Application of Natural Deep Eutectic Solvents (NADES) for Sappan Wood (Caesalpinia sappan L.) extraction to test for inhibition of DPP IV activity

Heri SETIAWAN 1, Ivanna L. ANGELA 2, Nela ROHMAH 2, Ofiati WIJAYA 2, Abdul MUN’IM 2,3* 5

1 Laboratory of Pharmacology, Faculty of Pharmacy, Health Sciences Cluster Building, Universitas Indonesia, Depok, 16424 Jawa Barat, Indonesia.
2 Laboratory of Pharmacognosy-Phytochemistry, Faculty of Pharmacy, Health Cluster Sciences, Universitas Indonesia, Depok, 16424 Jawa Barat Indonesia.
3 Graduate Program of Herbal Medicine, Faculty of Pharmacy, Universitas Indonesia, Depok, 16424, Jawa Barat Indonesia.
* Corresponding Author. E-mail: munim@farmasi.ui.ac.id (A.M.); Tel. +62-7884-90 01.

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ABSTRACT: Some natural deep eutectic solvents (NADES) can replace organic solvents that are toxic and harmful to the environment. The main flavonoid in sappan wood (Caesalpinia sappan L.) is brazilin. The aims of this study were to extract brazilin from sappan wood by using NADES and to evaluate the effect of the extract on inhibition of dipeptidyl peptidase IV (DPP IV) activity. The selected NADES compositions used choline chloride and betaine as hydrogen bond acceptors (HBA) with glycerol, xylitol, sorbitol, lactic acid, malic acid, and citric acid as various hydrogen bond donors (HBD). Ultrasound-assisted NADES extraction of brazilin was performed, and the content was measured by high-performance liquid chromatography (HPLC). Between selected NADES, betaine–lactic acid gave the highest brazilin content of 4.49 mg/g. This result was comparable to conventional methods by a reflux using 96% ethanol that gave brazilin content of 5.43 mg/g. However, the reflux required 3 hours for three extraction cycle, while optimal extraction conditions by betaine–lactic acid were 30 min. The sappan wood extract using choline chloride–lactic acid had a percent inhibition of 5.72% (2.03 ppm), and that using betaine–lactic acid had a percent inhibition of 7.74% (2.08 ppm) for DPP IV activity. These inhibitory activities were comparable to the brazilin standard which was 8.93% (2.2 ppm). Our results showed that two NADES compositions could extract brazilin from sappan wood and it showed potential DPP IV inhibitory activity.

KEYWORDS: Brazilin; Caesalpinia sappan L.; dipeptidyl peptidase IV; natural deep eutectic solvents; ultrasound-assisted extraction.

1. INTRODUCTION

Natural deep eutectic solvents (NADES) can be used as alternatives to replace organic solvents that are toxic, flammable, and harmful to the environment. NADES use primary metabolites, such as organic acids, sugars, amino acids, and sugar alcohols, that are formulated as a eutectic mixture with an ammonium salt (such as choline chloride) and hydrogen bond donors (such as urea and glycerol) that give a NADES melting point lower than each component’s melting point [1]. NADES has various advantages, such as biodegradability, sustainability, simple preparation, low toxicity and low cost [2].

Sappan wood (Caesalpinia sappan L.) is a Brazilian plant that is referred to as brazil wood. The wood is grown in India, Malaysia, and Indonesia. Sappan can be found in tropical climates at an altitude of 500–1000 m above sea level. Dried sappan wood is known as Sappan Lignum, is odorless, and has a slightly astringent taste [3]. Brazilin, sappan wood major compound and important bioactive, have long been utilized in food and pharmaceutical industry, its domestic and global market demand is established. Brazilin is an antioxidant compound that has a catechol in its chemical structure. The chemical formula of brazilin is C_{16}H_{24}O_{5}, and it has a crystal-like shape. Brazilin is slightly soluble in water; easily soluble in hot water, alcohol, and ether; and soluble in alkaline hydroxy solutions. Traditionally, water extract of sappan wood has been used to treat...
diabetes complications in Southeast Asia. It has been reported that brazilin has hypoglycemic activity through several biomolecular mechanisms [4].

Regarding diabetes, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotrophic polypeptide (GIP) stimulate insulin release in a glucose-dependent manner, thus it helps to maintain normal glucose levels. However, their half-lives in the body are short because of degradation by dipeptidyl peptidase IV (DPP IV). DPP IV inhibitors can prolong the half-lives of GLP-1 and GIP; therefore, inhibition of DPP IV activity is one of the targets for treatment of diabetes type 2 [5]. In a previous study, 100 ppm of sappan wood extract had DPP IV inhibition activity of 84.24% [6].

In this study, NADES was used as a solvent to extract sappan wood by ultrasound-assisted extraction (UAE). Brazilin content from NADES sappan wood extract (NADES-UAE) was compared with brazilin content obtained from conventional methods’ extractions. The NADES-UAE extract of sappan wood was then used to assess the inhibition of DPP IV activity. We also describe the validation of a high-performance liquid chromatography (HPLC) method for determining brazilin content in the extract.

2. RESULTS AND DISCUSSION

2.1. HPLC method validation

Method validation holds an important aspect of the quality assurance of pharmaceutical products. In Table 1, validation of HPLC method by linearity, precision, stability, and recovery were shown. The linear regression equation for the brazilin standard was \( y = 928.3 + 21446x \), with a correlation coefficient \( r = 0.9994 \). The \( r \)-value was \( \geq 0.9990 \), demonstrating a high degree of correlation and good linearity of the method. The percentage of recovery was 106.44\%%, while the intra-day and inter-day precision had %RSD values of 0.66\% and 0.73\% respectively. Signal-to-noise ratio of 3 for LOD was 0.24 µg/mL, and ratio of 10 for LOD was 0.79 µg/mL. The results indicated that the HPLC method used was sensitive and reliable for our current purpose and sample analysis. Moreover, the percentage RSD values of the retention times, peak areas and tailing factor were < 2\%, indicating an acceptable symmetry of the analyte peak and good precision for repeated injections. The chosen method suitability parameters complied with the International Conference on Harmonization [7] and AOAC guidelines [8].

![Table 1. Validation parameters of the HPLC method.](https://doi.org/10.35333/jrp.2020.160)

| Validation Parameter | Result             |
|----------------------|--------------------|
| Calibration Curve    | \( y = 928.3 + 21446x \) |
| \( R^2 \)            | 0.9994             |
| LOD (µg/mL)          | 0.24               |
| LOQ (µg/mL)          | 0.79               |
| Recovery, \( n = 6 \) (Mean ± SD, %) | 106.44 ± 1.2       |
| Area (% RSD)         | 1.1                |
| Retention time (% RSD) | 0.47              |
| Tailing factor (% RSD) | 0.34              |
| Intra-day precision (% RSD) | 0.66            |
| Inter-day precision (% RSD) | 0.73            |

2.2. Screening of optimal NADES

Sappan wood was extracted by using NADES solvents and the UAE method with a liquid : solid ratio of 20 mL/g. In this study, nine types of NADES were used to extract brazilin from sappan wood (Table 2). UAE was chosen as an extraction method because it has been shown previously to be effective, simple, and fast in extracting products from natural ingredients [9].

Our screening results showed that NADES consisting of choline chloride–lactic acid showed that the highest brazilin content was 4.28 mg/g. However extraction with betain–lactic acid also showed a comparable high yield of brazilin at 4.09 mg/g (Figure 1.A). NADES-chloride–lactic acid provided high content because it had the lowest surface tension among the other NADES types. Low surface tension will enable a solvent to more easily penetrate the sample matrix [10]. Lactic acid has a simple chemical structure so it has small steric resistance. The more steric resistance during formation of NADES means that the NADES formed will have a high surface tension, so higher energy input is needed in the active compound to interact with the chloride.
ion in NADES. The NADES viscosity also affected the content of brazilin. The viscosity is higher if one of the NADES components has more hydroxyl groups that act as hydrogen bond donors, thus creating more hydrogen bonds formed in the eutectic mixture [11].

Table 2. Different types of NADES.

| No | NADES Combination       | Hydrogen bond acceptor | Hydrogen bond donor | Molar Ratio |
|----|-------------------------|------------------------|---------------------|-------------|
| 1  | Choline chloride        | Xylitol                |                     | 4:1         |
| 2  | Choline chloride        | Glycerol               |                     | 1:2         |
| 3  | Choline chloride        | Sorbitol               |                     | 1:2         |
| 4  | Choline chloride        | Lactic acid            |                     | 1:1         |
| 5  | Choline chloride        | Malic acid             |                     | 1:1         |
| 6  | Choline chloride        | Citric acid            |                     | 1:1         |
| 7  | Betaine                 | Lactic acid            |                     | 1:1         |
| 8  | Betaine                 | Malic acid             |                     | 1:1         |
| 9  | Betaine                 | Citric acid            |                     | 1:1         |

2.3. Analysis of brazilin in samples

The results of the analysis showed that the sappan wood extract (NADES-UAE) had several peaks at different retention times. The peak for brazilin in the sample had a retention time of 7.3 min, which matched the retention of brazilin in the standard chromatogram (Figure 1.B and 1.C).

Figure 1. (A) Brazilin content based on NADES type, chromatograms of (B) brazilin standard and (C) example NADES-UAE extract of sappan wood.

2.4. Optimization of extraction conditions

The extraction conditions involved two factors: the extraction time and the percentage of water added. The sample: solvent ratio was fixed at 1:20. The response data showing average brazilin contents obtained are presented in Table 3. The highest brazilin content was 4.49 mg/g extracted by betain-lactic acid under the conditions of 60% water addition and 30 min of extraction time (run 2). Extraction by choline chloride-lactic acid yielded similar brazilin content as the screening test with 40% of water addition and 30 min of extraction time (run 10).

In the experimental design software, an ANOVA test was also performed to determine the interaction between factors in the study. As shown in Table 4, the p-value of the model were 0.0018 for betain-lactic acid and 0.0003 for choline chloride-lactic acid. A p-value of <0.05 was taken to indicate that a factor gave a significantly different effect. The models that gave significantly different effects were A (extraction time), B (% addition of water), and B² (square of percentage water addition).

The p-value of the lack of fit test (0.3657 and 0.0612) were > 0.05, which indicated that the model was not significant. However, a non-significant model of a lack of fit was desired because that indicates that the chosen model was correct. The coefficient of determination (R²) obtained were 0.905 and 0.9428, which show...
that 90.5% and 94.28% of the sample variables were influenced by independent variables for extraction by betain-lactic acid and choline chloride-lactic acid respectively. The ideal three-dimensional model from RSM resembled the shape of an umbrella. On the basis of the picture shown in Figure 2, we concluded that there was no tendency for an interaction effect between two variables on the contents of brazilin in sappan extract.

Table 3. Brazilin content from sappan wood extracts obtained using choline chloride–lactic acid (left) and betain–lactic acid (right) as a solvent.

| Run | Factor 1 Water addition (%) | Factor 2 Extraction time (minutes) | Choline chloride-Lactic acid Brazilin content Mean ± SD (mg/g) | Factor 1 Water addition (%) | Factor 2 Extraction time (minutes) | Betain-Lactic acid Brazilin content Mean ± SD (mg/g) |
|-----|-----------------------------|-----------------------------------|-------------------------------------------------------------|-----------------------------|-----------------------------------|--------------------------------------------------|
| 1   | 60                          | 50                                | 3.97 ± 0.04                                                 | 50                          | 30                                | 3.98 ± 0.09                                      |
| 2   | 60                          | 30                                | 3.93 ± 0.01                                                 | 60                          | 30                                | 4.49 ± 0.004                                    |
| 3   | 40                          | 10                                | 2.86 ± 0.03                                                 | 60                          | 50                                | 3.90 ± 0.05                                    |
| 4   | 40                          | 50                                | 4.03 ± 0.07                                                 | 60                          | 30                                | 4.25 ± 0.12                                    |
| 5   | 20                          | 30                                | 1.74 ± 0.06                                                 | 70                          | 50                                | 4.11 ± 0.01                                    |
| 6   | 20                          | 50                                | 2.97 ± 0.04                                                 | 70                          | 30                                | 4.25 ± 0.03                                    |
| 7   | 20                          | 10                                | 1.43 ± 0.01                                                 | 50                          | 50                                | 3.67 ± 0.03                                    |
| 8   | 40                          | 30                                | 4.07 ± 0.01                                                 | 60                          | 30                                | 4.11 ± 0.03                                    |
| 9   | 40                          | 30                                | 4.15 ± 0.01                                                 | 60                          | 30                                | 4.14 ± 0.04                                    |
| 10  | 40                          | 30                                | 4.28 ± 0.01                                                 | 60                          | 10                                | 3.61 ± 0.01                                    |
| 11  | 40                          | 30                                | 3.89 ± 0.01                                                 | 70                          | 10                                | 3.36 ± 0.06                                    |
| 12  | 40                          | 30                                | 4.01 ± 0.001                                                | 60                          | 30                                | 4.07 ± 0.08                                    |
| 13  | 60                          | 10                                | 3.31 ± 0.01                                                 | 50                          | 10                                | 2.77 ± 0.013                                   |

Table 4. ANOVA results for brazilin content.

| Source of Variation | Sum of Squares | df | Mean Square | F-value | p-value |
|---------------------|----------------|----|-------------|---------|---------|
| Choline chloride-Lactic acid | 9.78 | 5 | 1.96 | 23.06 | 0.0003 | Significant |
| Model               | 4.22 | 1 | 4.22 | 49.73 | 0.0002 |         |
| A-Water Addition (%) | 1.89 | 1 | 1.89 | 22.32 | 0.0021 |         |
| B-Extraction Time (min) | 0.1936 | 1 | 0.1936 | 2.28 | 0.1745 |         |
| AB                  | 0.6338 | 1 | 0.6338 | 25.63 | 0.0015 |         |
| A²                  | 0.2437 | 1 | 0.2437 | 2.87 | 0.1338 |         |
| B²                  | 0.5936 | 7 | 0.0848 |  |  |         |
| Residual            | 0.2364 | 3 | 0.1692 | 7.87 | 0.0612 | Not significant |
| Lack of Fit         | 0.0860 | 4 | 0.0215 |  |  |         |
| Pure Error          |  |  |  |  |  |         |
| Betain-Lactic acid  | 2.25 | 5 | 0.4503 | 13.34 | 0.0018 | Significant |
| Model               | 0.3128 | 1 | 0.3128 | 9.26 | 0.0188 |         |
| A-Water Addition (%) | 0.6338 | 1 | 0.6338 | 18.77 | 0.0034 |         |
| B-Extraction Time (min) | 0.0055 | 1 | 0.0055 | 0.1616 | 0.6997 |         |
| AB                  | 0.1086 | 1 | 0.1086 | 3.22 | 0.1160 |         |
| A²                  | 0.7807 | 1 | 0.7807 | 23.12 | 0.0019 |         |
| B²                  | 0.2364 | 7 | 0.0338 |  |  |         |
| Residual            | 0.1210 | 3 | 0.0403 | 1.40 | 0.3657 | Not significant |
| Lack of Fit         | 0.1154 | 4 | 0.0288 |  |  |         |
| Pure Error          |  |  |  |  |  |         |
2.5. Comparison of brazilin content in conventional extraction and NADES-UAE

Conventional extraction was performed by applying the maceration method using 80% ethanol and 96% ethanol and performing a reflux method using 95% ethanol. The brazilin content was 3.12 mg/g from maceration using 80% ethanol, 4.58 mg/g from maceration with 96% ethanol, and 5.43 mg/g from reflux. The brazilin content of sappan wood extract from reflux gave the highest value. From Table 5, the brazilin content from reflux gave a higher value than that from the NADES-UAE extract, but considering the extraction time and amount of solvent used, the extraction using NADES-UAE was more effective and efficient. Regarding extraction time, the reflux took 3 h for three extraction cycles, whereas the NADES-UAE took only 30 minutes for extraction. The amount of solvent used for the NADES-UAE extraction was also less than that used in the reflux method.

![Figure 2: 3D model of interaction between experimental factors and responses, (A) choline chloride-lactic acid, and (B) betaine-lactic acid.](https://doi.org/10.35333/jrp.2020.160)

**Table 5.** Comparison of brazilin content from the extraction methods used.

| Extraction Method            | Extraction Time       | Solvent Amount for Experimental Scale | Brazilin Content Mean ± SD, mg/g |
|------------------------------|-----------------------|--------------------------------------|----------------------------------|
| UAE, choline chloride-lactic acid | 30 minutes           | 10 mL                                | 4.28 ± 0.09                      |
| UAE, betaine-lactic acid     | 30 minutes            | 10 mL                                | 4.49 ± 0.004                     |
| Reflux, 96% ethanol          | 1 hour × 3 cycles     | 500 mL × 3 cycles                    | 5.43 ± 0.02                      |
| Maceration, 80% ethanol      | 24 hours × 3 cycles   | 300 mL × 3 cycles                    | 3.12 ± 0.08                      |
| Maceration, 96% ethanol      | 24 hours × 3 cycles   | 300 mL × 3 cycles                    | 4.58 ± 0.04                      |

2.6. Inhibition of DPP IV activity

Sitagliptin used as a positive control gave 90.43% inhibition of DPP IV activity, and the brazilin standard (2.2 ppm) gave 8.93% inhibition. The DPP IV activity inhibition values by the sappan wood extract from choline chloride-lactic acid were 4.54%, 5.01%, and 5.72%, respectively, for three concentrations of the extract, and those by the extract from betaine-lactic acid gave inhibition values of 5.70%, 6.72%, and 7.74%, respectively, for three concentrations of the extract (Table 6). The percent inhibition of the extracts against DPP IV activity increased with increasing brazilin content of the extract included in the enzymatic reaction. Extracts obtained by NADES betaine-lactic acid provided higher percent inhibition values than those of the extract obtained by NADES choline chloride-lactic acid. Inhibition of DPP IV activity by the sappan wood extract is probably caused by an enzyme-inhibitor bond. The enzyme-inhibitor bond can cause a decrease in enzyme activity because the active site of the enzyme is occupied and cannot bind as much substrate [12].

The effect of NADES itself to the DPP IV need to be controlled, to avoid bias on the effect from sappan extract. Our result of DPP IV activity inhibition by sappan extract was obtained after subtraction of the NADES effect to the enzymatic reaction. NADES can have varying effects on enzyme activity depending on several factors, including protein structure and catalytic mechanisms [11]. In our study, by comparing the activity of the enzyme in the presence of the NADES and after the individual components of the NADES were added,
we concluded that these activation and stabilization effects were caused by the NADES itself rather than by the presence of its separate components [13].

### Table 6. Brazilin content of the extracts used in DPP IV activity inhibition test.

| Brazilin content (ppm) | Choline chloride–Lactic acid extract (% Inhibition) | Betaine–Lactic acid extract (% Inhibition) | Brazilin standard (% Inhibition) |
|------------------------|-----------------------------------------------|-----------------------------------------|-------------------------------|
|                        | Mean ± SD                                      | Mean ± SD                                | Mean ± SD                      |
| 0.60                   | 4.54 ± 0.01                                    | 0.73                                    | 7.74 ± 0.28                   |
| 1.60                   | 5.01 ± 0.04                                    | 1.61                                    | 6.72 ± 0.29                   |
| 2.03                   | 5.72 ± 0.01                                    | 2.08                                    | 8.93 ± 1.26                   |

### 3. CONCLUSION

NADES consisting of betaine–lactic acid was able to extract brazilin from sappan wood with a higher content than the brazilin from other types of NADES. The optimal conditions for the extraction of sappan wood using NADES choline chloride–lactic acid were 60% addition of water and a 30 min extraction time, which gave a yield of 4.49 mg/g brazilin content. Sappan wood extracts from the two NADES, choline chloride–lactic acid and betaine–lactic acid, showed a high percentage of inhibition against DPP IV activity and were comparable to the brazilin standard.

### 4. MATERIALS AND METHODS

#### 4.1. Materials

Dried powder of sappan wood (Caesalpinia sappan L.) was obtained from Badan Penelitian Tanaman Rempah dan Obat (BALITTRO), Bogor, West Java. The following other materials were used: choline chloride (Xi’an Rongsheng, China), betaine (Shandong Ruihong Technology, China), glycerol (PT Molex Ayus, Indonesia), sorbitol (Dow Chemical, Singapura), xylitol (Zhejiang Huakang Pharmaceutical, China), lactic acid (Brataco, Indonesia), malic acid (Brataco), citric acid (Brataco), demineralized water (Brataco), aqua pro injection (IKA Pharmaceutical, Germany), methanol HPLC grade (Merck, Germany), acetonitrile HPLC grade (Merck), glacial acetic acid (Merck), brazilin standard (Sigma Aldrich, USA), 96% ethanol (Brataco), and DPP IV kit (Cayman Chemical, USA).

#### 4.2. Equipment

Ultrasonic bath (Krisbow), digital scales (Vibra HT), hotplate stirrer (IKA C-MAG), HPLC (Shimadzu LC-20AT, Japan), column C18 4.6 mm × 150 mm with pore size10 nm (Inertsil, Japan), micropipette 10–100 μL and 100–1000 μL (Socorex, Switzerland), microsyringe (Hamilton, USA), microplate reader (GloMax Promega, USA), rotary vacuum evaporator (Buchi, Indonesia), centrifuge (Hettich Zentrifugen, German), micropore 0.45 μm (Whatman, USA), syringe filter (Agilent, USA), and glassware (Iwaki Pyrex, Japan).

#### 4.3. Preparation of NADES

In this study, NADES was prepared by heating the hydrogen bond acceptor and hydrogen bond donor followed by stirring on a hotplate stirrer [14]. The NADES combinations used were choline chloride–xylitol at a ratio of 4:1; choline chloride–glycerol and choline chloride–sorbitol at a ratio of 1:2; and choline chloride–lactic acid, choline chloride–malic acid, choline chloride–citric acid, betaine–lactic acid, betaine–malic acid, and betaine–citric acid at a ratio of 1:1. The temperatures used for heating were 50 °C–80 °C. The molar ratio and temperature used in making NADES were those used in studies by Espino et al, 2016 [2].

#### 4.4. Conventional extraction

Sappan wood powder was extracted by using reflux with 95% ethanol, maceration with 80% ethanol, and maceration with 96% ethanol. In the reflux method, 25 g of sappan wood powder was extracted with 500 mL of 95% ethanol. In the maceration method, 15 g of sappan wood powder was extracted with 300 mL of 80% ethanol, and 10 g of sappan wood powder was extracted with 220 mL of 96% ethanol. Furthermore, the extraction filtrates were collected and evaporated on a rotary vacuum evaporator [4; 6; 15].
4.5. Experimental design

Response surface methodology (RSM) Design Expert 11.0 software (Stat Ease, USA) was used to design this experiment. The experimental factors are shown in Table 7. All experimental factors were input to the software to generate the experimental design with different conditions in total 13 extractions (13 runs).

| Extraction Condition | Level     |
|----------------------|-----------|
|                      | Low       | Medium  | High    |
| Water addition (%)   | 20        | 40      | 60      |
| Extraction time (minute) | 10       | 30      | 50      |

4.6. Extraction using NADES-UAE under RSM conditions

Sappan wood powder inside a vial was mixed with NADES at a 1:20 sample-to-solvent ratio. The sample mixture was extracted by using an ultrasonic bath with different extraction times according to the conditions given in the experimental design. The extraction solution was centrifuged at 4500 rpm for 17 min. The extract was filtered through filter paper and then stored in a vial.

4.7. Chromatographic conditions

In this study, the chromatographic system used was a Shimadzu LC-20AT HPLC with 0.3% acetic acid : acetonitrile (85.5:14.5) as the mobile phase. Ultraviolet–visible detection at a wavelength of 280 nm, an Inertsil C18 column (4.6 × 150 mm), a flow rate of 1 mL/min, and an injection volume of 20 μL were also used [6].

4.8. HPLC method validation

4.8.1. System suitability test

A standard solution of brazilin was injected six times, and the percentage relative standard deviations (%RSDs) for retention time, peak area, and tailing factor were calculated.

4.8.2. Linearity

Brazilin standard solutions were prepared in 80% ethanol at six concentrations (0.5, 0.75, 1, 1.5, 4, and 8 ppm). The linearity of the standard concentration range was determined from the correlation coefficient (r) value obtained from the equation derived from the standard calibration curve. The calibration curve was made by plotting the area of the brazilin peak from each standard solution on the chromatogram on the y-axis and standard concentrations on the x-axis.

4.8.3. Accuracy

Aliquots from the standard solution were added (spiked) to the sample. The spiked samples' concentrations were compared with those of the unspiked samples. The six spiked samples and six unspiked samples were analyzed by HPLC.

4.8.4. Precision

The inter-day and intra-day precision of the concentrations of the six samples were determined. The intra-day precision test was performed by analyzing the brazilin content in six samples on each of 2 days.

4.8.5. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ values were calculated according to the following equation.

\[
LOD = \frac{(3 \times Sb)}{Sl}
\]

[Eq. 1]

\[
LOQ = \frac{(10 \times Sb)}{Sl}
\]

[Eq. 2]

where Sl was the slope (b in the linear regression equation \( y = a + bx \)) and Sb was the standard deviation of the blank analytical response.
4.9. Brazilin determination

Standard solutions were injected (20 μL) into the HPLC system. The areas of the different standard solution concentrations were plotted to obtain a calibration curve from which a linear regression equation was derived. The standard solutions were made by dissolving 100 μg standard into 1 mL of 80% ethanol to obtain 100 ppm stock solution. The stock solution was diluted to make a 10 ppm solution and then diluted to give various concentrations (0.5, 0.75, 1, 1.5, 4, and 8 ppm).

A total of 100 μL NADES extract of sappan wood was dissolved in 80% ethanol to give a total volume of 10 mL. The solution was shaken until homogeneous and filtered through a 0.45 µm syringe filter and stored in a tightly closed vial protected from light. Conventional extract (±1 mg) dissolved in 80% ethanol to 10 mL, shaken until homogeneous, and then filtered through a 0.45 µm syringe filter.

4.10. DPP IV activity inhibition test

This study used a DPP IV Inhibitor Screening Assay Kit (Cayman Chemical, USA). The DPP IV activity test was performed by using fluorogenic methods with a glycine-proline-amino methyl coumarin substrate. Fluorescence was measured by using a Glomax at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Details of the composition and volume of the solution included in the well are followed the instruction manual of the kit. The rate of measured normal activity minus inhibited activity, divided by the rate of normal activity of a given object. It is expressed as a percentage, which is expressed as

\[
\text{Inhibition (\%)} = \frac{(\text{normal activity} - \text{inhibited activity})}{(\text{normal activity})} \times 100\% \quad \text{[Eq. 3]}
\]

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