samples were kept in air-tight containers and stored at -20°C until use.

Fruit specimens used in pili pulp oil production were also obtained from the same manufacturer. The botanical identity of the plant material was authenticated by the curator of the University of Santo Tomas Herbarium, Manila, Philippines.

Microbiological analysis

Assays were performed following the protocols described in the Bacteriological Analytical Manual (BAM) by U.S. Food and Drug Administration. Aliquots from serial dilutions were aseptically transferred onto appropriate selective media. Total yeast and mold count was determined on Potato Dextrose Agar (PDA) plates (Tournas *et al*., 2001). Total aerobic bacteria count was conducted using Plate Count Agar (PCA) (Maturin and Peeler, 2001). Total coliforms and *E.coli* were enumerated using the Most Probable Number (MPN) technique on lactose broth. Positive tubes were subcultured to Brilliant Green Lactose Bile (BGLB) and *E. coli* (EC) broths. EC tubes producing gas were finally streaked onto Eosine Methylene Blue (EMB) agar to obtain discrete colonies (Feng *et al*., 2002). Isolation of Staphylococcus aureus was performed on Baird-Parker (BP) agar (Bennett and Lancette, 2001). For Salmonella detection, Rappaport Vassiliadis (RV) and Tetrathionate (TT) broths were used as enrichment media. Samples from these enrichment cultures were subsequently streaked onto Hektoen enteric (HE), Bismuth Sulfite (BS) and Xylose Lysine Dexoxycholate (XLD) agar plates (Andrews *et al*., 2007). All experiments were carried out in duplicate.

Heavy metals analysis

Sampling and digestion procedures were performed based on AOAC methods (2012). Appropriate blanks were prepared and analysed simultaneously. Determination of arsenic was carried out by atomic fluorescence spectroscopy (AFS). Mercury content was quantified by atomic absorption spectroscopy (AAS) – cold vapour technique. Levels of cadmium and lead were analysed by inductively coupled plasma- atomic absorption spectroscopy (ICP-AAS). Elements were measured against a series of working standards of different concentrations. Recovery rates of metals ranged from 94% to 111%. Coefficient correlation (r) values of calibration plots were between 0.998 to 0.999. Results obtained from triplicate readings were expressed in ug/g.

Preparation of plant extract

Ground sample of lyophilized defatted pili pulp meal residue (400 grams) was initially soaked overnight in 2.5L of ethanol with occasional stirring at ambient temperature. The immersion process was repeated twice with fresh solvent to ensure complete extraction. Each mixture was filtered through a cotton plug. Filtrates were combined and concentrated under reduced pressure at 40°C. Residue remaining after evaporation was weighed and stored in a clean glass container at 4°C until use. This crude ethanolic extract was used directly in phytochemical and acute toxicity studies.

Phytochemical screening

Qualitative phytochemical analysis was done to detect presence of bioactive compounds according to standard methods described by Aguinaldo *et al*., (2005). Thin layer chromatographic (TLC) profiling was carried out on pre coated silica gel 60 F254 sheets (Merck, Germany). Chromatogram was developed using ethyl acetate as solvent and visualized under visible and ultraviolet light (UV254 nm and UV366 nm). Various spray reagents were used to characterize the class of compounds present in the extract.

Acute oral toxicity

Female ICR mice between 5 and 6 weeks of age, weighing 29-32 grams were employed in the study. All were purchased from the Industrial Technology Development Institute - Standards and Testing Division (Taguig, Philippines). Experiment was conducted in an accredited animal house facility by trained personnel. The animals were housed in cages and kept under standard environmental conditions of temperature (21 + 2°C), relative humidity (30-70%) and light (12-h light-dark cycle). They were fed with a standard diet and provided with water ad libitum throughout the course of the experiment. The animals were acclimatized for 7 days before treatment and were fasted overnight prior to dosing. Experimental protocols were in accordance with the Code of Practice for the Care and Use of Laboratory Animals of the Philippine Association for Laboratory Animal Science (PALAS, 2002) and approved by the local Institutional Animal Care and Use Committee (IACUC).

Acute oral toxicity procedure was executed as per Organization of Economic Cooperation and Development (OECD) 401 guidelines (OECD, 1993). Animals were randomly distributed to control and treated groups; each composed of 10 mice. The control group was given distilled water alone while the treated group received a single dose of the test

Table 1. Mean microbial counts of defatted pili pulp meal residue and standard limits

Test Parameters	Results	Microbial Limit (cfu/g)			
		Phil. FDA[a]	AHPA[b]	WHO[c]	USP[d]
Aerobic Plate Count (cfu/g)	2500	10^7	10^7	10^7	10^5
Yeast & Mold Count (cfu/g)	140	10^4	10^5	10^5	10^3
Coliform Count (MPN/g)	9.2	*10^4	**10^4	*10^4	*10^3
Escherichia coli (MPN/g)	Negative (< 3)	Negative	Not detected in 10g	10 in 1g	Absence in 10g
Staphylococcus aureus (cfu/g)	Negative (< 10)	Negative	NA	NA	NA
Salmonella spp (per 25g)	Negative	Negative	Not detected in 25g	Absence in 1g	Absence in 10g

[a] Food and Drug Administration Philippines limits for herbal food products – plant materials that will undergo pre-treatment;
[b] American Herbal Products Association limits for dried unprocessed herbs for use as ingredients in dietary supplements
[c] World Health Organization limits for raw herbal material intended for further processing
[d] United States Pharmacopeia limits for dried or powdered botanicals
*Enterobacterial Count
** As total coliforms (cfu/g)
NA – Not Assigned

extract. Doses were administered to animals by oral gavage using a gastric feeding tube attached to a 1-ml syringe. Food and water were withheld for the next 3-4 hours. Observations were carried out at 30, 60, 120, 180 and 240 minutes post administration and daily for a period of 14 days. Body weight and mortality were recorded. Behavioural and clinical manifestations of toxicity were likewise carefully monitored. On day 15, all animals were sacrificed and subjected to gross necropsy.

Liver and kidneys were collected for histopathological examinations. Following 18 hours of fixation in 10% neutral-buffered formalin, organs were embedded in paraffin. Sections of 5µm were cut and stained with hematoxylin and eosin for microscopic evaluation.

Statistical analysis

Experimental results were expressed as mean + standard error of mean (SEM). Student's t-test was applied to determine difference between body weights in mice. P values < 0.05 were deemed significant. Statistical analysis was performed using SPSS version 20.

Results

Microbiological analysis

The average microbial concentrations from defatted pili pulp meal residue are presented in Table 1. The microbial criteria for herbal materials developed by various regulatory bodies are likewise shown in Table1. Aerobic plate count (APC) shows the level of microorganism in a product (Maturin and Peeler, 2001). Oftentimes, APCs are used to gauge if HACCP (Hazard Analysis Critical Control Point) plans are carried out effectively in a food plant (Hong *et al*., 2008). Results for APC did not exceed the limit for herbal products imposed by regulatory agencies. Total yeast and mold count (TYMC) is a test to measure fungal load. TYMC level of defatted pili pulp meal residue complied with the stated microbial specifications.

The coliform group consists of the genus Escherichia, Citrobacter, Enterobacter, and Klebsiella. But unlike other coliforms, Escherichia coli is more specific to indicate fecal contamination (Gerba, 2008). Microbial examination of defatted pili pulp meal residue showed that coliform organisms were present in the product. However, the number of coliform bacteria was relatively few. The absence of E.coli indicates that the product conformed to the microbiological standards. According to the set microbial guidelines on herbal products, Salmonella spp and Staphyloccocus aureus should not exist. In this study, the tested material adhered to this requirement since both pathogens were not detected.

Heavy metals analysis

Results revealed that defatted pili pulp meal residue is relatively free of mercury and contains very low residual levels of arsenic, cadmium and lead. A look at the data shows that the concentration of all analysed metals falls within the tolerable limits established by national and international regulatory authorities (Table 2). This implies that the use of this

Table 2. Mean concentration of heavy metals in defatted pili pulp meal residue compare with published standards

Element	Level (µg/g)	Permissible Limit (ppm)				
		Phil. FDA[a]	U.S. FDA[b]	WHO[c]	USP[d]	ASEAN[e]
Arsenic	0.0008 ± 0.000	0.3	10	10	3	5
Cadmium	<0.05 ± 0.000	0.3	0.3	0.3	3	0.3
Lead	0.35 ± 0.032	10	10	10	10	10
Mercury	ND*	0.5	1	1	3	0.5

Values are mean + SEM, n =3

[a] Food and Drug Administration Philippines maximum limits for herbal food products – dried plants;

[b] United States Food and Drug Administration permissible limits in herbal drugs

[c] World Health Organization permissible limits for herbal ingredients

[d] United States Pharmacopeia limits for nutritional supplements

[e] Association of South East Asian Nations maximum limits for traditional medicines and health supplements

* None-detected at the method detection limit of 0.10 µg/g for Mercury

Table 3. Phytochemical compounds in ethanolic extract of *Canarium ovatum*, *Engl.*

Constituent	Test Reagent	Result
Phenols	Potassium ferricyanide-ferric chloride	+
Flavonoids		+
Tannins		+
Steroids	Antimony (III) chloride	-
Alkaloids	Dragendorff's	+
Indoles	Van Urk-Salkowski	+
Sterols and Terpenes	Vanillin-sulfuric acid	+
Cardenolides	Kedde	-
Sugars	a-Naphthol-sulfuric acid	-
Anthraquinones	Borntrager	+
Coumarins		-
Anthrone		-

+: present; -: absent

product is unlikely to be associated with heavy metal toxicity.

Phytochemical screening

Ethanolic extraction resulted to a green thin fluid with reddish-orange upper layer. The extractive yield from defatted pili pulp meal residue was 65.1 grams (16.28%). The pH and specific gravity values of the crude extract were 6.57 and 0.9264, respectively. TLC analysis revealed the occurrence of various secondary metabolites as summarized in Table 3.

The absence of steroids, cardenolides, coumarins, anthrone and sugars was recorded. On the other hand, pili extract contains a wealth of polyphenol compounds such as flavonoids, tannins and anthraquinones. The results conformed to an unpublished work of Del Rosario (2007) on antioxidant components of *Canarium ovatum*. According to this report, pili extracts tested positive on phenolic compounds. Total phenolic and flavonoid contents were greater in the pulp and peel extracts than in the nut. Further analysis of these phenolic compounds using TLC, UV/IR spectroscopy and HPLC revealed the presence of vitamin E, cyanidin and ferulic acid in pili.

These findings are consistent with previous reports conducted on other Canarium species. The fruit of *Canarium odontophyllum* was found to be a rich source of phenolic acids, flavonoids and anthocyanins (Azrina *et al*., 2010; Shakirin *et al*., 2010; Chew *et al*., 2012; Khoo *et al*., 2013; Ali-Hassan *et al*., 2013). Several of these phenolic constituents have been isolated and identified (Khoo *et al*., 2012; Mokiran *et al*., 2014). Tannins and flavonoids were detected in different solvent extracts of *Canarium schweinfurthii* fruit pulp (Shaba *et al*., 2013; Wahab *et al*., 2015). Appreciable amount of tannins in the mesocarp of *Canarium schweinfurthii* has likewise been determined by Nyam *et al*. (2014) and Ehirim *et al*. (2015). Meanwhile, studies on *Canarium album* fruit showed high phenolic and flavonoid contents (Guo *et al*., 2008; Liu *et al*., 2008). The structures of some phenolic compounds isolated from *Canarium album* fruit have been elucidated by He and Xia (2007), He *et al*. (2008), He and Xia (2008), He *et al*. (2009), Xiang *et al*. (2010) and Xiang *et al*. (2012).

Pili extract also showed positive results for

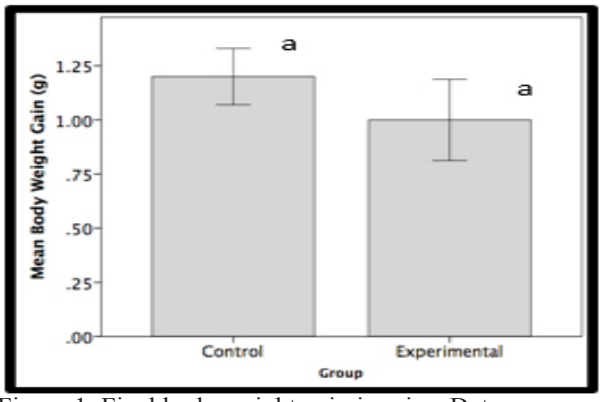

Figure 1. Final body weight gain in mice. Data are mean + SEM (n=10). Bars labelled with same letter are not significantly different at $P < 0.05$ level.

indoles, alkaloids, sterols and terpenes. These results are in accordance to literature data reporting alkaloid contents of *Canarium schweinfurthii* fruits (Shaba *et al*., 2013; Ehirim *et al*., 2015; Wahab *et al*., 2015). Earlier studies by Prasad *et al*. (2011) and Ali-Hassan *et al*. (2013) revealed that *Canarium odontophyllum* fruit contains lutein and ß-carotene. Carotene concentration of peel was higher compared to pulp and seeds. Xiang *et al*. (2012) has isolated a triterpenoid saponin from the n-butanol fraction of *Canarium album* dried fruits.

Acute oral toxicity

Ethanolic extract of *Canarium ovatum* fruit residue caused no deaths in mice within the 14-day study period. Single oral administration of the test extract did not result in any observable toxic effect. Normal body weight gains were recorded during the test period. In both groups, increase in body weight before and after treatment was significant ($p < 0.05$). As shown in Figure 1, body weight gain after 14 days was not statistically different ($p > 0.05$) between the control and treated groups.

Gross necropsy performed at termination revealed no abnormal findings in the vital organs of all animals. Histopathological examination of liver and kidneys in the control and treatment groups did not display significant morphological changes.

Findings indicate that the acute LD50, if any, of *Canarium ovatum* extract is in excess of 5g/kg body weight. Following the Hodge and Sterner (1943) toxicity scale, such substance can be classified as practically nontoxic. Similarly, Kennedy *et al*. (1986) stated that any substance with LD50 value greater than 5000mg/kg via oral route may be regarded as practically nontoxic.

Discussion

Defatted pili pulp meal residue represents a considerable proportion of the raw material used in pili pulp oil manufacture. Phytochemical analysis confirmed that this fruit residue is a valuable source of bioactive substances which have been reported to exert multiple biological actions to promote human health. Many flavonoids have been reported to have antioxidative, hepatoprotective, anti-inflammatory, antibacterial, and anticancer activities, whereas some possess antiviral activities (Kumar and Pandey, 2013). Tannins have shown cardioprotective effects and function as vasodilator, anti-carcinogenic, anti-allergic, anti-inflammatory, antibacterial, and antiviral agents (Rosales-Castro *et al*., 2014). Anthraquinones have been recognized to exert diuretic, cathartic, vasorelaxing, anticancer, anti-inflammatory, antimicrobial and phytoestrogen activities (Chien *et al*., 2015). Alkaloids are known to exhibit a broad spectrum of biological effects such as anti-cholinergic, antihypertensive, emetic, antitussigen, antitumor, diuretic, sym-pathomimetic, antiviral, miorelaxant, hypnoanalgesic, antidepressant, antimicrobial and anti-inflammatory activities (Aberoumand, 2012). Terpenes have potent action against malaria, cancer, inflammation and a variety of infectious diseases caused by viruses and bacteria (Wang *et al*., 2005). Phytosterols block cholesterol absorption and offer protection against oxidation, cancer and inflammation (Bartnikowsk, 2009).

Bacteria, fungi and viruses represent a wide variety of microbiological contaminants that may be associated with medicinal plants (Kunle *et al*., 2012). Microbial contamination of plant materials takes place in all stages of horticultural production namely growth, harvesting and postharvest handling (Vidovic *et al*., 2013). Aerobic bacteria and fungi often found in soil make up most of the natural microflora in plants. Additional contaminants like *Escherichia coli* or *Salmonella* spp. may arise as a result of faulty harvesting cleaning, drying, handling, and storage techniques (Kunle *et al*., 2012).

Applying the criteria mentioned in Table 1, the microbiological quality of defatted pili pulp meal residue was found to be satisfactory. From a microbiological point of view, it is deemed safe, provided further processing treatments (i.e. boiling, steeping etc.) are applied prior to consumption. The said plant material was not free from microbial contamination. Observed numbers of APC, TYMC and coliforms provide evidence of contamination at one or more points along the production line. Introduction of microbial impurities in plant foods

may originate from different factors such as poor worker hygiene, inadequate equipment sanitation, the use of contaminated irrigation or process water and application of biosolids or manure as fertilizers (Beuchat, 1996). A closer examination of agricultural and manufacturing practices is needed in order to come up with effective strategies towards reduction of microbial contamination of defatted pili pulp meal residue.

Food crops supply energy and essential nutrients in the human diet. Plant species, as well as different plant parts, vary widely in their ability to accumulate heavy metals (Ul Islam *et al*., 2007; Naser *et al*., 2011). Consumption of contaminated food is a major route of heavy metal exposure in humans and ingestion of toxic elements can lead to serious illness or death. Arsenic, cadmium, lead and mercury are the most lethal and widespread heavy metals (Morais *et al*., 2012) that are commonly associated to major human health problems and poisoning (Hutton 1987; Karayil *et al*., 2013). High cadmium intake damages the kidneys that can cause profound renal dysfunction (Bernard, 2008). Mercury is known to have deleterious effects on the central nervous system leading to behavioural changes and cognitive disorders. In addition, mercury is correlated with cardiotoxicity (Azevedo *et al*., 2012). Arsenic, a recognized human carcinogen, is mostly linked to skin and lung cancers. Elevated dose of arsenic results to skin lesions, cardiovascular, neurological and endocrine disorders (Guha Mazumder, 2008; Martinez *et al*., 2011). Lead toxicity has negative impact on multiple body systems namely the hematopoietic, renal, reproductive, and central nervous system (Flora *et al*., 2012).

Median lethal dose, also known as LD_{50}, is defined as the amount of chemical that is fatal to 50% of the test animals. It is a useful tool in the safety evaluation of herbal medicines. LD_{50} measurements are made because these values generally serve as indicators of acute toxicity of a substance (Saad and Said, 2011). Acute oral toxicity is defined as the adverse effects resulting from a single or multiple doses of a substance that occur immediately within 24 hours. Acute toxicity testing aims to acquire information about the biologic activity of a chemical and obtain understanding regarding its mechanism of action (Waler, 1998). Histopathological alterations in different tissues can provide further evidence of toxicity. In this study, histopathological examinations were performed on the two main organs of detoxification – liver and kidneys. Nuclear features and tissue integrity did not differ between the control and treated group suggesting that an acute intake of the test extract above 5 g/kg does not cause structural damage to these organs in mice. Single oral dose of this plant extract appears to be non-toxic as seen in this in vivo animal study.

Conclusion

In conclusion, the pili pulp mixture may not present any potential public health risk when used as a component for food and drug products. Although microbial quality was adequate to guarantee safety, there is a call for stringent implementation of proper agricultural and sanitation practices in order to minimize microbiological contamination. Further studies on chronic toxicity, carcinogenicity, mutagenicity and genotoxicity, are necessary to gain a deeper insight about the safety of this plant material. The present study is the first attempt to provide key evidence for the safe use of defatted pili pulp meal residue as ingredient in food and medicinal preparations.

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