Antioxidant and Enzyme Inhibitory Activities of *Areca catechu*, *Boesenbergia rotunda*, *Piper betle* and *Orthosiphon aristatus* for Potential Skin Anti-aging Properties

Z.A. Mat Yasin, A.S. Khazali, F. Ibrahim, N. Nor Rashid and R. Yusof
Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603, Kuala Lumpur, Malaysia and Department of Biomedical Engineering, Faculty of Engineering, University of Malaya, 50603, Kuala Lumpur, Malaysia

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Medicinal plants have been traditionally used for various applications including skin care. In this study, *Areca catechu*, *Boesenbergia rotunda*, *Piper betle* and *Orthosiphon aristatus* were tested for antioxidant and anti-aging properties. These plant extracts were subjected to 1,1-diphenyl-picrylhydrazyl (DPPH) and 2,2ʹ-azinobis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) radical scavenging activity analyses. The extracts were then assessed for collagenase, elastase and hyaluronidase inhibition and subjected to toxicity study in normal human dermal fibroblast cells. Lastly, bioactive compounds in these plant extracts were identified using high performance liquid chromatography. We found that *Piper betle* and *Areca catechu* possessed high total flavonoid and phenolic contents. These two extracts showed the best IC$_{50}$ values for DPPH and ABTS radical scavenging activities and also demonstrated the highest elastase and collagenase inhibition when compared to the other two plant extracts. These crude plants extracts were also non-toxic to normal human dermal fibroblast cells. Our HPLC results identified several potential bioactive compounds in these plant extracts that could be crucial in mediating the observed effects. Based on our results, these plant extracts especially *Piper betle* and *Areca catechu* extracts possess significant antioxidant and anti-aging properties and could be utilized in the development of safe and cost-effective anti-aging treatments.

**Keywords:** Antioxidant, Collagenase, Elastase, Hyaluronidase, Skin aging

**Corresponding Author:** Dr. N. Nor Rashid, Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603, Kuala Lumpur, Malaysia; Tel: +6 03-79677549; Fax: +6 03-79674957; Email: nurshamimi@um.edu.my

**INTRODUCTION**

The extracellular matrix (ECM) of the skin is composed of fibrous proteins such as collagens, elastic fibres and glycosaminoglycans that are linked together to strengthen and maintain the appearance of the skin. Collagen type I is the most abundant type of collagens found in the skin. Collagens are arranged into fibrils to increase skin integrity and the rubber-like elastin is intertwined with collagen to promote the resilience and elasticity of the skin. Hyaluronic acid is one of the most common glycosaminoglycans found in the skin and is important in maintaining tissue hydration and osmotic balance. However, exposure of the skin to UV radiation, chemical and pollution can lead to extrinsic skin aging (Ganceviciene et al., 2012). In particular, absorption of UV radiation by the skin could elevate the production of reactive oxygen species (ROS) which may cause skin damage due to lipid, protein and DNA degradation (Sharma et al., 2012). The accumulation of ROS was previously shown to trigger Activator Protein-1 (AP-1) transcription factor to increase the expression of matrix metalloproteinase (MMP) 1, 3 and 9, resulting in the degradation of collagen fibres in the skin (Sahu et al., 2013). In addition, ROS could also activate several cytokines, leading to the secretion of elastase by neutrophilic granulocytes and dermal ECM degradation (Chang et al., 2008). Finally, continuous or chronic exposure to UV radiation have been shown to reduce the level of hyaluronic acid in the epidermis (Ganceviciene et al., 2012). In short, disruption or downregulation of these ECM components, through elevated ROS activity upon exposure to UV radiation, can lead to visible signs of skin aging.
Inhibition of collagenase, elastase and hyaluronidase is one of the common approaches used in skin aging study. Natural-based or plant-based anti-aging products are more preferable than synthetic inhibitors especially in cosmetic products presumably due to safety concerns. In Malaysia, *Areca catechu*, *Boesenbergia rotunda*, *Piper betle* and *Orthosiphon aristatus* have been traditionally consumed or applied topically to reduce the signs of aging or improve individuals’ well-being. Additionally, *A. catechu*, *B. rotunda*, *P. betle* and *O. aristatus* extracts have been reported to possess various pharmaceutical values. For example, *A. catechu* nut extract was found to exhibit anti-depression activity by increasing serotonin and noradrenaline levels (Abbas et al., 2013). Recently, cardamonin (2ʹ,4ʹ-dihydroxy-6ʹ-methoxylchalcone), a compound isolated from *B. rotunda*, was shown to potentiate anti-inflammatory activity by inhibiting the release of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) (Voon et al., 2017). Hydroalcoholic extract of *P. betle* leaves exhibited significant analgesic and anti-inflammatory effects in mice (Reddy et al., 2016). Lastly, Orthosiphol A, a compound isolated from *O. aristatus*, was reported to have anti-diabetic activity by inhibiting the intestinal malate activity (Damsud et al., 2014). Although a myriad of studies has been performed on these plant extracts for various medicinal properties, anti-aging properties of these plant extracts have not been properly established. Therefore, the aim of the present study was to investigate the ability of *A. catechu*, *B. rotunda*, *P. betle* and *O. aristatus* crude extracts in protecting the skin against free radicals and inhibiting collagenase, elastase and hyaluronidase activities.

**MATERIALS AND METHODS**

**Plant Materials and Preparation of Extracts**

Dried nuts of *A. catechu*, rhizomes of *B. rotunda* and leaves of *P. betle* and *O. aristatus* were purchased from local markets around Kuala Lumpur, Malaysia. The plant materials were washed under running tap water, chopped into small fragments and dried in the oven at 60°C. After drying, the plants were grinded with mechanical grinder into fine powder. The powder of each plant was extracted with 1000 mL of absolute ethanol at room temperature for 24 h. The extract was filtered through Whatman filter paper No. 1, and the filtrate was concentrated under reduced pressure at 40°C. The extracts were stored at −20°C in storage vials for experimental use.

**Total Phenolic Content**

The total phenolic content of the extracts was determined using a colorimetric assay. Briefly, 100 µL of 1 mg/mL crude extracts were made up to 1 mL using distilled water and mixed thoroughly with 100 µL of Folin-Ciocalteu reagent (Sigma Aldrich). After 5 min of incubation at room temperature, 400 µL of 7% (w/v) sodium carbonate (Na₂CO₃) were added and the mixture was allowed to stand for further 90 min in the dark and absorbance was measured at 750 nm using the Epoch™ microplate reader (BioTek Instruments, Winooski, VT, USA). Gallic acid was used as the standard. The total phenolic content was calculated from the calibration curve, and the analyzed data were expressed as micrograms per milliliter of gallic acid equivalent (µg/mL of GAE).

**Total Flavonoid Content**

The total flavonoid content of *A. catechu*, *B. rotunda*, *P. betle* and *O. aristatus* were analysed using aluminium chloride (AlCl₃) colorimetric method (Baba and Malik, 2015). In brief, 100 µL of crude extracts, 300 µL of distilled water, 30 µL of 5% sodium nitrite (NaNO₂) and 20 µL of 10% aluminium chloride (AlCl₃) were mixed and allowed to stand at room temperature for 5 min. Then, 200 µL of 1 mM sodium hydroxide (NaOH) and 340 µL of distilled water were added and further incubated for 5 min at room temperature. The absorbance was measured at 510 nm using the Epoch™ microplate reader (BioTek Instruments, Winooski, VT, USA). Quercetin was used as the standard and ethanol was used as the blank. The total flavonoid content was calculated from the calibration curve, and the results were expressed as a µg/mL of Quercetin equivalents (Qu).

**1,1-Diphenyl-picrylhydrazyl (DPPH) Radical Scavenging Assay**

The free radical scavenging activity of the ethanolic extract of *A. catechu*, *B. rotunda*, *P. betle* and *O. aristatus* was measured using 1,1-diphenyl-2-picryl hydrazyl (DPPH). In brief, 600 µL of *A. catechu*, *B. rotunda*, *P. betle* and *O. aristatus* extracts were mixed with 200 µL DPPH solution and incubated in the dark at room temperature for 30 min. The absorbance was measured at 517 nm using the Epoch™ microplate reader (BioTek Instruments, Winooski, VT, USA). Ascorbic acid was used as the standard compound or the positive control at the same amount and concentration. The percentage of DPPH scavenging effect from the extracts was calculated based on the following equation:

\[ \text{DPPH scavenging effect} (\%) = \frac{A_0 - A_1}{A_0} \times 100 \]

where A₀ is the absorbance of control reaction; A₁ is the absorbance of test or standard sample.

**2,2’-azinobis(3-ethylbenzothiazoline)-6-Sulphonic Acid (ABTS) Radical Scavenging Assay**

For the ABTS radical scavenging assay, the radical cation was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulphate (K₂S₂O₈). The mixture was allowed to react for 16 h in the dark at 25°C until the reaction was complete and the absorbance was stable. The ABTS solution was diluted with distilled water to an absorbance of 0.700 ± 0.05 at 734 nm. The photometric assay was conducted by mixing 345 µL of ABTS solution with 5 µL of *A. catechu*, *B. rotunda*, *P. betle* and *O. aristatus* extracts and the absorbance values were measured at 734 nm using the Epoch™ microplate reader (BioTek Instruments, Winooski, VT, USA). The percentage of anti-oxidative activity of the plant extracts was calculated using the following equation:

\[ \text{ABTS scavenging effect} (\%) = \frac{A_0 - A_1}{A_0} \times 100 \]

where A₀ is the absorbance of control reaction; A₁ is the absorbance of test or standard sample.

**Cell Viability Assay**

Cell viability was monitored using CellTiter 96® AQueous One Solution Reagent MTS dye. The crude extracts of *A. catechu*, *B. rotunda*, *P. betle* and *O. aristatus* were dissolved in dimethyl sulphoxide (DMSO) and diluted in cell culture medium. The final concentration of DMSO was 0.1%. Normal human fibroblast cells...
were plated into 96-well plate at concentration of $5 \times 10^4$ cells/well in 100 µL DMEM medium per well. After 24 h of pre-conditioning, the culture medium was replaced with fresh medium and the cells were treated with several concentrations of the extracts (6.25, 12, 25, 50 and 100 µg/mL) for 24 h. Subsequently, 10 µL of MTS dye (1 mg/mL) was added to cell culture and further incubated for 2 h at 37°C. The index of cell viability was calculated by measuring the optical density (OD) of color produced by MTS dye reduction at 490 nm.

Collagenase Inhibition Assay

Collagenase activity was performed according to the protocol of Barrantes and Guinea (2003) with minor modifications. Briefly, 1.1 U/mL of C. histolyticum collagenase (ChC) and 1 mM of N-[3-(2-furyl) acryloyl]-L-leucyl-glycyl-L-prolyl-L-alanine (FALGPA) were prepared in 0.05 M tricine buffer [N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl) glycine] containing 0.4 M NaCl and 0.01 M CaCl$_2$; pH 7.5. A 25 µL aliquot of each extracts was transferred into a 96-well plate and incubated with 25 µL of ChC solution and 25 µL tricine buffer for 15 min at 37°C. Then, 50 µL of the FALGPA solution (Sigma Aldrich) was added to initiate the reaction. Water and oleanolic acid at the same amount and concentration were used as the negative and positive controls respectively. The mixtures were incubated at 37°C for 30 min and measured at 340 nm using the Epoch™ microplate reader (BioTek Instruments, Winooski, VT, USA). The inhibition percentage was calculated according to the following formula:

$$\text{Enzyme inhibition activity (\%)} = \frac{1 - B}{A} \times 100$$

where $A$ is the enzyme activity without extract and $B$ is the enzyme activity in the presence of the extract or control.

Elastase Inhibition Assay

The effect of A. catechu, B. rotunda, P. betle and O. aristatus extracts on elastase activity was assayed according to Kim et al. (2009) with some modifications. Briefly, an aliquot of 100 µL of 0.2 M Tris-HCl buffer pH 8.0, 25 µL of 10 mM N-(methoxy succinyl)-ala-ala-pro-val-4-nitroanilide (MAAPVN) and 50 µL of 100 µg/mL extracts were mixed and incubated for 15 min. Then, 25 µL of 0.3 units/mL elastase was added and incubated for another 15 min. Blanks consisted of all of the components except the enzyme. Oleanolic acid was used as the positive control at the same amount and concentration. The inhibition rate was measured at 410 nm using the Epoch™ microplate reader (BioTek Instruments, Winooski, VT, USA) and calculated as follows:

$$\text{Enzyme inhibition activity (\%)} = \frac{1 - B}{A} \times 100$$

where $A$ is the enzyme activity without extract and $B$ is the enzyme activity in the presence of the extract or control.

Hyaluronidase Inhibition Assay

The hyaluronidase inhibition assay was carried out based on the method described by Yahaya and Don (2012) with slight modifications. A 5 µL aliquot of A. catechu, B. rotonda, P. betle and O. aristatus extracts was pre-incubated with 75 µL bovine hyaluronidase (1.50 U) in 25 µL of buffer containing 20 mM (pH 7.0) sodium phosphate buffer, 77 mM sodium chloride, and 0.01% bovine serum albumin (BSA) for 10 min at 37°C. The assay was initiated by adding 100 µL of hyaluronic acid sodium salt from rooster comb (0.03% in 300 mM sodium phosphate, pH 5.35) to the mixture and incubated for further 45 min at 37°C. The undigested hyaluronic acid (Sigma Aldrich) was precipitated with 1 mL acid albumin solution composed of 0.1% BSA in 24 mM sodium acetate and 79 mM acetic acid (pH 3.75). The solution was allowed to stand at room temperature for 10 min, and the absorbance was measured at 600 nm using the Epoch™ microplate reader (BioTek Instruments, Winooski, VT, USA). Oleanolic acid served as the positive control at the same amount and concentration. The percentage of hyaluronidase inhibition were calculated by:

$$\text{Enzyme inhibition activity (\%)} = \frac{1 - B}{A} \times 100$$

where $A$ is the enzyme activity without extract and $B$ is the enzyme activity in the presence of the extract or control.

High-performance Liquid Chromatograph Analysis

The identification of the compounds present in these plant extracts was performed on a Shimadzu Prominence HPLC, using Zorbax Eclipse ODS C18 reverse phase column (150 mm × 4.6 mm ID, 5 µm) (Agilent, Germany). 0.1% phosphoric acid in water: acetonitrile was used as the mobile phase with 1 min/mL flow rate and the UV detector was set at 280 nm. Samples were filtered through a 0.22 µm nylon membrane and 200 µL were injected into the system. Compounds from each sample were identified by comparing their relative retention time with the chromatogram of a mixture of standards. All standard solutions were injected under the same conditions. All chemicals and solvents used for analysis were of HPLC grade.

Statistical Analysis

The mean values were calculated from a total of three replicates and the standard error (SE) of the mean was determined. One-way ANOVA and Duncan’s multiple range test ($P < 0.05$) was applied to determine the significance of the result among the extracts using SPSS version 20 for Windows.

RESULTS AND DISCUSSIONS

High Phenolic and Flavonoid Contents in P. betle and A. catechu

Plants produce secondary metabolites including phenolics, terpenes, steroids and alkaloids. These organic compounds have been studied for various medicinal properties. In order to characterize antioxidant and anti-aging properties of several traditional plants in Malaysia, we first quantified the total phenolic and flavonoid contents in these extracts. As presented in Figure 1, P. betle demonstrated the highest content of phenolic (16.67 GAE µg/g DW) and flavonoid (68.77 QE µg/g DW) followed by A. catechu. O. aristatus showed the lowest total phenolic content which was 3.39 GAE µg/g DW whereas B. rotunda showed the lowest total flavonoid content (6.92 QE µg/g DW). Free radical scavenging ability is mediated by the hydroxyl groups of the phenolic compounds (Maestri et al., 2006). Therefore, total phenolic concentration could be used as an indicator for potential antioxidant activity. In addition to high phenolic content, P. betle also possessed the highest flavonoid content. Hence, we predicted P. betle to possess the best antioxidant properties compared to the other plant extracts.
Plant Extracts with High Phenolic Content Exhibit Significant Antioxidant Properties

To verify our findings, the antioxidant activities of these plant extracts were analysed by measuring DPPH and ABTS radical scavenging activity. For DPPH radical scavenging activity, *P. betle* showed the lowest IC\textsubscript{50} value at 0.0017 ± 0.41 µg/mL, followed by *A. catechu* and *B. rotunda*. DPPH radical scavenging activity by *O. aristatus* was not detectable (Table 1). *P. betle* also showed the lowest IC\textsubscript{50} value for ABTS radical scavenging activity at 22.9 ± 0.35 µg/mL, followed by *A. catechu*, *B. rotunda* and *O. aristatus* (Table 1). However, at 100 µg/mL, *A. catechu* and *P. betle* showed similar activity of DPPH radical scavenging, at 89% and 85% respectively. This could be due to saturated extract concentration since the results were close to the scavenging activity of the positive control (Fig. 2). *P. betle* also showed the highest ABTS radical scavenging activity at 35% (Fig. 2).

Plant Extracts are Not Toxic to Normal Human Cells

Next, we examined the toxicity of the extracts. The cytotoxic effects (IC\textsubscript{50}) of these plant extracts in normal human fibroblast cells are shown in the Table 1. No cytotoxic effects of *P. betle* and *A. catechu* extracts on normal human fibroblasts were observed within the tested concentrations. *B. rotunda* and *O. aristatus* showed some cytotoxicity but at a high concentration range. In short, these plant extracts are not toxic to normal human cells and should be safe, at least, for topical application.

*P. betle* and *A. catechu* Inhibit Collagenase and Elastase but Not Hyaluronidase

Next, we investigated the effects of these plant extracts against collagenase, elastase and hyaluronidase activities. A fixed concentration of the crude extract (100 µg/mL) was chosen for the enzyme inhibition assays. *P. betle* demonstrated the highest percentage of collagenase inhibition (75.09%) whereas *B. rotunda* and *O. aristatus* could not inhibit collagenase activity (Fig. 3). *P. betle* also showed the highest percentage of elastase inhibition (38.48%), followed by *A. catechu* and *O. aristatus* (Fig. 3). Lastly, only *B. rotunda* and *O. aristatus* showed considerable hyaluronidase inhibition (Fig. 3). Previously, it was reported that two fractions of grape pomace extract possessed high inhibition of elastase activity even though the polyphenol contents were low (Wittenauer et al., 2015). However, another report showed that the root extracts of *Pueraria candollei*, which contains higher phenolic content than *Coccinia grandis* fruit extract, demonstrated higher elastase inhibition activity (Chattuwatthana et al., 2015). Thus, phenolic content may not be a suitable indicator for the inhibitory action against aging-related enzymes. Based on this study, different plant extracts have diverse antioxidant, anti-elastase, anti-collagenase and anti-hyaluronidase activities. The enzymatic inhibitory effects may depend on multitude of factors such as extraction methods, the amount and types of active compounds present in the plant, plant part of extraction and cultivation condition of the plant. Nonetheless, similar to our antioxidant results, *A. catechu* and *P. betle* extracts showed promising anti-aging potential as they exhibited significant inhibition on collagenase and elastase but not hyaluronidase whereas enzymes inhibition by *B. rotunda* and *O. aristatus* extracts were negligible. Optimal mixtures of these extracts may be required to compensate for the lack of hyaluronidase inhibition in *A. catechu* and *P. betle* extracts.

Plant Extracts Contain Various Antioxidant and Anti-aging Compounds

Lastly, the bioactive compounds in these extracts that may be responsible for their antioxidant and enzyme inhibitory properties were identified and listed in Table 2. Ellagic acid was the...
main compound detected in *A. catechu* while quercetin and eugenol were detected in *P. betle* (Fig. 4). Ellagic acid has been shown to deactivate various free radicals, presumably through its anion. Interestingly, ellagic acid could provide continuous protection against oxidative stress as it is not reduced after being metabolized (Galano et al., 2014). Quercetin is a known anti-aging/antioxidant compound and is abundantly present in various foods. Quercetin was also reported to mediate protection against osteoporosis, some forms of cancers, pulmonary and cardiovascular diseases and aging (Boots A et al., 2008). Interestingly, quercetin could rejuvenate senescent fibroblasts and improved cell survival and longevity (Chondrogianni et al., 2010). Eugenol is a phenolic compound that has several medicinal properties such as antioxidant, anti-inflammatory and anti-carcinogenic effects (Bezerra et al., 2017 and Barboza et al., 2018). This study verifies the beneficial effects of these plant extracts containing several bioactive compounds as potential anti-aging agents. However, there is limited information and studies done on the isolated compounds from potential plant extracts for the development of anti-aging agents. Therefore, intensive studies are crucial to improve and elucidate the molecular mechanisms of these compounds as anti-aging agents.

### Table 2 | Bioactive compounds detected in crude plant extracts.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Compounds</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. catechu</em></td>
<td>Ellagic acid</td>
<td>14.66</td>
</tr>
<tr>
<td><em>B. rotunda</em></td>
<td>Pinocembrin</td>
<td>35.53</td>
</tr>
<tr>
<td></td>
<td>Pinostrobin</td>
<td>41.026</td>
</tr>
<tr>
<td><em>P. betle</em></td>
<td>Quercetin</td>
<td>25.506</td>
</tr>
<tr>
<td></td>
<td>Eugenol</td>
<td>32.830</td>
</tr>
<tr>
<td><em>O. aristatus</em></td>
<td>Rosmarinic acid</td>
<td>20.84</td>
</tr>
<tr>
<td></td>
<td>Rutin</td>
<td>21.146</td>
</tr>
</tbody>
</table>

**FIGURE 3** | Percentage of collagenase, elastase and hyaluronidase activities by *A. catechu*, *B. rotunda*, *P. betle* and *O. aristatus* crude extracts at concentration of 100 µg/mL. Same letter denotes non-significant difference according to Duncan Multiple Range Test (*P* < 0.05).
CONCLUSION
This study was undertaken to demonstrate the anti-aging properties in some of the traditional plants in Malaysia. Our findings show that these plants, especially *A. catechu* and *P. betle*, possess substantial antioxidants and anti-aging effects. Further experiments are warranted to characterize the antioxidant and anti-aging activities of the individual bioactive compounds from these plant extracts and to determine the optimum extracts cocktail. These results may facilitate the development of safe and cost-effective anti-aging products.

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CONFLICT OF INTERESTS
The authors have no conflict of interests.

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