Extraction, Profiling and Characterization of Phytosterols and Triterpenoids from Pili (Canarium ovatum Engl.) Pulp Oil Exhibiting Antioxidant and Antibacterial Properties

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Abstract

Pili (Canarium ovatum Engl.), an indigenous tree found in the Philippines, is highly regarded for its fruit due to its high economic value. During processing, the pulp is often discarded as waste but contains considerable amounts of oil and bioactive minor lipid components. The present study explored the antioxidant and antibacterial properties of saponified diethyl ether extract of pili pulp oil and relate this activity to the nature of compounds present in the extract thru GCMS. The extract indicated the elution of 18 major compounds which are mostly cyclic triterpenic (α- and β-amyrin, lupenone β-amyrone) and phytosterol (β-sitosterol, brassicasterol, stigmasterol) class of compounds. Characterization of the bioactivity of the extract showed high antioxidant activities measured by DPPH radical scavenging and lipid peroxidation inhibition activities that were comparable with that of α-tocopherol. Moreover, an observed bacteriocidal activity was demonstrated by the extract against E. coli and S. typhi with MIC values of 40 and 35 µg/mL, respectively. The observed bioactivity of the pili pulp oil extract can be attributed to these compounds which has high potential for the development and utilization in the food sector.

Extraction, Profiling and Characterization of Phytosterols and Triterpenoids from Pili (*Canarium ovatum* Engl.) Pulp Oil Exhibiting Antioxidant and Antibacterial Properties

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ABSTRACT

Pili (*Canarium ovatum* Engl.), an indigenous tree found in the Philippines, is highly regarded for its fruit due to its high economic value. During processing, the pulp is often discarded as waste but contains considerable amounts of oil and bioactive minor lipid components. The present study explored the antioxidant and antibacterial properties of saponified diethyl ether extract of pili pulp oil and relate this activity to

the nature of compounds present in the extract thru GCMS. The extract indicated the elution of 18 major compounds which are mostly cyclic triterpenic (α- and β-amyrin, lupenone β-amyrone) and phytosterol (β-sitosterol, brassicasterol, stigmasterol) class of compounds. Characterization of the bioactivity of the extract showed high antioxidant activities measured by DPPH radical scavenging and lipid peroxidation inhibition activities that were comparable with that of α-tocopherol. Moreover, an observed bactericidal activity was demonstrated by the extract against *E. coli* and *S. typhi*with MIC values of 40 and 35 µg/mL, respectively. The observed bioactivity of the pili pulp oil extract can be attributed to these compounds which may provide desirable health benefits.

Keywords: pili pulp oil, phytosterol, triterpenoids, β-sitosterol, stigmasterol, amyrin, antibacterial, antioxidant

INTRODUCTION

Canarium ovatum Engl., locally known as pili, is an indigenous tree commonly found in the Philippines which is cultivated for its edible fruit (Coronel, 1996). Pili nut kernel is the most valuable part of the fruit due to its high economic value owing to its increasing competitiveness in the global confectioneries market (Catelo and Jimenez, 2016). In pili nut processing, its pulp are often discarded as waste but it contains an appreciable amounts of oil and important minor lipid species such as carotenoids, phytosterols and tocopherols (Pham and Dumandan, 2015; Tugay et al., 2020). These nutritionally beneficial minor lipid compounds have gained considerable interest particularly on its bioactivity which plays an important role in the development of high-value products. Antioxidants and antimicrobial agents play a significant role in the food sector primarily because bacterial growth and lipid oxidation are the main factors that determine food quality loss and shelf-life reduction. Oftentimes, synthetic additives such as BHA/BHT are commonly added in food products to inhibit the process of lipid oxidation and microbial growth, and to extend their shelf-life. However, a shift to naturally derived compounds is seen and increasingly being sought by many companies due to possible adverse effects associated with long-term intake of synthetic compounds (Román et al., 2017; Zhong et al., 2018). Phytosterols, including other cyclic triterpenes which constitutes majority of unsaponifiable fractions of seeds oils, are known to have several bioactive properties linked to various implications on human health, including anti-inflammatory, antioxidative, antimicrobial, cholesterol-lowering and anti-carcinogenic activities (Jiang et al., 2019; Marangoni and Poli, 2010; Nzogong et al., 2018; Ostlund, 2004; Vilahur et al., 2019). On the other hand, triterpenoid extracts that are are rich in lupeol, betulinic acid and amyrin have shown to inhibit growth of food-borne pathogenic bacteria particularly the methicillin resistant *S. aureus*, *E. feacalis* and *P. aeruginosa*as studied by (Amoussa et al., 2016; Nzogong et al., 2018). Hence, the present study explored the potentials of minor lipid components in pili pulp oil as a source of phytosterols and cyclic triterpenoids with antioxidant and antibacterial properties. The interest in these naturally derived compounds is not only due to their biological activity, but also to maximize the economic potentials of pili pulp oil.

MATERIALS AND METHODS

Materials

Pili (*Canarium ovatum* Engl.) fruits were obtained from a local market at Goa, Camarines Sur, Philippines. Microbial strains (*E. coli, P. aeruginosa, S. tyhpi, S. aureus*, and *B. cereus*) used for the antibacterial activity assay were obtained from the Philippine National Collection of Microorganisms (PNCM), BIOTECH, UPLB, College, Los Baños, Laguna, Philippines. All other chemicals and standards were purchased commercially.

Sample Preparation and Oil Extraction

Pili fruits were manually depulped by blanching in a lukewarm water for about 15-20 min. The pulps were then collected and dried in a convection-type oven at 70 °C overnight or until moisture content reached about less than 3%. Extraction of oil was carried out using *n*-hexane at 1:4 ratio of dried pulp weight (g) to solvent volume (mL). After 12 h of extraction at room temperature with constant agitation, the oil was recovered by solvent evaporation using a rotary evaporator.

Saponification and Fractionation

The unsaponifiable fraction of oil was obtained by saponification following the method of Almeida et al. (2020) with some modifications. A 0.3 g of the oil sample was saponified using 10 mL 3% w/v ethanolic potassium hydroxide at 50 °C for 3 h. Then, the solution was cooled by adding 10 mL distilled water. Subsequent fractionation of the phytosterol and triterpenoids was done by repeated liquid-liquid extraction using 10 mL diethyl ether for three times. The organic layers were then combined, washed twice with 10 mL distilled water, and dried over an anhydrous sodium sulfate. The saponified diethyl ether extract (SDEE) of pili pulp oil was then collected upon filtration and solvent evaporation under stream of nitrogen gas.

GCMS Profiling

Profiling of SDEE was performed on a Shimadzu GCMS-QP2020 equipped with Shimadzu AOC-20i Plus auto injector (Shimadzu Corp., Kyoto, Japan) under electron impact ionization at 70 eV. Separation of components was done in SH-Rxi-5Sil MS capillary column with dimensions of 30 m x 0.25 mm ID x 0.25 µm film thickness (Shimadzu Corp., Kyoto, Japan). The initial oven temperature was held at 190 °C for 1 min then raised to 300 °C at 15 °C/min and kept constant for 10 min. Helium was used as the carrier gas with constant flowrate of 1 mL/min. The injector, MS ion source and MS interface temperatures were set at 310, 230 and 280°C, respectively. A sample injection of 1 µL was performed in a split mode of 10:1 and peaks were detected in full scan acquisition mode from m/z 50 to 500. Identification of the individual components was done by NIST mass spectral library on the basis of the mass fragment and e/z values of each component. Relative concentration of each peak was computed based on total ion count.

DPPH Radical Scavenging Activity

The scavenging activity of SDEE against DPPH radical was evaluated using the method of (Wang et al., 2011) with some modifications. Briefly, 3.8 mL of 0.07 mM DPPH solution in chloroform was mixed with 0.2 mL of sample with varying concentrations. The mixture was incubated in the dark at room temperature for 30 min. Chloroform and α-tocopherol were used as blank and positive control, respectively. The absorbance of the resulting mixture was measured at 516 nm and the percent radical scavenging activity was calculated based on the equation:

$$DPPH\ Radical\ Scavenging\ Activity = \left[1 - \frac{A_{\text{sample}}}{A_{\text{blank}}}\right]\ x\ 100$$

The EC_{50} value is defined as the concentration of sample required to scavenge 50% of DPPH radical under the assayed conditions.

Lipid Peroxidation Inhibition Activity

A mixture of 0.1 mL of 0.1-10 µg/mL of SDEE (in chloroform) and 0.5 mL of 10% egg yolk homogenate (w/v in distilled water) was mixed in a screw capped tube and the volume was made up to 1.0 mL by adding distilled water. Then, 0.05 mL 0.07 M $FeSO_4$ (in distilled water) was added, and the mixture was incubated at 37 °C for 30 min to induce lipid peroxidation. A 0.05 mL of 20% (w/v) trichloroacetic acid solution (TCA) in distilled water and 1.0 mL 0.1 % (w/v) thiobarbituric acid solution (TBA) in distilled water was added to the mixture, vortexed, and heated in a water bath at 80 °C for 30 min. After cooling, 3 mL of butanol was added, and the mixture was centrifuged at 3000 rpm for 5 min. The absorbance of the upper layer was measured against 3 mL butanol at 512 nm. Chloroform and α-Tocopherol were used as blank and positive controls, respectively. The lipid peroxidation inhibition activity was calculated using the equation:

$$\text{Lipid peroxidation inhibition}\ \ \% = \left[1 - \left(\frac{A_{512\ sample}}{A_{512\ blank}}\right)\right] x100$$

The EC_{50} value is defined as the concentration of sample required to inhibit 50% of lipid peroxidation under the assayed conditions.

Antibacterial Activity

Disk Diffusion Assay

Qualitative antimicrobial activity of SDEE against *S. aureus, B. cereus, E. coli, S. typhi* and *P. aeruginosa* was carried out by disk diffusion method (Wikler, 2006). Briefly, a suspension of the test microorganism standardized to 0.5 McFarland with approximately 1.5 x 10^8 CFU/mL was uniformly spread onto individual solid media plates of Muller-Hinton agar using sterile cotton swab. Disks of 6 mm in diameter were impregnated, until saturation, with 0.1 mg/mL of the extract. The disks were then allowed to dry placing in the inoculated agar. Chloramphenicol and chloroform served as positive and negative controls, respectively. The inoculated plates were incubated at 37 °C for 18-24 h. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms.

Minimum Inhibitory Concentration Assay

The MIC of the extract exhibiting sensitivity to the tested microorganism based on the disk diffusion assay was determined following the methods outlined by Eloff (1998) with some modifications. First, a stock solution of the extract (1 mg/mL) was prepared in Meuller Hinton broth supplemented with 0.02% Tween 80. The solution was then sonicated for 30 s and vortex homogenized for 2 min to obtain a stable emulsion. Serial dilutions of the extract in broth were prepared in micro tubes of 1 mL with a concentration range from 0.1 to 100 µg/mL. Then, 295 µL of each dilution were transferred into a 96-well microplate. A 5 µL of test bacterial suspension (1.0 x 10^6 CFU/mL) was inoculated to obtain a final concentration of 1.67 x 10^4 CFU/mL and a final volume of 300 µL per well. The inoculum (positive control) and culture medium (negative control) were put into the first column of the microplate, and the chloramphenicol antibiotic control ranging from 0.5 to 75 µg/mL in the final column. Each plate was wrapped loosely with cling film to prevent dehydration during incubation for 18-24 h at 37 °C. Subsequently, 10 µL of bacterial growth indicator, resazurin at 6.75 mg/mL, was added to wells, which were then incubated for 30 min at 37 °C. The lowest concentration of the extract that visually showed no growth was determined as MIC.

Statistical Analysis

All experiments and measurements were carried out in triplicate. The data were presented as mean ± SEM and were analyzed using one-way ANOVA. Means were compared using Tukey's post hoc comparison test using R software (version 4.2.1, R Foundation for Statistical Computing, Vienna, Austria). Significant differences between means were determined at$P< 0.05$.

RESULTS AND DISCUSSION

Oil Extraction, Fractionation and GCMS Profiling of the Diethyl Ether Unsaponifiable Extract of Pili Pulp Oil

Extraction of oil revealed 23.92 ± 0.21% yield, expressed in dry weight basis of pulp. The unsaponifiable fraction of oil obtained from diethyl ether extraction showed 1.0294 ± 0.0501%. These values were slightly lower than our previous findings (Pham and Dumandan, 2015) which can be attributed to the varietal differences of pili fruit used, for which this was also observed in the report of Tugay et al. (2020) with 0.47 to 0.53% UM content depending on the fruit's variety. Analysis of the chromatogram obtained from the SDEE extract indicated the elution of 18 major compounds which are mostly triterpenic and phytosterol class of compounds (Fig. 1, Table 1).**Figure 1.Table 1.** In terms of relative abundance with respect to total ion count (TIC), β-Amyrin showed highest abundance of around 34.75%, followed by α-amyrin and β-sitosterol with 20.92 and 15.78%, respectively. Other phytosterols identified in SDEE includes brassicasterol, stigmasterol, campesterol, and fucosterol (Fig. 2). These were consistent with our previous study where the major sterols present are stigmasterol and campesterol (Pham and Dumandan, 2015). In addition, it also contains other pentacyclic triterpenes such as lupenone, amyrone, cycloartenol and betulinaldehyde (Fig. 3).**Figure 2.Figure 3.** Amyrin, both α- and β- isomers are generally found in different plant part extracts such as in *C. tramdenum* bark which contains 0.03 mg/g β-amyrin (Quan et al. 2019). Areal parts of *M. barteri* and*S. longifolia* also contains high amounts of α-amyrin as reported by Ogwuche et al. (2014) and

Saeidnia et al. (2016). Other than plant parts, amyrin along with other sterols were also detected in oil samples such as in olive, flaxseed, and camellia seed oils (Varvouni et al., 2021; Wang et al., 2017).

Antioxidant Activity

The antioxidant activity of the SDEE were analyzed in terms of scavenging activity against DPPH radical and the ability to inhibit lipid peroxidation in egg yolk system. As shown in Table 2, SDEE showed comparable EC_{50} values of 3.02 µg/mL with that of α-tocopherol (2.92 µg/mL), a known antioxidant compound, though a moderate activity was observed in terms of its DPPH radical scavenging activity with slightly higher EC_{50} value of 74.45 µg/mL in SDEE as compared to 65.91 µg/mL in control.**Table 2.** Terpenoids and sterols, in general, have shown to exert antioxidant properties owing to its hydroxyl group that participates via hydrogen atom transfer or single electron transfer mechanism in quenching reactive oxygen species (Kedare and Singh, 2011; Yoshida and Niki, 2003). It has been demonstrated that phytosterol components such as campesterol, β-sitosterol and stigmasterol have shown to exhibit scavenging activities against DPPH radical and provide hepato- and neuro-protection of hydrogen peroxide-induced oxidative stress levels (Prommaban et al., 2020; Yoshida and Niki, 2003). Isomers of amyrin isolated from *Myrcianthes pungens* leaves revealed high antioxidant activity with antioxidant protection equivalent to the Trolox of 137 and 129%, for α- and β-amyrin, respectively, at 500 µg/mL (Cardoso et al. 2018).

Antibacterial Activity

The antibacterial potential of SDEE against 5 common food pathogens are shown in Table 3. The extract was found to exhibit significant inhibitory activity against *E. coli* and *S. typhi* with 10.3 and 11.7 mm zone of inhibition, respectively, at 0.1 mg/mL. The MIC showed that 40 and 35 ug/mL of the extract was able to inhibit the growth of *E. coli* and *S. typhi*, respectively. The extract was not active, or did not show antibacterial activity to *S. aureus*, *B. cereus*, and *P. aeruginosa* at 0.1 mg/mL.**Table 3.**The antibacterial activity of SDEE can be attributed to the high amyrin content. which was also observed by Abdel-Raouf et al. (2015) in several algal extracts containing β-amyrin exerting antibacterial activities against the 5 pathogens tested in this study. In a separate study, hexane extract of *Manilkara subsericea* fruit which are mostly α-and β-amyrin acetate showed MIC value of 250 µg/mL against *S. aureus* (Fernandes et al. 2013). Aside from amyrin, Alawode et al. (2021) reported that phytosterols, specifically stigmasterol and β-sitosterol that were isolated from *Icacina trichantha*, showed high antimicrobial properties against *B. subtilis*, *E. coli*and *C. albicans*. The regulatory activity of amyrin including other triterpenoids in disrupting pathways responsible for cell division and protein synthesis, as well as destabilization of bacterial cell membrane and inhibition of cell growth are some of the plausible mechanisms of action of these compounds (Chung et al., 2013). Furthermore, these compounds also cause disorganizing effects on cardiolipin-rich domains present in the membrane of *E. coli* as demonstrated by Broniatowski et al. (2015).

CONCLUSION

Extraction, saponification, and diethyl ether fractionation of the unsaponifiable matter of pili pulp oil showed strong antioxidant properties as measured by its scavenging activity against DPPH radical and lipid peroxidation inhibition activities and are found to be comparable to α-tocopherol, a known antioxidant compound. The extract also exerts antibacterial activities against *E. coli* and *S. typhi* which are bacterial pathogens of concern especially in food preparation. GCMS profiling revealed 18 compounds majority of which are cyclic triterpenes and phytosterols such as α- and β-amyrin, lupenone, β-sitosterol, brassicaterol, and stigmasterol. Our results suggest that these bioactive compounds are responsible for the observed bioactivities which may provide desirable health benefits.

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AUTHORSHIP

N.G.D. conceived and design the experiment, and wrote the first draft of the manuscript, A.C.T.K., R.D.P.A., and C.R.T. conducted the experiment. L.J.P. provided technical expertise in the design of experiment and provided insights in the preparation of manuscript. All authors contributed to and approved the final draft of the manuscript.

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