RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2023.059

ScienceAsia 49 (2023): **618–626**

The antioxidant activities and inhibitory effects on α-glucosidase and α-amylase of ethanolic and aqueous extracts from various parts of Thai *Caesalpinia sappan* L.

Apaporn Boonmee[a,b], Nuntaporn Moonrungsee[a,b], Teerapich Kasemsuk[a,b], Winyou Puckdee[c], Pratya Komonpanich[c], Chutapa Kunsook[c], Na-monrug Khamchatra[c], Sorasak Nakeim[c], Attakorn Khamchutra[d], Raksa Suninthaboonrana[e], Phoom Chairatana[f], Borvornwat Toviwek[g], Prapasiri Pongprayoon[g,h,*], Sunisa Suwancharoen[a,b,*]

[a] Department of Chemistry, Faculty of Science and Technology, Rambhai Barni Rajabhat University, Chanthaburi 22000 Thailand
[b] Herbal Product for Health and Beauty Research and Development Center, Rambhai Barni Rajabhat University, Chanthaburi 22000 Thailand
[c] Department of Biology, Faculty of Science and Technology, Rambhai Barni Rajabhat University, Chanthaburi 22000 Thailand
[d] Department of Environmental Science, Faculty of Science and Technology, Rambhai Barni Rajabhat University, Chanthaburi 22000 Thailand
[e] Khao Soi Dao Silvicultural Research Station, Chanthaburi 22140 Thailand
[f] Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700 Thailand
[g] Department of Chemistry, Faculty of Science, Kasetsart University, Bangkok 10900 Thailand
[h] Center for Advanced Studies in Nanotechnology for Chemical, Food and Agricultural Industries, KU Institute for Advanced Studies, Kasetsart University, Bangkok 10900 Thailand

[*]Corresponding authors, e-mail: sunisa.s@rbru.ac.th, fsciprpo@ku.ac.th

Received 4 Nov 2022, Accepted 18 Apr 2023
Available online 28 Jul 2023

ABSTRACT: *Caesalpinia sappan* (CS) has been commonly used in beverage and folk medicine in China and ASEAN countries because of its various therapeutic properties. Only the heartwood has long been used in traditional medicines due to the presence of high concentration of brazilin (one of the most important bioactive compounds), whereas the other parts are limited in use. Thus, this work aimed to investigate ways of utilizing other parts of CS. This information is important for further use of other CS parts to promote zero waste and hence sustainable resource utilization. Herein, the crude ethanolic (CEE) and crude aqueous (CAE) extracts from twelve parts of CS were evaluated for their biological activities for the first time. Our work demonstrates that not only the heartwood, but also other parts of CS exhibit interesting biological activities. CAE from heartwood of branches exhibits the highest antioxidant activity, which is higher than that of the positive control, butylated hydroxytoluene. CEE from florets shows the highest inhibitory effect against α-amylase, while CAE of barks shows better α-glucosidase inhibitory activity than the existing glucosidase inhibitor (acarbose). Molecular docking of brazilin (key bioactive compound) to both α-amylase and glucosidase can also confirm the tight binding of brazilin to α-glucosidase.

KEYWORDS: *Caesalpinia sappan*, brazillin, molecular docking, α-glycosidase, α-amylase

INTRODUCTION

Caesalpinia sappan (CS) or sappan wood is distributed in China and Southeast Asia. Its heartwood is used as a red pigment and an additive in food due to no unique flavor [1–3]. The dried heartwood of CS has long been used in traditional medicines for emmenagogue, anti-inflammation, hemostasis, and improving blood flow [4–6]. The heartwood was reported to be rich in homoisoflavonoids and brazilins [5, 7, 8]. Especially, brazilin was reported to show the antioxidant [9], anti-inflammatory [10], anti-acne [11], and antimicrobial [12] activities. Brazilin is one of the most important bioactive compounds found in CS and has a wide range of industrial applications including textile, food, and pharmaceutical area [5, 13]. Recently, CS has been reported to reduce and control blood glucose levels [14, 15], suggesting that CS may be used as an alternative treatment for diabetes. Nonetheless, all studies only focused on the heartwood extract where the pharmaceutical activities of other parts remain unknown. Sappan wood is often planted as a living hedge due to its easy growth and dense growth characteristics. In Thailand, Sappan wood acts as the land boundaries for protecting plantations against wild animals. Only heartwood is utilized for a medicinal propose. CS heartwood extracts have been recognized as safe plant extract and long been used as medical ingredient due to their high bioactive properties [5]. To get the heartwood, it is required to cut down as a whole where the rest are left as waste. To date, there is no information on biological activities of other CS parts. This information is important for further use of other CS parts to promote zero waste and hence

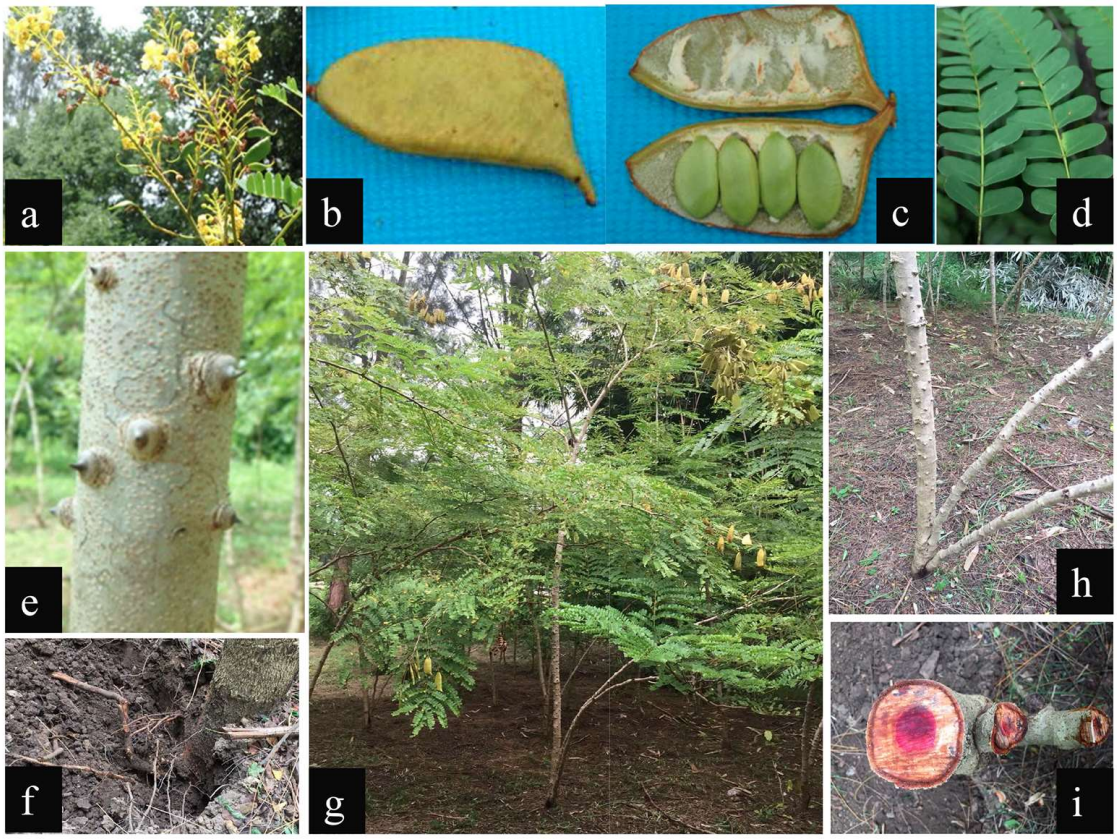

Fig. 1 Photographs of *Caesalpinia sappan* a: yellow flowers, b–c: hard and indehiscent pod fruit with a hard recurved beak at the upper angle, d: compound leaves, e: stem with scattered spines, f: tap root system, g: shrub, h: trunk split into 2 branches, and i: dark red heartwood.

sustainable resource utilization.

Thus, we aimed to investigate the biological activities of other parts of Thai CS. We first obtained the crude ethanolic (CEE) and crude aqueous (CAE) extracts from twelve parts of Thai CS, which are barks, bark of branches, seeds, heartwood, heartwood of branches, leaves, sapwood, sapwood of branches, twigs, florets, pods, and roots (Fig. 1). Then, the antioxidant activities, α-amylase and α-glucosidase inhibitory activities, and cytotoxicity against Vero cells of all parts were determined. The amount of bioactive brazilin in each part was also investigated in this work. These results are useful for improving resource utilization and reducing resource damage.

MATERIALS AND METHODS

Sample preparation and extraction

CS wood was collected from Khao Soi Dao silvicultural research station, Chanthaburi, Thailand. The identification and voucher specimens (No.02010) were deposited in the natural trails located in Plant Genetic Conservation Project under the Royal initiative of Her Royal Highness Princess Maha Chakri Sirindhorn at Rambhai Barni Rajabhat University. Twelve parts of CS were finely chopped and weighed. Then, the samples were oven-dried at 60 °C and weighed. All dried samples were kept in zip-lock bags in a presence of silica gel. Water or ethanol was then used to get crude extracts from the samples. For CAE, one of the twelve dried parts of CS (30 g) was mixed with 450 mL of distillate water and refluxed at 100–140 °C for 1 h. After cooling down, the extract was filtered with sterile straining cloth and then with filter paper no. 1 (11-μm pore). After that, the filtrate was lyophilized to obtain dried crude extract. The dried extract was weighed to calculate the yield percentage. For CEE, a similar protocol to aqueous extract preparation was used, but 95% ethanol (Daejung Chemical & Metals, Korea) was added to the round-bottomed flask instead of water.

General cell culture method

Vero cells were cultured in Dulbecco's modified eagle medium (DMEM, Gibco, USA) with 10% fetal bovine serum (FBS, HyClone, UK). To cells with ∼90% confluency, 0.05% trypsin-EDTA was added to detach the cells. Then, the cells were centrifuged at 1,200 rpm at

4 °C. The cells were resuspended in 1 ml of DMEM, and the number of cells was determined by using a manual hemocytometer. The cell concentration was adjusted to achieve the desired concentration.

Cytotoxicity assay

The cytotoxicity of twelve CS parts against Vero cells was assayed. First, Vero cells (15,000 cells) were added into 96-well plates and incubated at 37 °C and 5% CO_2 for 24 h. With this cell density, the monolayers were ∼100% confluent at the time of the experiment. With cells at this confluency, the supernatant was discarded and washed twice with fresh DMEM. CS extracts were then added to the wells, and the plates were incubated at 37 °C and 5% CO_2 for 72 h. The cell viability was determined by MTT assay. First, the supernatant was removed, and 20 µl of MTT reagent (2 mg/ml, Bio Basic, Canada) was added. The plates were incubated at 37 °C and 5% CO_2 for 4 h. Then, DMSO (100 µl) was added to dissolve Formazan crystals. The absorbance at 540 and 630 nm was measured by a microplate reader (Biochrom, UK). The cell viability was calculated using the following formula:

$$\text{Viability}\,(\%) = \frac{\text{A540}_\text{t} - \text{A630}_\text{t}}{\text{A540}_\text{c} - \text{A630}_\text{c}} \times 100,$$

where A540_t = absorbance at 540 nm of the CS extract-treated wells, A630_t = absorbance at 630 nm of the CS extract-treated wells, A540_c = absorbance at 540 nm of the control wells, and A630_c = absorbance at 630 nm of the control wells.

The 50% cytotoxic concentration (CC_{50}) of each CS extract was determined by Probit analysis.

Antioxidant activity by DPPH assay

To make a standard solution of butylated hydroxytoluene (BHT, Panreac Sintesis, Spain) and gallic acid (1000 µg/ml, Sigma-Aldrich, Germany), 0.0010 g of the compound was mixed with 1 ml of ethanol. These two solutions were used as positive controls. The stock solution of BHT was diluted with ethanol to achieve the concentrations of 0.02, 0.04, 0.06, and 0.08 µg/ml. The stock solution of gallic acid was diluted with ethanol to achieve the concentrations of 0.002, 0.004, 0.006, and 0.008 µg/ml. Each solution (100 µl) was added to a 96-well plate. Ethanol was used as a control solution. Then, 100 µl of 200 µM 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich) was added to each well except the blank control, in which 100 µl of ethanol was used instead. The plate was incubated at room temperature in the dark. After 30-min incubation, the absorbance at 490 nm was measured by a microplate reader. The experiments were performed in triplicate. The antioxidant activity percentage (% Inhibition) was calculated by using the following formula:

$$\%\,\text{Inhibition} = \frac{(\text{Abs}_\text{control} - \text{Abs}_\text{sample})}{\text{Abs}_\text{control}} \times 100,$$

where $\text{Abs}_\text{control}$ = absorbance at 490 nm of the control wells and Abs_sample = absorbance at 490 nm of the samples.

Then, a linear correlation of sample concentration (x axis) and % Inhibition (y axis) was plotted, and the IC_{50} values were determined for each compound.

The antioxidant activities of CAE and CEE were determined by two-fold serial dilution. To 0.0010 g of the extract, 200 µl of DMSO was added, and the solution was diluted with 800 µl of ethanol to achieve the concentrations of 2, 1, 0.5, 0.25, and 0.125 mg/ml, respectively. Then, a 100-µl aliquot of each concentration was added to a 96-well plate and followed by adding 100 µl of 200 µM DPPH except the blank control, in which 100 µl of ethanol was used instead. The plate was incubated at room temperature in the dark. After 30-min incubation, the absorbance at 490 nm was measured by a microplate reader. The experiments were performed in triplicate. The antioxidant activity percentage (% Inhibition) was calculated by using the above formula, and the 50% inhibitory concentration (IC_{50}) values were determined for each extract.

Antioxidant activity by FRAP assay

The protocol for Ferric Reducing Antioxidant Power (FRAP) assay was adapted from a previous work [14]. In this assay, an antioxidant reduces ferric tripyridyltriazine (Fe^{3+}-TPTZ) to ferrous tripyridyltriazine (Fe^{2+}-TPTZ), which is purple and absorbs light at 600 nm.

To obtain a standard curve, 0.001 g of ferrous sulfate (Ajax Finechem, Australia) was dissolved in Milli-Q water to achieve a concentration of 1 mg/ml and then was diluted to achieve working concentrations of 50, 100, 250, 350, and 500 µg/ml. A 20-µl aliquot of the standard solutions was added to a 96-well plate and followed by the FRAP reagent (180 µl). The plate was incubated at room temperature in the dark for 8 min. Then, the absorbance at 600 nm was measured by a microplate reader. A plot of ferrous sulfate concentration as a function of the absorbance at 600 nm was generated and used as a standard curve for ferrous sulfate. Another standard curve for Trolox (Acros organics, Belgium) was obtained by repeating the above protocol.

To determine the FRAP values, CAE and CEE with a concentration of 1 mg/ml were prepared. A 20-µl aliquot of each extract was added to a 96-well plate with a FRAP reagent (180 µl). The plate was incubated at room temperature in the dark for 8 min. Then, the absorbance at 600 nm was measured by a microplate reader. Each condition was performed in triplicate, and each experiment was repeated in triplicate. The FRAP value was determined by using the standard curves of

ferrous sulfate and Trolox. The results were expressed as mg Fe (II)/g (FRAP values) and mg Trolox/g of extract sample (TEAC values). The positive controls were BHT and gallic acid as mentioned in the DPPH assay.

α-Amylase inhibitory assay

The protocol was adapted from a literature [16]. Acarbose, a current glucosidase inhibitor, was used as a reference in this section. To prepare a standard solution of acarbose at a concentration of 10 mg/ml, 50-mg Glucobay (Bayer Pharma AG, Germany) was ground finely and dissolved in 3 ml of DMSO. The mixture was then vortexed and centrifuged for 10 min. The supernatant was collected and adjusted to 5 ml with DMSO. The stock solution was diluted with phosphate buffered saline (PBS) at pH 7.0 to obtain concentrations of 0.005, 0.010, 0.020, and 0.030 mg/ml, respectively.

To prepare a 50-mg/ml stock solution, 0.0500 g of each extract (CAE and CEE) was dissolved in 1 ml of DMSO and sonicated for 20 min. The stock solution was then diluted with buffer to achieve concentrations varying from 3 to 30 mg/ml. A 20-µl aliquot of the diluted solution was mixed with 20 µl of 1% starch in a 96-well plate. Then, a 30-µl aliquot of α-amylase was added, and the plate was incubated at room temperature for 5 min. After the incubation, the reaction was quenched with 20 µl of HCl (1 M) and 80 µl of a 1% iodine solution. The absorbance at 650 nm was measured by a plate reader. The assay was conducted in triplicate. The inhibition percentage was calculated by using the following formula:

$$\%\,\text{Inhibition} = \frac{[(\text{A}-\text{B})-(\text{C}-\text{D})]}{(\text{A}-\text{B})} \times 100,$$

where A = absorbance at 650 nm of control, B = absorbance at 650 nm of control blank, C = absorbance at 650 nm of sample, and D = Absorbance at 650 nm of sample blank.

A correlation of % Inhibition and concentration was plotted, and the IC_{50} was determined.

α-Glucosidase inhibitory assay

The protocol was adapted from a literature [16]. Preparation of a 10 mg/ml standard solution of acarbose was the same as described in α-Amylase inhibitory assay. The stock solution was diluted with phosphate buffered saline (PBS) at pH 7.0 to achieve concentrations of 0.375, 0.750, 1.50, and 3.00 mg/ml.

To prepare a 10-mg/ml stock solution, 0.0100 g of CS extracts was dissolved in 1 ml of DMSO and sonicated for 20 min. The stock solution was then diluted with buffer to achieve concentrations varying from 0.375 to 3.00 mg/ml. A 20-µl aliquot of the diluted solution was added to a 96-well plate and followed by a 50-µl aliquot of a 5-mM solution of 4-nitrophenyl β-D-glucopyranoside (PNPG, Tokyo Chemical Industry, Japan) and a 60-µl aliquot of α-glucosidase from *Saccharomyces cerevisiae* (0.3 U/ml, Sigma-Aldrich). The plate was incubated at room temperature for 30 min. After the incubation, the absorbance at 405 nm was measured by a plate reader. The assay was conducted in triplicate. The inhibition percentage was calculated by using the following formula:

$$\%\,\text{Inhibition} = \frac{[(\text{A}-\text{B})-(\text{C}-\text{D})]}{(\text{A}-\text{B})} \times 100,$$

where A = absorbance at 405 nm of control, B = absorbance at 405 nm of control blank, C = absorbance at 405 nm sample, and D = absorbance at 405 nm sample blank.

A correlation of % Inhibition and concentration was plotted, and the IC_{50} was determined.

Brazilin concentration analysis in the CS extracts

To prepare a 200-µg/ml standard solution, brazilin (0.002 g, Sigma-Aldrich) was dissolved in 10 ml of 99.9% methanol. The stock solution was diluted with a mixture of 0.3% acetic acid and acetonitrile (88.5% and 11.5%, respectively) to achieve concentrations varying from 0.05 to 20 µg/ml. The resulting solution was filtered through a 0.45 µm filter and stored in a HPLC vial.

To prepare a 500 µg/ml solution, the CS extract (0.005 g) was dissolved in 10 ml of 0.3% acetic acid and acetonitrile mixture (88.5% and 11.5%, respectively). The nonpolar compounds were removed from the solution by solid phase extraction (SPE, C8). The resulting solution was filtered through a 0.45 µm filter and stored in a HPLC vial.

For qualitative analysis of brazilin in the CS extracts, 10 µl of the filtered solutions was injected into a HPLC with the following conditions: C18 (4.6 mm × 250 mm) column, the mobile phase composition of 0.3% acetic acid/acetonitrile (88.5/11.5), detecting wavelength at 280 nm, and flow rate of 1.00 ml/min with isocratic mode. The peaks and their retention times were compared between standard brazilin and the CS extracts.

For quantitative analysis of brazilin in the CS extracts, a standard solution of brazilin at concentration varying from 0.05–20 µg/ml was injected into HPLC. A correlation of concentration and area under the peak was plotted. Then, the solution of the CS extracts was injected into HPLC, and the concentration of brazilin in each CS extract was determined by using the area under the peak. The experiments were conducted in triplicate.

Molecular docking

The crystal structures of α-glucosidase (PDB code: 3WY2) and α-amylase (PDB code: 1SMD) were downloaded from the PDB databank (https://www.rcsb.

Table 1 Cytotoxicity of the ethanolic and aqueous CS extracts to Vero cells. Different superscript letters in each column denoted significant difference ($p < 0.05$). a–n represents the order of significant difference values by "a" representing the highest value and "n" representing the lowest value.

Part	Cytotoxicity CC_{50} (μg/ml)	
	CEE	CAE
Bark	217.99 ± 23.94^{f}	579.55 ± 49.66^{f}
Bark of branch	139.47 ± 9.52^{bc}	473.59 ± 19.81^{d}
Seed	137.93 ± 2.77^{bc}	355.87 ± 37.05^{c}
Heartwood	145.09 ± 4.17^{cd}	110.19 ± 28.83^{a}
Heartwood of branch	104.85 ± 7.21^{a}	318.73 ± 34.85^{c}
Leaf	101.21 ± 2.58^{a}	724.21 ± 32.85^{g}
Sapwood	239.38 ± 12.69^{g}	234.67 ± 7.10^{b}
Sapwood of branch	166.38 ± 16.31^{e}	546.01 ± 41.38^{ef}
Twig	159.35 ± 4.67^{de}	389.78 ± 6.24^{cd}
Floret	224.19 ± 4.33^{f}	422.22 ± 1.63^{cd}
Pod	126.92 ± 3.71^{b}	$1{,}152.39 \pm 176.07^{h}$
Root	159.78 ± 3.66^{de}	218.80 ± 5.86^{b}

org). All hydrogen atoms were added into both structures. The three-dimensional Brazilin structure and its parameters were generated using Discovery Studio v21.1.0.20298 [17]. Brazilin was docked into the active sites of both α-glucosidase and α-amylase using GOLD 5.1 software [18]. The best binding mode and energy were computed for each protein.

Statistical analysis

Data are expressed as mean ± SD from three separate observations. For *in vitro* biological activities and brazilin content, one way ANOVA test followed by pairwise comparisons ($p < 0.05$) were used to analyze the differences among activities of various CS parts. The regression analysis was used to estimate the relationships between brazilin content and biological activities.

RESULTS AND DISCUSSION

Biological activities of CAE and CEE extracts

CAE and CEE extracts were prepared from twelve parts of CS collected from Khao Soi Dao silvicultural research station, Chantaburi province. The percentage yield is listed in Supplementary data. Five biological activities (cytotoxicity to Vero cells, antioxidant activity by DPPH and FRAP, α-amylase inhibition activity, and α-glucosidase inhibition activity) of the CS extracts were determined. The cytotoxicity of the CS extracts is needed to be determined to acquire the optimal concentrations of the extracts that are not toxic to the cells [19, 20]. Since CS parts have already been used in many local products and traditional medicines, the cytotoxicity tests of each CS part become important for further use.

The cytotoxicity of CAE and CEE was evaluated using cell viability assay on Vero cells and shown as CC_{50} (Table 1). The CC_{50} values of CEE from different parts are ranged from 101.21–239.38 μg/ml, while those of CAE are in the range of 110.19–1,152.39 μg/ml. Overall, it is clear that CAE shows less toxicity to cells than CEE. Therefore, CAE seems to be more practical to be used than CEE due to its lower cytotoxicity. For CEE, leaves and heartwood of branches exhibited the highest cytotoxicity (101.21 ± 2.58, and 104.85 ± 7.21 μg/ml, respectively), whereas that from sapwood displayed the lowest cytotoxicity (239.38 ± 12.69 μg/ml). In case of CAE, it is interesting that CAE from heartwood displayed the highest toxicity, while the pods showed the least cytotoxic effect ($1{,}152.39 \pm 176.07$ μg/ml). These cytotoxic results demonstrate that the overuse of the heartwood can produce harmful effect to human health. This also highlights the importance of this work to determine the biological activities of other parts to use as the heartwood substituent. For example, CAE from pods with the least cytotoxicity can serve as one of the possible alternatives. It is interesting that CEE from heartwood exhibited a comparable degree of cytotoxicity to its corresponding CAE, while CEE from heartwood of branches showed approximately 3-fold higher toxicity than that of CAE. We speculated that the difference may arise from their different compositions of phenolic components, including xanthone, coumarin, chalcones, flavones, homoisoflavonoid, brazilin, and so on. Moreover, these small molecules possess different solubilities in water and ethanol [5].

Furthermore, the antioxidant activities of CAE and CEE were also investigated (Table 2). Based on DPPH assay, both CAE and CEE show a comparable degree of antioxidant activities. CEE extracted from heartwood of branches, roots, heartwood, barks, sapwood of branches, and sapwood seems to show high antioxidant activities (IC_{50} of 19.03 ± 1.84, 19.73 ± 1.63, 24.37 ± 2.06, 32.07 ± 6.46, 33.10 ± 2.04, and 45.20 ± 2.42 μg/ml, respectively) which are also higher than the standard antioxidant, BHT (48.37 ± 4.65 μg/ml). On the contrary, only two CAE from heartwood of branches and heartwood displayed higher antioxidant activity than BHT (IC_{50} of 18.1 ± 5.86, and 29.17 ± 6.33 μg/ml). In previous studies, the heartwood extract was reported to show high antioxidant activity [21, 22]. Here, not only the heartwood extract, but also the heartwood of branches demonstrated the high antioxidant activity. Moreover, it is interesting that the heartwood of branches also showed a comparable degree of free-radical scavenging activity to the heartwood extract (Table 2). These findings imply the potential use of heartwood of branches as an alternative source for a medicinal use. Nevertheless, all CS extracts still exhibited lower antioxidant activities than gallic acid, another antioxidant standard (5.37 ± 0.29 μg/ml) (Table 2).

Moreover, based on the FRAP assay, seven CEEs (heartwood of branches, heartwood, sapwood

Table 2 Antioxidant activities of CS extracts. Different superscript letters in each column denoted significant difference ($p < 0.05$). a–n represents the order of significant difference values by "a" representing the highest value and "n" representing the lowest value.

Part	Antioxidant Activity					
	DPPH (IC_{50} μg/ml)		FRAP value (μg Fe (II)/mg)		TEAC value (μg Trolox/mg)	
	CEE	CAE	CEE	CAE	CEE	CAE
Bark	32.07 ± 6.46^{a}	83.67 ± 7.77^{de}	411.19 ± 0.28^{f}	480.86 ± 0.41^{f}	267.48 ± 0.33^{f}	277.21 ± 0.72^{f}
Bark of branch	56.20 ± 2.33^{a}	67.27 ± 1.29^{d}	196.50 ± 0.33^{h}	585.45 ± 0.48^{e}	122.05 ± 0.19^{h}	337.13 ± 0.27^{e}
Seed	304.43 ± 20.12^{c}	230.13 ± 19.10^{h}	50.11 ± 0.54^{m}	214.37 ± 0.37^{k}	22.88 ± 0.37^{m}	123.72 ± 0.21^{k}
Heartwood	24.37 ± 2.06^{a}	29.17 ± 6.33^{b}	513.54 ± 0.21^{d}	857.16 ± 0.25^{b}	336.83 ± 0.21^{d}	494.15 ± 0.29^{c}
Heartwood of branch	19.03 ± 1.84^{a}	18.10 ± 5.86^{ab}	615.32 ± 0.46^{b}	985.53 ± 0.43^{a}	405.58 ± 0.24 b	568.03 ± 0.25 b
Leaf	975.17 ± 48.38^{d}	317.83 ± 18.40^{i}	20.42 ± 0.36^{n}	103.49 ± 0.41^{n}	2.77 ± 0.24^{n}	59.82 ± 0.24^{n}
Sapwood	45.20 ± 2.42^{a}	164.70 ± 5.41^{g}	405.16 ± 0.48^{g}	413.67 ± 0.16^{g}	263.40 ± 0.39^{g}	238.49 ± 0.14^{h}
Sapwood of branch	33.10 ± 2.04^{a}	151.13 ± 1.29^{g}	523.02 ± 0.46^{c}	244.48 ± 0.51^{j}	343.51 ± 0.22^{c}	140.99 ± 0.18^{j}
Twig	145.73 ± 2.85^{b}	95.70 ± 0.60^{ef}	110.54 ± 0.57^{j}	437.25 ± 0.47^{h}	63.83 ± 0.33^{j}	252.16 ± 0.23^{g}
Floret	270.73 ± 63.58^{c}	110.30 ± 7.05^{f}	77.59 ± 0.44^{k}	273.44 ± 0.25^{i}	41.50 ± 0.31^{k}	157.68 ± 0.18^{i}
Pod	300.70 ± 59.53^{c}	307.93 ± 25.51^{i}	75.85 ± 0.23^{l}	118.24 ± 0.47^{m}	40.32 ± 0.16^{l}	68.24 ± 0.40^{m}
Root	19.73 ± 1.63^{a}	67.30 ± 2.46^{d}	440.79 ± 0.08^{e}	817.52 ± 0.11^{c}	287.54 ± 0.05^{e}	471.30 ± 0.06^{d}
Positive control						
BHT	48.37 ± 4.65^{a}	48.37 ± 4.65^{c}	153.60 ± 0.14^{i}	153.60 ± 0.14^{l}	85.72 ± 0.11^{i}	85.72 ± 0.11^{l}
Gallic acid	5.37 ± 0.29^{a}	5.37 ± 0.29^{a}	785.72 ± 0.28^{a}	785.72 ± 0.28^{d}	577.37 ± 0.22^{a}	577.37 ± 0.22^{a}

Table 3 The α-amylase and α-glucosidase inhibitory activities of CS extracts. Different superscript letters in each column denoted significant difference ($p < 0.05$). a–n represents the order of significant difference values by "a" representing the highest value and "n" representing the lowest value.

Part	Enzyme inhibitory activity, IC_{50} (μg/ml)			
	α-amylase		α-glucosidase	
	CEE	CAE	CEE	CAE
Bark	226.67 ± 5.86^{f}	$2{,}405.33 \pm 51.16^{g}$	122.00 ± 2.65^{b}	59.00 ± 8.00^{a}
Bark of branch	199.67 ± 4.04^{e}	$1{,}563.33 \pm 152.22^{d}$	158.67 ± 5.13^{e}	143.67 ± 3.51^{c}
Seed	172.33 ± 11.59^{d}	$1{,}483.67 \pm 67.31^{d}$	136.00 ± 3.61^{c}	171.33 ± 20.11^{d}
Heartwood	280.67 ± 15.95^{g}	$2{,}105.33 \pm 67.04^{f}$	69.67 ± 5.13^{a}	90.67 ± 5.51^{b}
Heartwood of branch	271.67 ± 11.37^{g}	$2{,}021.67 \pm 58.59^{f}$	81.00 ± 5.57^{a}	97.33 ± 6.66^{b}
Leaf	167.00 ± 6.00^{d}	842.33 ± 26.31^{c}	138.00 ± 4.36^{c}	150.67 ± 4.04^{c}
Sapwood	170.67 ± 8.62^{d}	$1{,}998.67 \pm 97.60^{f}$	132.00 ± 2.00^{c}	144.33 ± 2.52^{c}
Sapwood of branch	168.00 ± 8.54^{d}	$1{,}774.33 \pm 94.32^{e}$	129.00 ± 4.58^{bc}	144.33 ± 1.93^{c}
Twig	169.33 ± 6.51^{d}	$3{,}181.00 \pm 180.80^{h}$	156.67 ± 3.51^{de}	282.67 ± 10.02^{g}
Floret	128.33 ± 6.35^{b}	394.33 ± 1.15^{b}	288.33 ± 2.08^{h}	256.67 ± 4.73^{f}
Pod	149.67 ± 6.03^{c}	858.33 ± 17.16^{c}	148.33 ± 14.64^{d}	153.33 ± 6.03^{c}
Root	151.00 ± 13.11^{c}	$2{,}069.33 \pm 227.85^{f}$	178.67 ± 2.52^{f}	211.67 ± 5.86^{e}
Positive Control				
Acarbose	3.00 ± 0.00^{a}	3.00 ± 0.00^{a}	219.50 ± 5.13^{g}	213.83 ± 11.69^{e}

of branches, roots, barks, sapwood, and barks of branches) exhibited promising ferric-reducing activities when compared with BHT. So do ten CAEs (heartwood of branches, heartwood, roots, barks of branches, barks, twigs, sapwood, florets, sapwood of branches, and seeds). Interestingly, the heartwood of branches showed the highest ferric-reducing activity in both CEE and CAE with the FRAP values of 615.32 ± 0.46 and 985.53 ± 0.43 μg Fe (II)/mg, respectively (or Trolox equivalent antioxidant capacity (TEAC) of 405.58 ± 0.24 and 568.03 ± 0.25 μg Trolox/mg, respectively). These values are higher than that of BHT and are comparable to that of gallic acid.

Moreover, the heartwood extracts were found to be able to treat diabetes by reducing blood sugar [14, 15, 23, 24]. α-amylase and α-glucosidase were reported to control postprandial hyperglycemia and reduce the risk of developing diabetes [25–27]. Controlling activities of both enzymes were found to benefit diabetes treatment and management [28–30]. Although the heartwood extract was reported to inhibit both enzymes, no data on other CS parts are available. Thus, the inhibitory effect of crude extracts from different CS parts on α-amylase and α-glucosidase was studied (Table 3). Both extracts seemed to show inhibition ability against both enzymes, but CS extracts appeared to perform better in the inhibition of α-glucosidase. Seemingly, CS extract is more effective

Table 4 Brazilin content of all extracts from CS. Different superscript letters in each column denoted significant difference ($p < 0.05$). a–n represents the order of significant difference values by "a" representing the highest value and "n" representing the lowest value. The symbol "–" refers to undetectable.

Part	Brazilin Content (mg/100g sample)	
	CEE	CAE
Bark	1.57 ± 0.15^{d}	1.93 ± 0.12^{c}
Bark of branch	1.33 ± 0.23^{d}	4.03 ± 0.21^{d}
Seed	–	–
Heartwood	16.17 ± 3.35^{b}	13.80 ± 0.17^{b}
Heartwood of branch	20.50 ± 1.87^{a}	15.00 ± 0.10^{a}
Leaf	–	–
Sapwood	1.00 ± 0.26^{d}	0.30 ± 0.00^{e}
Sapwood of branch	1.33 ± 0.86^{d}	0.50 ± 0.00^{e}
Twig	0.57 ± 0.25^{d}	–
Floret	–	–
Pod	4.23 ± 0.15^{c}	–
Root	1.53 ± 0.42^{d}	–

for α-glucosidase than α-amylase. Importantly, CS extracts clearly showed greater inhibitory effect against α-glucosidase than acarbose (a commercial diabetic inhibitor). This suggests CS extracts as one of herbal medicine candidates for diabetes treatment. Among all parts, the CEE and CAE from florets and CAE from twigs demonstrated the lowest activities against α-glucosidase. The heartwood extracts surprisingly exhibited strong inhibitory activity on α-glucosidase. The promising inhibitory ability of the heartwood extracts against α-glucosidase can imply their feasibility to reduce the blood sugar and consequentially control diabetes. Nonetheless, the high cytotoxicity of heartwood extracts reported must be aware. Promisingly, not only the heartwood, but other parts also displayed significant α-glucosidase-inhibiting activity. Especially, CAE from barks ($IC_{50} = 59.00 \pm 8.00$ μg/ml) and heartwood of branches ($IC_{50} = 97.33 \pm 6.66$ μg/ml) showed a comparable degree of inhibitory effect to heartwood CAE ($IC_{50} = 90.67 \pm 5.51$ μg/ml). Both seem to be more encouraging to be used due to their lower toxicity when compared to the heartwood extracts. Seemingly, other CS parts can also be used as α-glucosidase inhibitors leading to the control of blood sugar level. This finding becomes vital for the community in terms of improving resource utilization and reducing waste production.

Qualitative and quantitative analysis of Brazilin in the CS extracts

Brazilin is the major bioactive compounds found in CS and displays antidiabetic, antioxidant, and anti-inflammatory activities [5, 22, 31], and therefore the brazilin level in each CS part was also determined. HPLC was used to identify the presence of brazilin in each sample. The brazilin solution standard was used as a reference, and its peak was observed at retention time (T_R) of 14.273 min (Fig. S1a). During the injection of CS extracts into HPLC, a peak with T_R of 14.789 min was identified as brazilin due to its retention time being close to the brazilin standard (Fig. S1b). Then, the brazilin standard was added to the CS extracts, and the mixture was analyzed by HPLC. The increased intensity of the peak with T_R of 14.789 min reflects the presence of brazilin.

Next, the amount of brazilin in CS extracts and the percent yield were evaluated using the area under the brazilin peak (Table 4). Our results agree with many previous studies that the heartwood is the major source of brazilin [5, 22, 32]. Furthermore, the ethanolic condition seems to promote better brazilin extraction than water. Interestingly, not only in the heartwood, but brazilin is also found in other CS parts. Especially, the heartwood of branches provides a high content of brazilin (20.50 ± 1.87 mg in CEE and 15.00 ± 0.10 mg in CAE). In contrast, the undetectable level of brazilin is found in seeds, leaves, and florets (Table 4). The regression analysis between biological activities (dependent variable, y) and brazilin content (independent variable, x) was carried out (Table S2). The brazilin level in each CS part appears to be correlated to the biological activity. The CS parts with high inhibitory ability against α-glucosidase appear to have high brazilin contents (Table 4) except the bark extract, which shows good ability to inhibit α-glucosidase, but the insignificant brazilin content was identified. The inhibition ability found in barks may be due to other active compounds. A further study is required to identify such key compounds.

Molecular docking of brazilin to α-amylase and α-glucosidase

Several studies demonstrate the ability of CS extracts on treating diabetes by reducing blood sugar [14, 15, 23, 33], but no molecular details can explain this scenario. Also, recent studies have reported the ability of brazilin in sappan wood to decrease blood sugar and α-glucosidase activity [23, 33]. Thus, in this work, the main active compound, brazilin, was selected to study its binding affinity to α-amylase and α-glucosidase which are two important enzymes for controlling hyperglycemia. Molecular docking was carried out to explore the microscopic view of the brazilin binding. The orientations of docked brazilin in the binding pocket of both enzymes are shown in Fig. 2.

The higher fitness score for α-glucosidase demonstrates brazilin prefers α-glucosidase (56.42) to α-amylase (47.66). In Fig. 2, the binding poses of brazilin in both enzymes are revealed. In a α-glucosidase system, brazilin can form five hydrogen bonds with Asp62, Asp202, Glu271, Asp333, and Arg400, respectively. In contrast, brazilin seems to form a weaker interaction network to α-amylase.

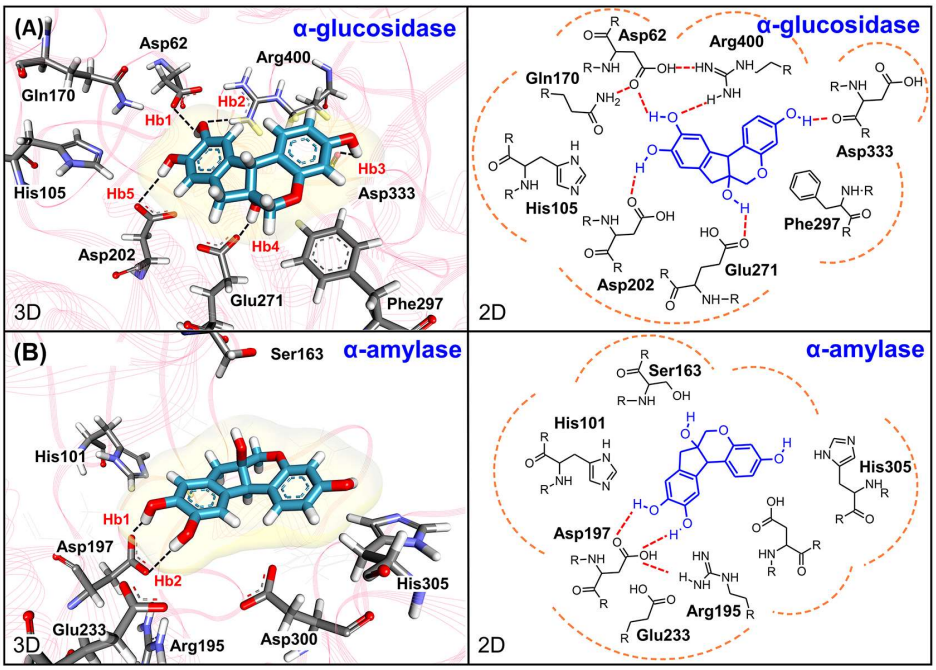

Fig. 2 Orientations of bound brazilin in the active site of α-glucosidase (A) and α-amylase (B). The 2D conformations of both systems are shown on the right. The red dashed lines indicate a presence of hydrogen bonds calculated from Discovery studio software.

Only an interaction with Asp197 is identified here. All hydroxyl groups on brazilin can interact with α-glucosidase, while only hydroxyl groups on *o*-66 side can interact with α-amylase. This can explain why brazilin shows the higher inhibition affinities for α-glucosidase than α-amylase in the experimental part. The docking results also reveal the importance of hydroxyl groups of brazilin in binding. Especially, double hydroxyl groups on *o*-dihydroxylbenzene side appear to generate a significant hydrogen bond network.

CONCLUSION

In this work, the possibility of using other CS parts as an alternative for medicinal use is investigated. Most CS parts (heartwood of branches, heartwood, roots, barks of branches, barks, twigs, sapwood, florets, sapwood of branches, and seeds) exhibited higher antioxidant activity than standard agents. It is interesting that the heartwood of branches provides the highest antioxidant activity. Furthermore, not only the heartwood, but heartwood of branches also contains a high level of brazilin. This finding is important for future use of other CS parts to reduce waste and sustain resource utilization; however, the high cytotoxicity of this part must be considered before use. Moreover, CS is found to show high inhibition activities against α-amylase and especially α-glucosidase. Brazilin seems to prefer α-glucosidase to α-amylase due to higher number of glucosidase-brazilin hydrogen bonds. The findings in this study will be useful for alternative diabetes treatment. An insight here can serve as a guideline for sustainable resource utilization in the community.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2023.059.

Acknowledgements: We would like to thank the National Science and Technology Development Agency (NSTDA, Grant no. P-17-50720) and Kasetsart University Research and Development Institute (KURDI, Grant no. FF(KU) 25.64) for financial support.

REFERENCES

1. Bae IK, Min HY, Han AR, Seo EK, Lee SK (2005) Suppression of lipopolysaccharide-induced expression of inducible nitric oxide synthase by brazilin in RAW 264.7 macrophage cells. *Eur J Pharmacol* **513**, 237–242.
2. Jin SK, Ha SR, Choi JS (2015) Effect of *Caesalpinia sappan* L. extract on physico-chemical properties of emulsion-type pork sausage during cold storage. *Meat Sci* **110**, 245–252.
3. Kim Y, Shim H, Kim K (2016) Antimicrobial effect of *Caesalpinia sappan* L. extract on foodborne bacteria. *J Korean Soc Food Sci Nutr* **45**, 1026–1034.
4. Wan Y-J, Xu L, Song W-T, Liu Y-Q, Wang L-C, Zhao M-B, Jiang Y, Liu L-Y, et al (2019) The ethanolic extract of

Caesalpinia sappan heartwood inhibits cerebral ischemia/reperfusion injury in a rat model through a multi-targeted pharmacological mechanism. *Front Pharmacol* **10**, 29.
5. Nirmal NP, Rajput MS, Prasad RG, Ahmad M (2015) Brazilin from *Caesalpinia sappan* heartwood and its pharmacological activities: a review. *Asian Pac J Trop Med* **8**, 421–430.
6. Yan Y, Chen Y-c, Lin Y-h, Guo J, Niu Z-r, Li L, Wang S-b, Fang L-h, et al (2015) Brazilin isolated from the heartwood of *Caesalpinia sappan* L induces endothelium-dependent and-independent relaxation of rat aortic rings. *Acta Pharmacol Sin* **36**, 1318–1326.
7. Li CQ, Wang SF, Lu CC, Kang WY, Wang JM (2016) Research progress on chemical compounds and pharmacological effects of *Caesalpinia*. *Zhongguo Zhong Yao Za Zhi* **41**, 1773–1786.
8. Zhao H, Bai H, Wang Y, Li W, Koike K (2008) A new homoisoflavan from *Caesalpinia sappan*. *J Nat Med* **62**, 325–327.
9. Sasaki Y, Hosokawa T, Nagai M, Nagumo S (2007) *In vitro* study for inhibition of NO production about constituents of Sappan Lignum. *Biol Pharm Bull* **30**, 193–196.
10. Washiyama M, Sasaki Y, Hosokawa T, Nagai M (2009) Anti-inflammatory constituents of Sappan Lignum. *Biol Pharm Bull* **32**, 941–944.
11. Nirmal NP, Panichayupakaranant P (2014) Anti-*Propionibacterium acnes* assay-guided purification of brazilin and preparation of brazilin rich extract from *Caesalpinia sappan* heartwood. *Pharm Biol* **52**, 1204–1207.
12. Puttipan R, Chansakaow S, Khongkhunthian S, Okonogi S (2018) *Caesalpinia sappan*: a promising natural source of antimicrobial agent for inhibition of cariogenic bacteria. *Drug Discov Ther* **12**, 197–205.
13. Nathan VK, Rani ME (2021) Natural dye from *Caesalpinia sappan* L. heartwood for eco-friendly coloring of recycled paper based packing material and its *in silico* toxicity analysis. *Environ Sci Pollut Res* **28**, 28713–28719.
14. Sudirman J, Sampara N, Mawang S, Passe R, Aswan R, Ahmad M (2020) The analysis of reducing blood glucose levels of diabetics with diabetes mellitus by giving a secang wood stew (*Caesalpinia sappan* L.) to menopausal women in Makassar city. *Enferm Clin* **30**, 506–509.
15. Wediasari F, Nugroho GA, Fadhilah Z, Elya B, Setiawan H, Mozef T (2020) Hypoglycemic effect of a combined andrographis paniculata and caesalpinia sappan extract in streptozocin-induced diabetic rats. *Adv Pharmacol Pharm Sci* **20**, 8856129.
16. Chalopagorn P, Klomsakul P (2017) α-amylase and α-glucosidase inhibitory activities of *Caesalpinia sappan*, ficus foveolate and eurycoma longifolia extracts. *Phranakhon Raj Res J (Sci Technol)* **12**, 63–73. [in Thai]
17. BIOVIA (2021) Dassault Systémes. In: *Discovery Studio Visualizer. v21.1.0.20298*, Dassault Systémes, San Diego, CA, USA.
18. Verdonk ML, Cole JC, Hartshorn MJ, Murray CW, Taylor RD (2003) Improved protein-ligand docking using GOLD. *Proteins* **52**, 609–623.
19. Ananil K, Hudson JB, de Souza C, Akpagana K, Tower GH, Arnason JT, Gbeassor M (2000) Investigation of medicinal plants of togo for antiviral and antimicrobial activities. *Pharm Biol* **38**, 40–45.
20. Soni MG, Taylor SL, Greenberg NA, Burdock GA (2002) Evaluation of the health aspects of methyl paraben: a review of the published literature. *Food Chem Toxicol* **40**, 1335–1373.
21. Arsiningtyas I (2021) Antioxidant profile of heartwood and sapwood of *Caesalpinia sappan* L. tree's part grown in Imogiri nature preserve, Yogyakarta. *IOP Conf Ser: Earth Environ Sci* **810**, 012040.
22. Nirmal NP, Panichayupakaranant P (2015) Antioxidant, antibacterial, and anti-inflammatory activities of standardized brazilin-rich *Caesalpinia sappan* extract. *Pharm Biol* **53**, 1339–1343.
23. Sakir NA, Kim JG (2019) The effect of sappan wood extracts in treating diabetes induced in mice. *Makara J Health Res* **23**, 9.
24. Arsiningtyas I, Kato E, Kawabata J (2015) Antihyperglycemic principles of *Caesalpinia sappan* L. wood, through *in vitro* inhibition of carbohydrate digestive enzymes: α-glucosidase and α-amylase. *Planta Medica* **81**, PM_42.
25. Gong L, Feng D, Wang T, Ren Y, Liu Y, Wang J (2020) Inhibitors of alpha-amylase and alpha-glucosidase: potential linkage for whole cereal foods on prevention of hyperglycemia. *Food Sci Nutr* **8**, 6320–6337.
26. Kazeem M, Adamson J, Ogunwande I (2013) Modes of inhibition of α-amylase and α-glucosidase by aqueous extract of *Morinda lucida* Benth leaf. *BioMed Res Int* **2013**, 527570.
27. Tundis R, Loizzo MR, Menichini F (2010) Natural products as alpha-amylase and alpha-glucosidase inhibitors and their hypoglycaemic potential in the treatment of diabetes: an update. *Mini Rev Med Chem* **10**, 315–331.
28. Van De Laar FA, Lucassen PL, Akkermans RP, van de Lisdonk EH, Rutten GE, van Weel C (2005) α-glucosidase inhibitors for patients with type 2 diabetes: results from a Cochrane systematic review and meta-analysis. *Diabetes Care* **28**, 154–163.
29. Alqahtani AS, Hidayathulla S, Rehman MT, ElGamal AA, Al-Massarani S, Razmovski-Naumovski V, Alqahtani MS, El Dib RA, et al (2020) Alpha-amylase and alpha-glucosidase enzyme inhibition and antioxidant potential of 3-oxolupenal and katononic acid isolated from *Nuxia oppositifolia*. *Biomolecules* **10**, 61.
30. Agarwal P, Gupta R (2016) Alpha-amylase inhibition can treat diabetes mellitus. *Res Rev J Med Health Sci* **5**, 1–8.
31. Wu SQ, Otero M, Unger FM, Goldring MB, Phrutivorapongkul A, Chiari C, Kolb A, Viernstein H, et al (2011) Anti-inflammatory activity of an ethanolic C*aesalpinia sappan* extract in human chondrocytes and macrophages. *J Ethnopharmacol* **138**, 364–372.
32. Ngamwonglumlert L, Devahastin S, Chiewchan N, Raghavan GSV (2020) Color and molecular structure alterations of brazilein extracted from *Caesalpinia sappan* L. under different pH and heating conditions. *Sci Rep* **10**, 12386.
33. Artanti N, Dewijanti ID, Muzdalifah D, Windarsih A, Suratno S, Handayani S (2023) Alpha-glucosidase inhibitory activity of the combination of *Caesalpinia sappan* L. and *Garcinia mangostana* extract. *J Appl Pharm Sci* **13**, 189–198.

Appendix A. Supplementary data

Table S1 The percent yield of the aqueous and ethanolic extracts from *C. sappan*.

Part of *C. sappan*	% Yield	
	Aqueous extract	Ethanolic extract
Barks	3.90	0.44
Barks of branches	5.69	0.37
Seeds	8.81	4.40
Heartwood	2.12	5.53
Heartwood of branches	3.47	5.93
Leaves	13.4	9.00
Sapwood	0.63	0.40
Sapwood of branches	0.70	0.40
Twigs	1.66	0.06
Florets	15.4	13.5
Pods	4.30	2.26
Roots	2.62	1.23

Table S2 Regression analysis between brazilin content and biological activities.

Biological activities	Solvent	a	b	Adjusted R^2
Cytotoxicity CC_{50} (μg/ml)	Ethanolic	−1914.1	160.28	0.59
	Aqueous	−3.8468	0.1508	0.93
DPPH (IC_{50} mg/ml)	Ethanolic	−1.6688	0.0525	0.92
	Aqueous	−8.3534	0.1388	0.83
TEAC value (μg Trolox/mg)	Ethanolic	10486	179.72	0.75
	Aqueous	20269	242.06	0.98
α-Amylase IC_{50} (mg/ml)	Ethanolic	5.0881	0.1811	0.90
	Aqueous	−3.8468	0.1508	0.93
α-Glucosidase IC_{50} (mg/ml)	Ethanolic	−3.8557	0.1479	0.85
	Aqueous	−3.8468	0.1508	0.93

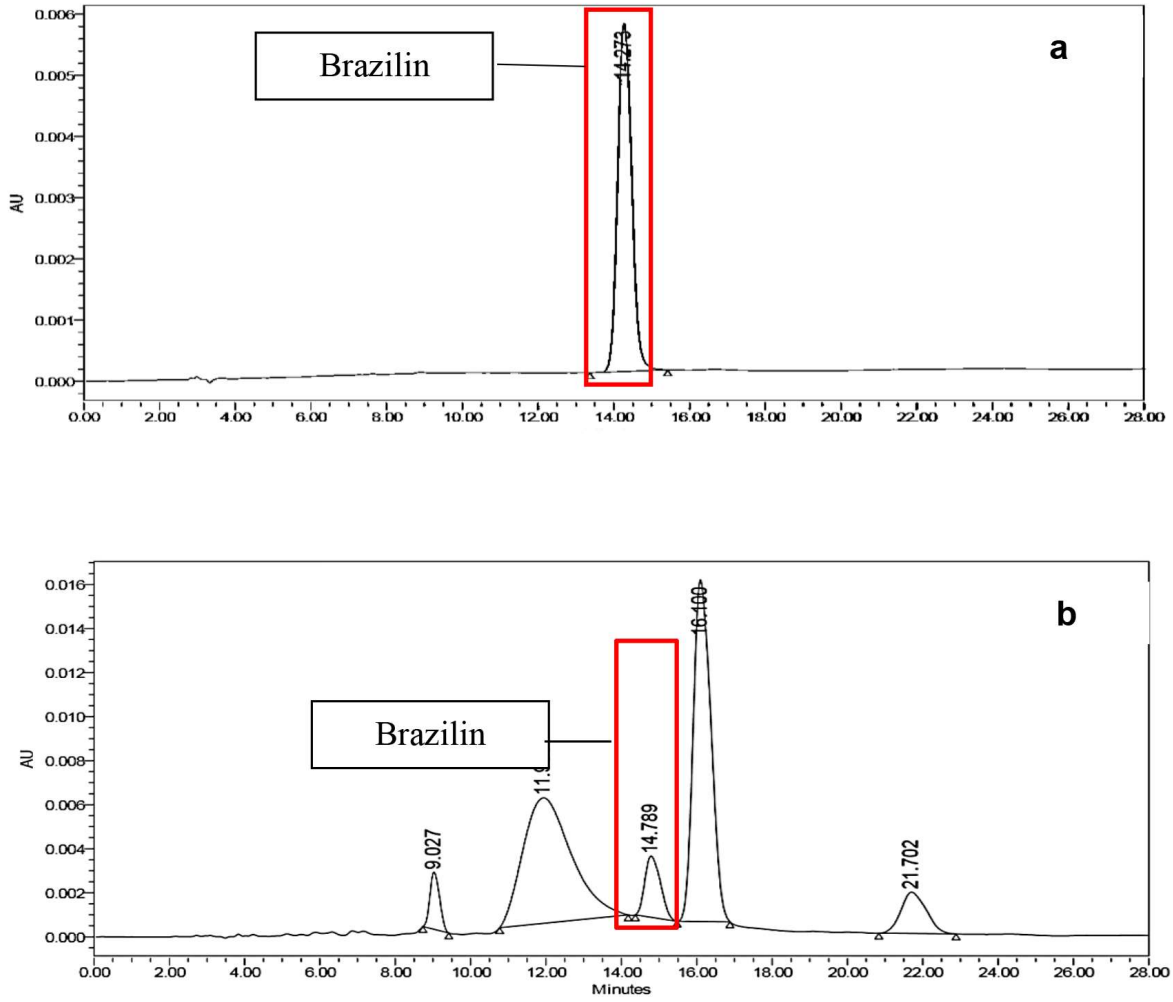

Fig. S1 Chromatogram of brazilin at concentration of 5 μg/ml (a) and chromatogram of the extract from heartwood of branches at concentration of 500 μg/ml (b).

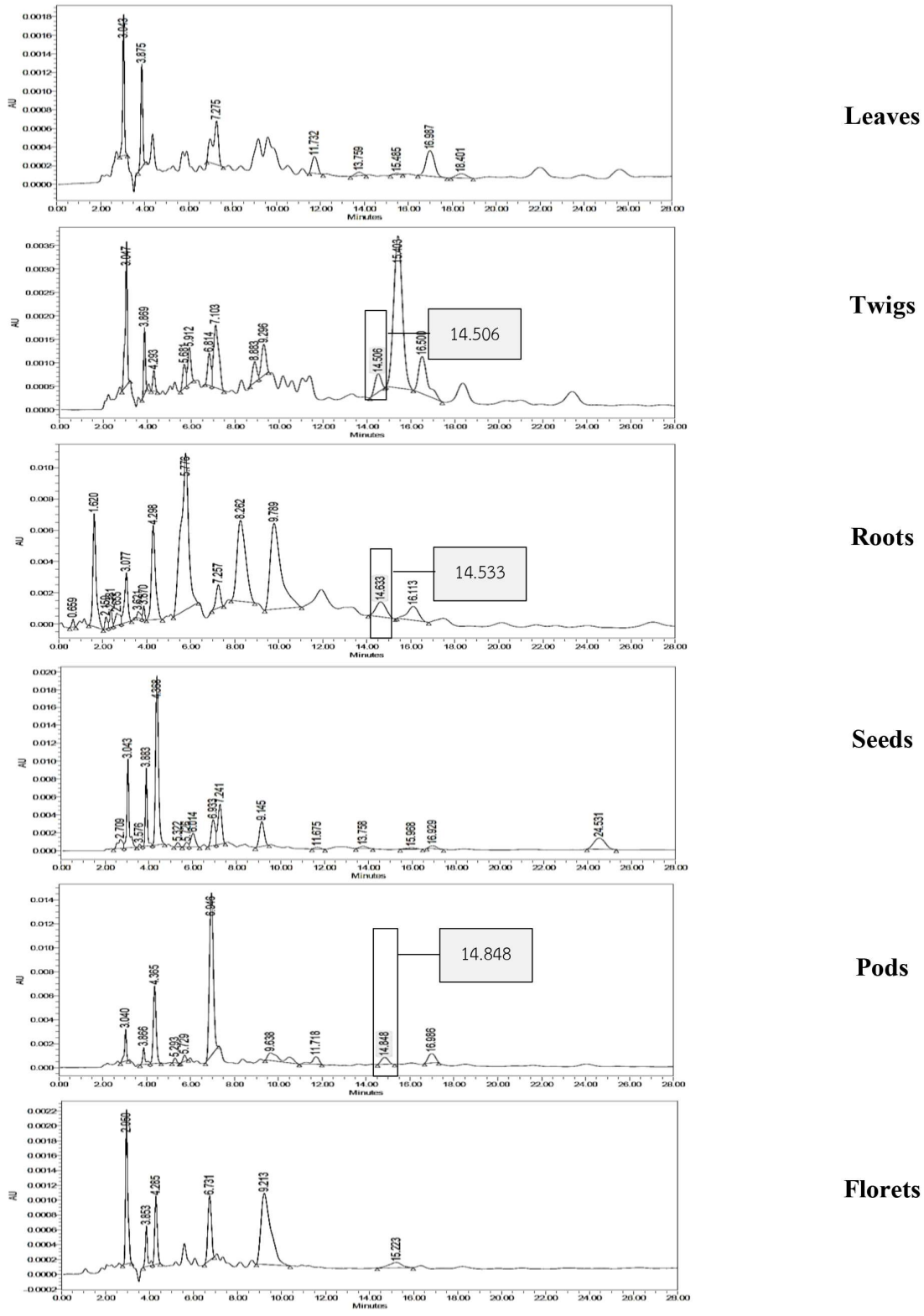

Fig. S2 Chromatogram of the crude ethanolic extract from leaves, twigs, roots, seeds, pods and floret.

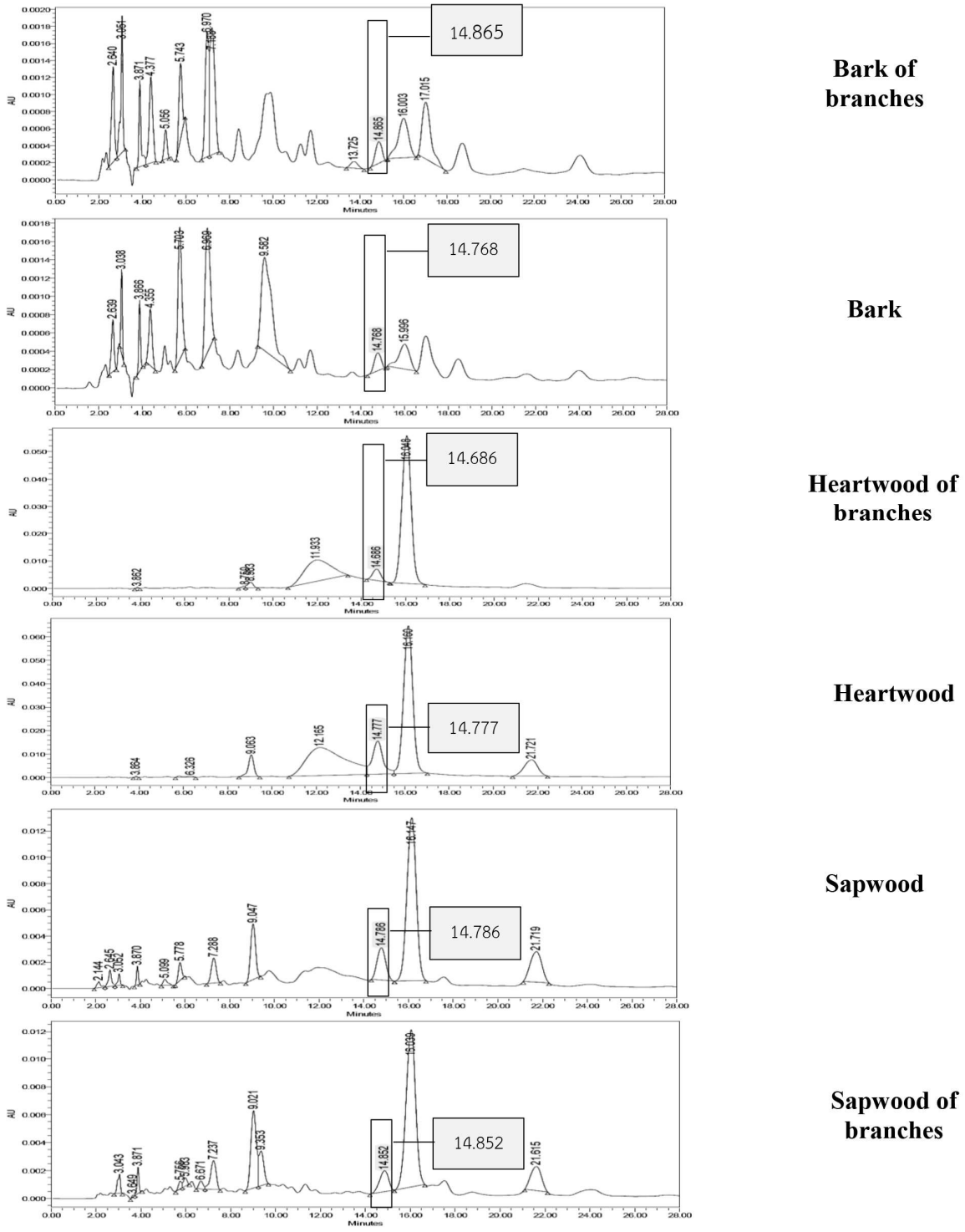

Fig. S3 Chromatogram of the crude ethanolic extract from bark of branches, bark, Heartwood of branches, Heartwood, Sapwood, Sapwood of branches.

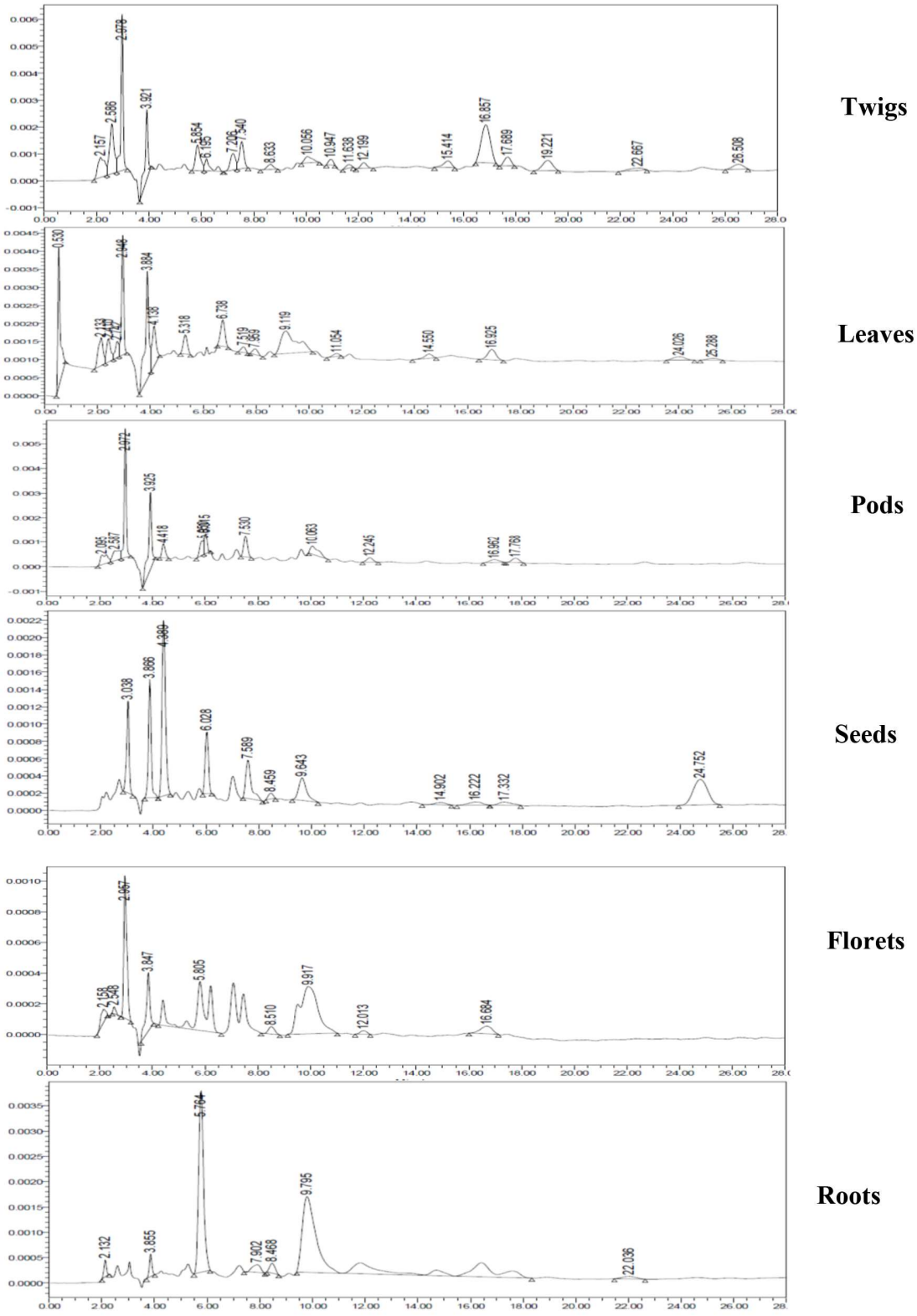

Fig. S4 Chromatogram of the crude aqueous extract from twigs, leaves, pods, seeds, florets and roots.

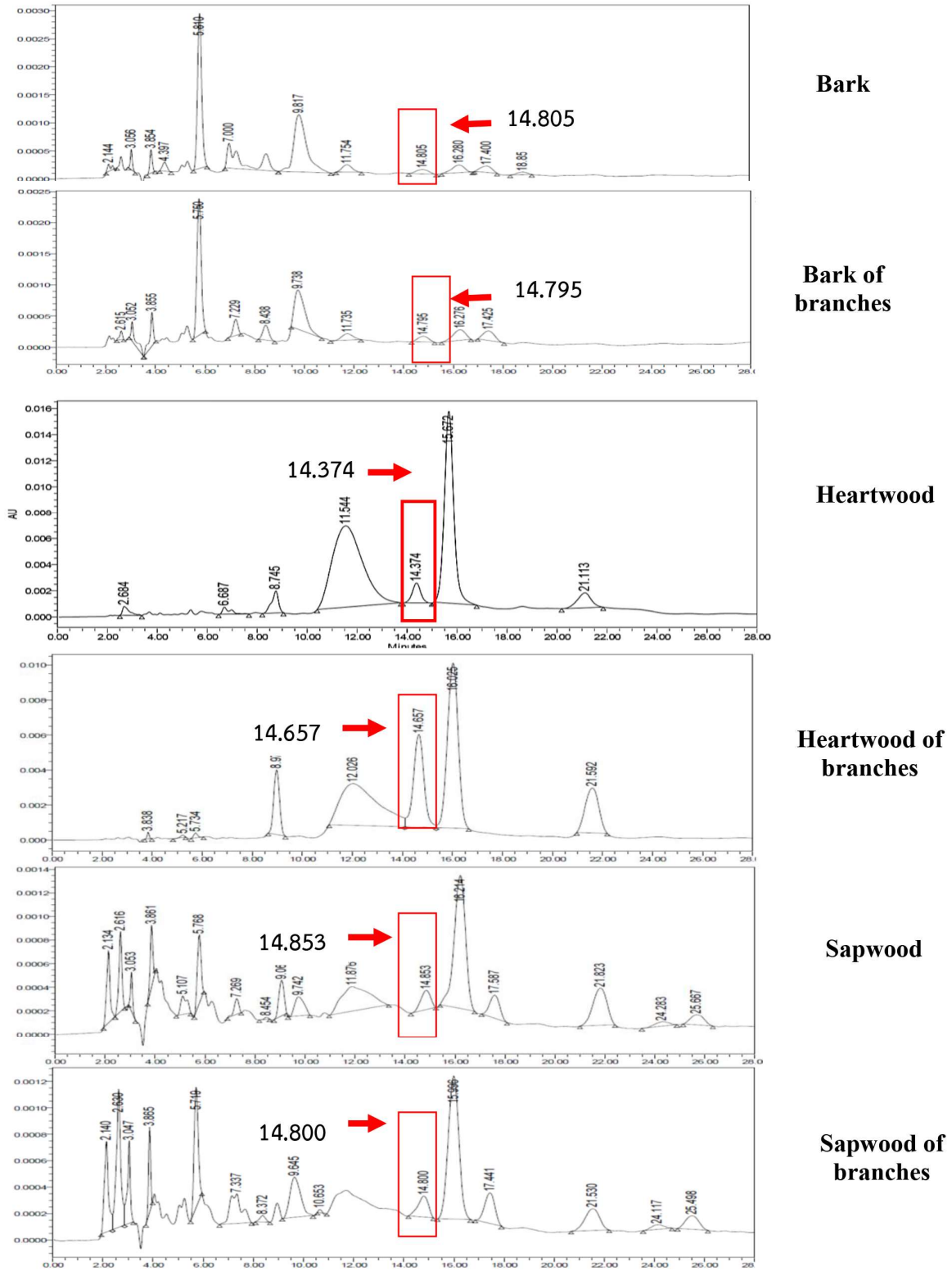

Fig. S5 Chromatogram of the crude aqueous extract from bark, bark of branches, heartwood, heartwood of branches, sapwood and sapwood of branches.