In vitro antidiabetic, antioxidation and cytotoxicity activities of ethanolic extract of Aquilaria crassna leaves and its active compound; mangiferin

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Received 03 March 2018; revised 14 December 2018

Degenerative diseases have been considered as a major public health problem in many countries, thus, finding medicines to treat these diseases without undesirable side effects is required. This study aimed to investigate the antidiabetic, anti-oxidation and cytotoxicity activities of the ethanolic extract of Aquilaria crassna leaves (ACE) and its active metabolite; mangiferin. The yeast α-glucosidase inhibitory assay was performed, and the IC50 of ACE and mangiferin were found to be 0.1840±0.0032 and 0.5714±0.0044 mg/mL, respectively. In addition, these samples were analyzed in term of the in vitro antioxidant activities using standard antioxidant assays. The results showed that ACE and mangiferin do possess anti-oxidant properties. Moreover, the cytotoxicity of ACE and mangiferin was also evaluated against three human cancer cell lines using MTT assay. The ACE could inhibit cell viability of MDA-MB-231; breast cancer cells (IC50 = 33.89±0.50 μg/mL) greater than HT-29; colorectal cancer cells (IC50 = 51.74±1.42 μg/mL) and HepG2; hepatic cancer cells (IC50 = 53.63±1.54 μg/mL) Mangiferin could also reduce the viability of these three cell lines, but the IC50 was greater than 100 μg/mL. In conclusion, our findings may provide some evidence for understanding the indigenous use of A. crassna leaves.

Keyword: Antioxidant, Aquilaria crassna leaves, Cytotoxicity, Ethanolic extract of Aquilaria crassna leaves (ACE), α-glucosidase inhibition

IPC Code: Int. Cl. A61P 17/18, A01H 5/12, A61K 38/00, A01H 5/12, C12P 19/16

In the human body, free radicals and oxidants can be generated from either normal cellular metabolism or external sources. These radicals can react with biomolecules resulting in cell injury, which leads to various degenerative diseases1. Lately, the use of medicinal plants has augmented the treatment options of degenerative disorders because such plants have been proved in terms of their medical properties by plentifully in vitro and in vivo experiments2. Thailand is a country which has a medical civilization dating back to prehistoric indigenous regional practices through to modern-day medicine at present. The use of herbal products has been promoted in the health care system, and ensuring the quality, efficacy as well as safety of medicines must be regulated before marketing.

Aquilaria crassna Pierre ex Lecomte (agarwood) belongs to the Thymelaeaceae family of flowering plants. Agarwood has been accredited as being used as part of Ayurvedic medicine, traditional chinese medicine and traditional Thai medicine for centuries. This plant is not only notable for aroma therapy purposes but is also known as a medicinal plant for the remedy of several symptoms, such as inflammation, pain, fever, and constipation. Mangiferin, the xanthone derivative, was reported as the compound that can be isolated from the leaves of A. crassna3. Mangiferin has been claimed as super-antioxidant from natural sources and was reported to exhibit several pharmacological effects including anti-allergic, antidiabetic, antimicrobial, and anti-inflammatory4. Even though agarwood leaf herbal tea has been claimed to provide benefits relating to anticancer and anti-hyperglycemic effects on a commercial level, scientific evidence is still needed to prove the biological activity and mechanism of action of such claims about the product. The present study aimed to quantify the mangiferin content in A. crassna leaves and to assess some biological activities of the ethanolic extract of A. crassna leaves (ACE) (and mangiferin). The findings from this research may support this medicinal plant’s capability and usefulness of application.
Materials and methods

Plant materials
*Aquilaria crassna* leaves were collected in Nan Province, Thailand, and authenticated by Associate Professor Nijsiri Ruangrungsi. The specimen was deposited at the College of Public Health Sciences, Chulalongkorn University, Thailand. The leaves were dried in hot air oven at 50°C, and pulverized for the extraction process.

Preparation of ACE
Three grams of ground leaf powder were exhaustively extracted with 95% ethanol by Soxhlet apparatus. The extract was filtered through filter paper and subjected to evaporation until dry. The dried ACE was accurately weighed and the percentage of yields was calculated and reported.

Mangiferin content in AC extract using TLC-densitometric method
The stock solution of mangiferin was prepared by dissolving in 85% ethanol in a volumetric flask at a concentration of 1 mg/mL. The concentration of working standard solutions ranged from 0.15 to 0.65 mg/mL, while the ACE was diluted with absolute ethanol to produce the test extract at a concentration of 3 mg/mL. The solutions of standard mangiferin and ACE were spotted on a silica gel 60 GF 254 TLC plate. The mobile phase comprised of ethyl acetate : water : formic acid, with a respective ratio of 17:3:2 v/v/v. The developed TLC plates were densitometrically scanned in the absorbance mode with a wavelength of 254 nm. The calibration curve was constructed to quantify the amount of mangiferin in ACE via the peak area. Finally, the mangiferin content was calculated based on the extract yield.

Alpha-glucosidase inhibitory assay
The yeast alpha-glucosidase inhibitory activity used the p-Nitrophenyl-β-D-glucopyranoside (PNPG) as a substrate with minor modified protocol.

Anti-oxidation assay
The 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay was performed, according to a previously described method. The NO' scavenging assay was carried out using the Griess reagent, according to a prior report. The capability of the test substances to quench O2' in the reaction mixture was measured via the reduction of nitrotetrazolium blue. Quercetin was used as a positive control. In addition, the measurement of the power of anti-oxidation properties was performed by a FRAP assay method with slight modifications. The reducing power capability was expressed in µM ferrous sulfate equivalents in milligrams per dried weight of the sample. The concentrations of the phenolic compound in ACE were determined using the Folin-Ciocalteu's reagent method.

Cytotoxicity testing
Cell viability was determined by a modified MTT assay, in accordance with a previously described method. Human colorectal adenocarcinoma (HT-29), human hepatocellular carcinoma (HepG2) and human breast cancer cells (MDA-MB-231) were obtained from the American Type Culture Collection and were cultured in complete medium. The complete medium contained 10% (v/v) FBS, 1% (v/v) penicillin-streptomycin in DMEM. Trypsinized cells were seeded into each well at a density of 1x10^5 cells/mL and in a humidified atmosphere of 5% CO2, 37°C. After 24 hours, the cells were exposed to various concentrations of the AC extract, mangiferin, or doxorubicin (positive control) for 24 hours. After that, the medium was replaced by the MTT solution (0.4 mg/mL) and incubated for 4 hours. Purple formazan crystals were dissolved in DMSO. The relative number of viable cells was assessed by measuring the absorbance of the formazan product at 570 nm with a microplate reader.

Statistical analysis
All data sets are reported as mean ± standard deviation from three independent experiments. Differences among the groups were compared to the control by a one-way analysis of variance, followed by Dunnett's test.

Results
Analysis of mangiferin in *Aquilaria crassna* leaves
The yield of ACE from Nan province was 13.33% of the dried weight. Densitogram of standard mangiferin and ACE under UV 254 nm is shown in Fig. 1. The calibration curves ranged from 450-1,950 ng/spot which has R² = 0.9992. The content of mangiferin in ACE determined using TLC densitometry was found to be 2.1130% of the dried weight.

α-glucosidase inhibitory activity of ACE and mangiferin
ACE, mangiferin and acarbose exhibited the inhibitory effect of yeast α-glucosidase activity in the concentration-response manner (Fig. 2) The IC₅₀ values of ACE, mangiferin, and acarbose were 0.1840±0.0032, 0.5714±0.0044, and 17.3947±0.0189 mg/mL, respectively.
**In vitro anti-oxidation determination**

The antioxidant activities of ACE and its metabolite; mangiferin are summarized in Table 1.

**DPPH scavenging activity**

The DPPH scavenging results of mangiferin and ACE are displayed in Fig. 3 and Table 1 as compared with quercetin. From the results, IC₅₀ values of mangiferin, ACE, and quercetin were calculated from the dose-response curve and found to be 0.64±0.005, 21.54 ± 0.17, and 3.46±0.09 mg/mL, respectively.

**Scavenging of nitric oxide radical (NO•)**

The mangiferin and ACE also displayed a dose-dependent elevation in NO• scavenging activity.

<table>
<thead>
<tr>
<th></th>
<th>ACE</th>
<th>Mangiferin</th>
<th>Quercetin</th>
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<tbody>
<tr>
<td><strong>DPPH scavenging</strong></td>
<td>21.54±0.17</td>
<td>0.64±0.005</td>
<td>3.46±0.09</td>
</tr>
<tr>
<td><strong>nitric oxide radical scavenging</strong></td>
<td>79.13±0.74</td>
<td>40.55±0.17</td>
<td>13.17±0.30</td>
</tr>
<tr>
<td><strong>Superoxide radical scavenging</strong></td>
<td>278.12±4.29</td>
<td>105.49±1.28</td>
<td>16.91±0.19</td>
</tr>
</tbody>
</table>

(*Mean ± standard deviation) n = 3
The percentage inhibition of mangiferin and ACE showed the IC\textsubscript{50} values at 40.55 ± 0.17 and 79.13 ± 0.74 μg/mL, respectively, while the IC\textsubscript{50} value of quercetin was 13.17±0.30 μg/mL.

Superoxide radical scavenging activity
The activity of superoxide radical scavenging of mangiferin and ACE was particularly increased with the augmented concentration (Fig. 5). According to the results, IC\textsubscript{50} values of mangiferin, ACE and quercetin were calculated and found to be 105.49 ± 1.28, 278.12 ± 4.29, and 16.91 ± 0.19 μg/mL, respectively.

Ferric reducing antioxidant power (FRAP)
The ferric ion reducing antioxidant power of the AC extract and mangiferin were evaluated in terms of its capability to reduce TPTZ-Fe\textsuperscript{3+} to TPTZ-Fe\textsuperscript{2+}. The FRAP values of mangiferin and AC extract, which were calculated from ferrous sulfate calibration curve (R\textsuperscript{2} = 0.9985), were found to be 11.82 μMFe (II)/mg and 2.52 μM Fe (II)/mg, respectively.

Total phenolic content (TPC)
The total phenolic content of the ACE, calculated based on the calibration curve (R\textsuperscript{2} = 0.999), was 120.18±0.50 mg gallic acid equivalents/g.

Cytotoxicity evaluation
The cytotoxic on human cancer cell lines of mangiferin and ACE, as compared to doxorubicin, was demonstrated with concentration-dependent (Fig. 6). ACE showed significant toxicity on all tested cancer cells and could be extrapolated to determine the IC\textsubscript{50} value. Mangiferin acted as a cytotoxic potential, but the percentage of cell viability at the highest concentration (100 μg/mL) was 62.93% for MDA-231, 67.85% for HepG2, and 63.52% for HT-29. IC\textsubscript{50} values for cytotoxic activities of ACE, mangiferin, and doxorubicin are presented in Table 2.

Discussion
In this study, mangiferin content in ACE was quantified using TLC -densitometry technique. The amount of substance was quantitated based on the wavelength of specific light absorption or emission and its intensity. The amount of mangiferin in this sample is nearly equal to the content in Aquilaria sinensis which quantitated using LC-MS\textsuperscript{12}.

Moreover, this research also investigated the effect of ACE and mangiferin on antioxidation activities, α-glucosidase inhibitory activity, and cytotoxic activity. α-glucosidase is a membrane-bounded intestinal enzyme that functions as the last step of starch hydrolysis to glucose. From our findings, ACE (IC\textsubscript{50}=0.1840 mg/mL) possessed a strong potential as an α-glucosidase inhibitor to the same degree as other Aquilaria species (A sinensis, A. hirta, and A. Malaccensis)\textsuperscript{13,14}. Moreover, mangiferin (IC\textsubscript{50}=0.5714 mg/mL; 1.3530mM) also showed α-glucosidase inhibitory properties greater than acarbose.
Table 2 — Cytotoxic activities of ACE, mangiferin, and doxorubicin against cancer cell lines

<table>
<thead>
<tr>
<th></th>
<th>MDA-231</th>
<th>HepG2</th>
<th>HT-29</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>33.89±0.50</td>
<td>53.63±1.54</td>
<td>51.74±1.42</td>
</tr>
<tr>
<td>Mangiferin</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.12±0.01</td>
<td>2.66±0.27</td>
<td>0.39±0.02</td>
</tr>
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</table>

(*Mean ± standard deviation) n = 3

(IC50=17.3947 mg/mL; 26.9432 mM) which is in accordance with previous reports13,15. Feng et al (2011) isolated compounds from the leaves of *A. sinensis* and all isolated compounds were subjected to determine the glucosidase inhibitory activity13. These eight isolated compounds (aquilarisinin, aquilarisin, hypolaetin 5-O-β-D-glucuronopyranoside, aquilarixanthone, iriflophenone 2-O-α-L-rhamnopyranoside, iriflophenone 3-C-β-D-glucoside, iriflophenone 3,5-C-β-D-diglucopyranoside, and mangiferin) could inhibit the α-glucosidase.
enzyme which was more potent than acarbose. However, A. crassna was classified in the same genus of A. sinensis. The ACE might be containing these compounds; therefore, it might greater perform the inhibitory activity. In addition, the computational simulation by a molecular docking technique revealed that 1,3-dihydroxybenzoanthon ring compound concurrently binds to the non-competitive site of yeast α-glucosidase. Therefore, this compound might exhibit a significant synergistic inhibition of glucosidase enzyme.

As afore mentioned, some degenerative disorders result from oxidative stress in human cells. The in vitro anti-oxidation assay signified the ability to scavenge the free radicals. In this study, ACE and mangiferin could scavenge the DPPH with IC50 values similar to previous reports. NO•, an unstable reactive biomolecules, leading to undesirable alterations. This study showed that various toxic reactive oxygen species which attack the biomolecules, leading to undesirable alterations. This study implied that ACE and mangiferin has the potential as a natural antioxidant by inhibiting RNS and ROS, which might due to its phenolic properties. Phenol compounds are comprehended to possess the high anion scavenging capability. The FRAP assay as well as the determination of total phenolic content were the techniques used for evaluation the anti-oxidation capacity of the testing sample. The capacity of ACE on the FRAP assay and TPC differed from prior research studies because of the extraction procedure, as well as the geographical location of plantation. In addition, the concentration of ethanol and the solid-to-solvent ratio of A. crassna leaf extraction had a significant effect on TPC and scavenging activities, whereas, the extraction time had an insignificant effect.

MTT assay is a precise and uncomplicated method that provides beneficial information on the anticancer, anti-proliferative and cytotoxic determination. This method quantifies the metabolic action of viable cells through the mitochondrial enzyme converting soluble yellow tetrazolium salt to dark blue water-insoluble formazan crystal. The cytotoxicity activity of A. crassna leaf extract has not been previously investigated, whereas the other parts; oil and stem bark, have been proved as having anticancer activity. ACE contained numerous substances, some of them might be acted as cytotoxicity properties or performed synergistic effects to decrease the viability of the cancer cell lines. From these findings, mangiferin could also decrease the cell viability of these three cancer cell lines but the IC50 was greater than 100 μg/mL. The previous research mentioned that the 300 μM (126.70 μg/mL) of mangiferin could inhibit the viability of breast cancer cells at a rate of 50%. Therefore, A. crassna leaves might be used as an indigenous source for cytotoxicity of colorectal cancer, hepatic cancer and breast cancer but it is still necessary to examine the in vivo biological activity in a further study in order to explore the possible use of this plant to treat illness.

Conclusion

ACE and its active metabolite; mangiferin, exhibited the capability as a α-glucosidase inhibitor with a higher ability than that of acarbose. In addition, ACE and mangiferin also showed an anti-oxidation capacity but one which was less potent than quercetin, a positive control. Ethanolic extract of A. crassna leaves showed significant cytotoxicity against these three cancer cell lines, while mangiferin performed less cytotoxicity. In conclusion, our findings may provide some useful evidence for understanding the indigenous use of A. crassna leaves.

Acknowledgement

This work was supported by the 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarships and the 90th Anniversary of Chulalongkorn University, Rachadapiseksomphot Endowment Fund from Graduate School. We would also like to thank the Chulalongkorn University Drug and Health Products Innovation Promotion Centre, Faculty of Pharmaceutical Sciences and College of Public Health Sciences, Chulalongkorn University for the use of laboratory facilities.

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