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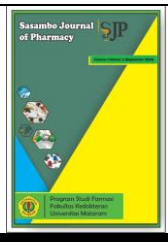

Influence of extraction method on total phenolic content and antioxidant activity of Sappan Wood (*Caesalpinia sappan* L.) extract

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Abstract: Sappan wood is a botanical species that contains a high concentration of phenolic compounds, which contribute to several pharmacological properties, including antidiabetic, antibacterial, antioxidant, and wound-healing activities. In order to achieve phenolic compounds of high quality and quantity, it is crucial to select an appropriate extraction procedure. Plant extraction is an important step for chemical isolation, chemical analysis, and evaluating the biological and pharmacological activities of plant compounds. Therefore, determining the most favorable extraction conditions is a critical undertaking in order to maximize both the quantity of active plant compounds and the extraction yield. This research aimed to compare extraction methods based on total phenolic content (TPC) and antioxidant activity (IC_{50}). The extraction techniques employed include maceration, stirring-assisted extraction (SAE), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE). The results show TPC values for maceration, SAE, UAE, and MAE, which are 132.85±1.44, 150.99±1.69, 206.70±6.56, and 115.70±1.44 mg GAE/g DE, respectively. The DPPH antioxidant activity (IC_{50}) values are 32.33±2.64, 25.01±0.34, 20.68±0.29, and 29.18±0.70 µg/mL, respectively. The research findings indicate that various extraction procedures can impact the extraction yield, total phenolic content (TPC), and antioxidant activity (IC_{50}) of sappan wood, and UAE is the best extraction method.

Keywords: Antioxidant; *Caesalpinia sappan*; Extraction method; Total phenolic content; Ultrasound assisted extraction.

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Introduction

Herbal beverages are rich in phytochemical antioxidants, which have the potential to mitigate the risk factors associated with certain diseases and aid in the treatment of non-communicable ailments (Suksaeree et al., 2022). A dietary intake of fruits and vegetables rich in antioxidants has been linked to a decreased risk of numerous chronic diseases, including cardiovascular disease and diabetes, according to a recent study. Phenolic compounds derived from plants have antioxidant properties (Nurul Mahmudati et al., 2022).

Sappan wood is an example of a phenolic plant. Sappan wood is a member of the Leguminosae family of plants. It is cultivated throughout Southeast Asia, Africa, and the Americas, where it is widely dispersed. C. sappan wood is composed of alkaloids, phenolics, flavonoids, and glycosides, among other phytochemicals (Suwan et al., 2018). Sappan wood (Sappan) is customarily ingested as a botanical beverage in Indonesia due to the perceived health advantages it offers. Antibacterial, blood tonic, expectorant, antimalarial, anti-inflammatory, and wound healing properties, as well as immunomodulatory, astringent,

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antitumor, expectorant, and antioxidant properties (Nguyen et al., 2020; Purba et al., 2023; Putri & Sabila, 2021; Vij et al., 2023).

Antioxidants are chemical compounds that possess the ability to impede, avert, or counteract the lipid oxidation process, which is detrimental to cellular integrity. Antioxidant compounds function by transferring electron pairs to atoms or molecules that possess unpaired electrons in exchange for one electron donation. Antioxidants also serve as compounds capable of forming bonds with free radicals within the body (Vardhani, 2019). Sappanchalcone, brazilin, and flavonoids, which were effectively isolated from secondary metabolites of *C. sappan* wood, exhibit antioxidant properties. These compounds chelate iron ions and reduce superoxide anion radicals (Arsiningtyas, 2021; Firdawati et al., 2022).

Plant extraction is an important step for chemical isolation, chemical analysis, and evaluating the biological and pharmacological activities of plant compounds. A considerable quantity of desired active plant compounds can be extracted under optimal conditions and extraction techniques while also preventing the degradation of certain sensitive compounds. Therefore, determining the most favorable extraction conditions is a critical undertaking in order to maximize both the quantity of active plant compounds and the extraction yield. Presently, a variety of conventional and contemporary techniques are being utilized to extract compounds from medicinal plants (Suksaeree et al., 2022).

During the extraction process, a material is isolated from a mixture by employing various solvents. Conventional extraction techniques, such as maceration, soxhlet, and hydrodistillation, utilize significant amounts of solvents and necessitate extended extraction durations (Ayu et al., 2020). Maceration is the immersion of simplicia powder in a suitable solvent at room temperature while being shielded from light to extract the active component. Extraction process in maceration, phytochemicals in plants are extracted by passive diffusion. In order to enhance the effectiveness of extracting bioactive compounds from plants, several novel extraction methods have been suggested.

One such method is stirring-assisted extraction (SAE), which combines heating with mechanics in the form of constant stirring at a certain rotation speed. The stirring process leads to a higher mass transfer coefficient and improves the convective mass transfer rate, thus facilitating the extraction process and leading to an increase in the extraction yields. In contrast to maceration which uses the principle of passive diffusion, in SAE an active diffusion process occurs which is caused by stirring in the system (Mohamad et al., 2013).

Ultrasound-assisted extraction (UAE) has gained increasing interest in recent times. UAE is a contemporary technique for extracting substances using ultrasonic vibrations. Ultrasonic waves used in extraction induce cavitation, resulting in the generation of fracture power and mechanical disruption of cell walls. This enables ultrasonic technology to minimize the need for chemical usage in the pretreatment process (Khasanah et al., 2021). The ultrasonic method offers several advantages, including obtaining more concentrated extracts, more active substances, and a shorter processing time. This is achieved through the use of ultrasonic waves, which assist in breaking down cell walls in the liquid phase below their boiling point. This process facilitates the formation of spontaneous bubbles and enhances the permeability of cell walls (Susiloningrum et al., 2023).

In addition to the UAE, microwave-assisted extraction (MAE) is a contemporary extraction method that can potentially increase the quantity of phytochemicals extracted from plants. The microwave process utilizes the combined effects of ionic conduction and dipole movement to heat material in the presence of microwaves (Bagade & Patil, 2021). The main reason for heating the plant matrix in a microwave assembly is the presence of moisture in the plant cell. In microwave-assisted extraction, the purpose of heating dried plant material is to target the small quantity of moisture within a plant cell. When the moisture inside the innermost cell of a plant is heated using a microwave, it evaporates and exerts significant pressure on the cell wall. The cell wall is weakened inside by this pressure, resulting in its rupture. Thus, the release of potential components from the damaged cell occurs, thereby enhancing the extraction efficiency of phytoconstituents (Vivekananda Mandal, Yogesh Mohan, 2007).

Materials and Methods

Tools and Materials

The tools employed in this study include a digital overhead stirrer (DLAB, Beijing, China), magnetic hotplate stirrer (DLAB, Beijing, China), sonicator bath (EECOO, China), microwave (SHARP, Karawang, Indonesia), UV-visible spectrophotometer (Shimadzu, Kyoto, Japan), micropipette (Socorex, Switzerland).

The materials used in this research include Sappan wood (Nano brothers herbal), distilled water (Rofa laboratory), gallic acid (Merck), 2,2-Diphenyl-1-picrylhydrazyl (Sigma Aldrich), methanol p.a (Merck), folin-ciocalteu's phenol (Merck), sodium carbonate anhydrous (Merck).

Methods

Determination of Sappan Wood

The dried sappan wood was obtained from local markets in Situbondo. The specimen was authenticated by the Herbarium Jatinangor, Padjadjaran University, Indonesia.

Extraction

1. Maceration

Sappan powder was subjected to a maceration process by Irfan et al., (2022), with some modifications. Extraction was conducted using distilled water as the solvent, with a sample-to-solvent ratio of 1:10 and at ambient room temperature. A succinct 50 g sample of Sappan powder was transferred into a specimen glass. Following this, distilled water was added to the sample-filled receptacle. The Beaker glass was covered with aluminum foil to prevent solvent evaporation during operation. Maceration was carried out for 3 days; every 24 hours, the mixture was stirred for 2 minutes. After 3 days, filter the extract using a filter cloth, then concentrate it using a water bath.

2. Stirring-assisted extraction (SAE)

The SAE method was carried out according to Setyawan & Kartini, (2023) with slight modifications. A receptacle is filled with 50 grams of sappan wood powder, followed by the addition of distilled water in a volumetric ratio of 1:10 (g/ml). An overhead agitator was utilized to facilitate the extraction process at a temperature of 50°C for 60 minutes at a speed of 200 rpm. The extract was filtered and then concentrated in a water bath.

3. Ultrasound-assisted extraction (UAE)

Extraction with UAE was conducted with several modifications following de Sousa et al. (2020). A quantity of 50 grams of sappan wood powder is combined with distilled water in a glass beaker at a ratio of 1:10 (g/ml). The sappan wood solution was extracted for 30 minutes at 50°C using a bath sonicator set at 40 kHz. Following filtration, the extract is concentrated in a water bath (de Sousa et al., 2020).

4. Microwave-assisted extraction (MAE)

The extraction process employed in MAE is derived from the work conducted by Bachtler and Bart (2021), with notable adjustments. A solution is prepared by combining powdered sappan wood and distilled water in a receptacle at a volumetric ratio of 1:10 (g/ml). The sappan wood solution was subsequently agitated for 5 minutes with a stir stick. Reheating the sappan wood solution at 540 watts for ten minutes in the microwave. Following filtration, the extract is concentrated (Bachtler & Bart, 2021).

Total Phenolic Content

The quantification of total phenolic content was conducted using the Folin-Ciocalteu (FC) method, as described by (Akmal et al., 2023). Sappan wood solution in methanol solvent (1000 µg/ml) was combined with 5 ml of FC reagent (1:10 in water for injection), and thereafter incubated at room temperature in a light-protected area. The sample was combined with 4 ml of a Na2CO3 solution (7.5% in distilled water) and incubated for an additional 45 minutes. The sample's absorbance was quantified using a UV-Vis spectrophotometer at a specific wavelength of 782nm. The standard utilized was gallic acid. A calibration curve was constructed using gallic acid solutions ranging from 10 to 50 µg/ml in concentration. The results were quantified as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g DE).

Antioxidant Activity

The efficacy of sappan wood extract in scavenging free radicals was assessed by determining its antioxidant activity by the DPPH (2,2-diphenyl-l-picrylhydrazyl) free radical technique. The assessment of antioxidant activity aligns with the methodology employed by (Akmal et al., 2023), with minor adjustments. A 1 ml of a methanolic solution containing sappan wood extract (at concentrations ranging from 6.25 to 100 µg/ml) was combined with 2 ml of a DPPH solution (at a concentration of 40 µg/ml). The mixture was then incubated for 40 minutes at room temperature while being kept away from light. The sample's absorbance was quantified at a wavelength of 516nm using UV-Vis spectrophotometry. The blank solution underwent identical processing as the sample, substituting sappan wood extract for water for injection.

Data Analysis

The experiments were conducted three times, and the findings were analyzed using analysis of variance (ANOVA) followed by the Tukey's Test using IBM SPSS Statistics 26.0 software (Da Porto et al., 2013).

Result and Discussion

The results and discussion will explain the influence of the extraction method on the extraction yield, total phenolic content, and antioxidant activity of sappan wood.

Extraction Yield

The extract yield of sappan wood extract from the smallest is 10.21, 11.57, 12.25, and 12.82% for SAE, UAE, MAE, and maceration, respectively (**Table 1**).

Table 1. Extraction yield of Sappan wood extract

Extraction Method	Extraction Yield (%)
Maceration	12.82 ± 0.06^{bcd}
Stirring-assisted Extraction (SAE)	10.21 ± 0.03^{acd}
Ultrasound-assisted Extraction (UAE)	11.57 ± 0.02^{ab}
Microwave-assisted Extraction (MAE)	12.25 ± 0.07^{ab}

All data are presented as means ± SD (n= 3).
The diverse superscript letters in the row indicate significant differences (P < 0.05)

According to the Indonesian Herbal Pharmacopoeia (2017), the extraction yield for sappan wood extract is not less than 8.1%. All extraction methods demonstrated extraction yields over 8.1%, indicating their appropriateness for extracting sappan wood. The extraction yield of all methods can exceed 8.1%, as the solvent utilized is water, in contrast to the Indonesian herbal pharmacopeia, which employs ethanol. Water and ethanol solvents have distinct polarity. Water has a higher degree of polarity in comparison to ethanol (Kementrian Kesehatan Republik Indonesia, 2017; Sun et al., 2015).

The results of the extraction yield indicated that the maceration procedure yielded the most significant results. The maceration method is a traditional technique based on the principle of diffusion. In the maceration procedure, the solvent infiltrates the cell cavity containing bioactive materials. As a result, the substance that enters the cell cavity will have a greater concentration than the solvent present outside the cell. The solvent within will come out or diffuse due to the difference in concentration. The maceration process can provide higher extraction yields than other methods due to its longer extraction duration, namely 3 days (Lamadjido et al., 2019; Wijaya et al., 2018).

The SAE, UAE, and MAE procedures exhibit reduced extraction yields but with a negligible disparity. Based on the duration of the extraction process, the three approaches are significantly briefer compared to maceration. Despite the limited time available, the extraction yield exceeds the standards set by the Indonesian Herbal Pharmacopoeia for the three procedures. SAE, UAE, and MAE received intervention in the form of elevated extraction temperatures. Increasing the extraction temperature results in a proportional increase in the extraction yield (Rifkia & Prabowo, 2020).

Total Phenolic Content (TPC)

Gallic acid was employed as a natural standard for the purpose of generating a calibration curve (**Figure 1**) during the assessment of the total phenolic content. The calibration curve for gallic acid concentration, ranging from 10-50 µg/ml, yielded a r^2 value of 0.9854.

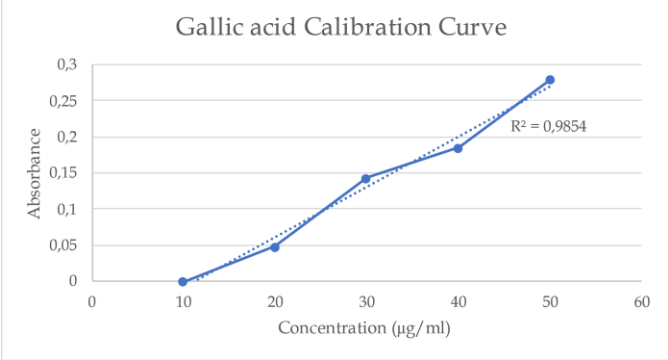

Figure 1. Gallic acid calibration curve

The research findings indicate that the extraction procedure has an impact on TPC. **Table 2** displays the TPC values for maceration, SAE, UAE, and MAE, which are 132.85±1.44, 150.99±1.69, 206.70±6.56, and 115.70±1.44 mg GAE/g DE, respectively.

Table 2. Total phenolic content of Sappan wood extract

Extraction Method	TPC (mg GAE/g DE)
Maceration	132.85 ± 1.44^{bcd}
SAE	150.99 ± 1.69^{acd}
UAE	206.70 ± 6.56^{abd}
MAE	115.70 ± 1.44^{abc}

All data are presented as means ± SD (n=3).
The diverse superscript letters in the row indicate significant differences (P < 0.05)

The extraction method with the highest TPC concentration is Ultrasonic Assisted Extraction (UAE). The UAE has notable benefits, particularly a substantially greater extraction rate and bioavailability rate. Ultrasonic waves have unique physical characteristics that allow them to efficiently disturb the structure of plant cell tissues, resulting in the breaking or distortion of cell walls. This enables a more comprehensive retrieval of the relevant elements, leading to much greater extraction efficiency in comparison to conventional approaches. Additionally, the UAE provides the benefit of reduced extraction durations while still achieving optimal extraction efficacy (Mnayer et al., 2017).

The UAE is an established force in the field of power ultrasonics. Power ultrasonics is a specialized field within ultrasonology that specifically explores the application of ultrasonic energy for the purpose of manipulating and transforming substances. The UAE technique employs heat, mechanical, and cavitation effects to extract bioactive substances. Ultrasonic activity causes the destruction of the cell wall and enhances the release and diffusion of cell components (Chemat et al., 2017).

The thermal effect of ultrasonic waves pertains to the phenomenon in which the vibrational energy of ultrasonic waves is assimilated by the medium and

transformed into thermal energy. As a result, the temperature of the medium increases proportionally. The calorific value is dependent upon variables such as the characteristics of the medium, ultrasonic power, and duration of exposure (Qiu et al., 2020).

The mechanical effect of ultrasonic refers to the phenomenon in which the application of ultrasonic waves to a medium causes the particles inside the medium to vibrate in response to the mechanical wave. Consequently, the movement of particles is intensified, resulting in a hastened process of mass transfer (Wen et al., 2018).

The cavitation effect is widely regarded as the most prominent among the numerous ultrasonic effects. Ultrasonic cavitation refers to the phenomenon where tiny bubbles (cavitation nuclei) in a liquid experience vibrations, expansion, and continuous energy accumulation due to the effect of ultrasonic waves (Akmal et al., 2024). Once the energy exceeds a particular threshold, the cavitation bubble rapidly collapses and seals tightly (Tiwari, 2015).

The extraction technique that yields the second-largest quantity of TPC is the employment of SAE. This approach employs the same temperature as that of the UAE. This discovery confirms the idea that higher temperatures can affect the total amount of phenolic substance that is removed. Additionally, the SAE goes through an agitating process where the turbulence brought about by the increased stirring speed enhances the interaction between the solid and the solvent. As a result, the rate at which mass is transferred increases, causing an increase in the movement of phenolic compounds from the sample's surface into the solvent. As a consequence, a greater amount of phenolic chemicals are extracted (Lucena, 2012).

The temperature-dependent extraction process is also observed in the MAE approach. However, in contrast to the UAE and SAE methods, the MAE technique yields the lowest total phenolic content (TPC) for sappan wood extract.

In general, the utilization of MAE results in an enhanced escape of substances from the desired matrix while decreasing the extraction duration and the reliance on solvents in comparison to traditional methodologies (Esquivel-Hernández et al., 2017).

Microwave-assisted extraction (MAE) utilizes nonionizing electromagnetic waves to disrupt or alter the cellular structure of a sample matrix. The processes of ionic conduction and dipole rotation, which frequently take place simultaneously in many MAE applications, facilitate the transmission of energy in MAE. Ionic conduction involves the movement of ions through a solution, which leads to the generation of uniform heat in the medium. This heat results from the solvent's resistance to the migration of ions when electromagnetic waves are applied. Dipole rotation refers to the realignment of molecules along the electric field, resulting in thermal agitation when the electromagnetic waves are no longer present as the molecules return to a disordered condition (Gomez et al., 2020).

The temperature in the system may contribute to the low TPC observed in the MAE approach. The temperature of the sample solution, following extraction with 540 watts of electricity for a duration of 10 minutes, reached a range of 80-87°C. In a previous study by Mokrani & Madani (2016), it was found that the total phenolic content (TPC) declined as the temperature increased, reaching a maximum decrease at 70°C. Due to this reason, the TPC MAE is lower as compared to the SAE and UAE.

Antioxidant Activity

The primary active chemical found in sappan wood is brazilin. Brazilin is a red pigment that imparts a distinctive red hue to sappan wood. It is a flavonoid belonging to the class of chemicals known as chalcones. Brazilin has been discovered to possess antioxidant properties (Vij et al., 2023). The antioxidant mechanism of blazilin involves the transfer of hydrogen atoms to free radicals. Consequently, the greater the efficiency of the brazilin structure in facilitating hydrogen transfer, the higher the antioxidant potency of brazilin will be (Hassanpour & Doroudi, 2023).

The DPPH assay was extensively employed to quantify the antioxidant capacity of materials in ethanol-based systems by measuring their ability to scavenge free radicals (Piang-Siong et al., 2017). The DPPH radical-scavenging activity was quantified by plotting the percentage against the sample concentration. The IC_{50} value, which represents the concentration of sample required to achieve 50% inhibition, was determined from this plot. A lower IC_{50} value indicates a greater scavenging activity of the material (Chen et al., 2020).

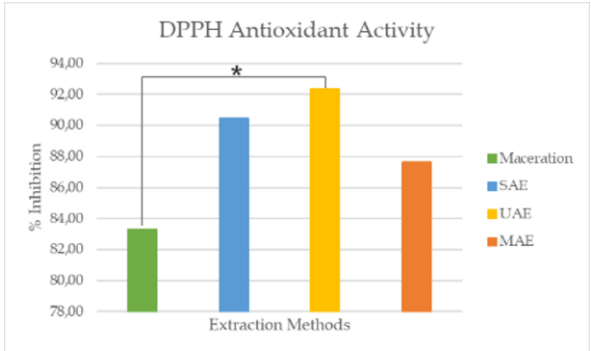

Figure 2. DPPH antioxidant activity. All data are presented in the manner of means (n=3). *Indicate significant difference ($P < 0.05$)

The free radical scavenger (100µg/mL) capabilities of maceration, SAE, UAE, and MAE are 83.30±5.35, 90.45±0.44, 92.39±0.39, and 87.64±2.52, respectively (**Figure 2**). The DPPH antioxidant activity (IC_{50}) values are 32.33±2.64, 25.01±0.34, 20.68±0.29, and 29.18±0.70 µg/mL, respectively, as shown in **Table 3**. The results indicate that all extraction procedures yield extracts with highly potent antioxidant properties in very strong categories.

Table 3. DPPH antioxidant activity of Sappan wood extract

Extraction Method	IC_{50} (µg/mL)	Category
Maceration	32.33 ± 2.64^{bc}	Very strong
SAE	25.01 ± 0.34^{acd}	Very strong
UAE	20.68 ± 0.29^{abd}	Very strong
MAE	29.18 ± 0.70^{bc}	Very strong

All data are presented as means ± SD (n=3).
The diverse superscript letters in the row indicate significant differences ($P < 0.05$)

The evaluation of antioxidant activity with the DPPH approach indicates that the UAE method exhibits the highest percentage of free radical inhibition and the most favorable antioxidant activity value (IC_{50}). The inhibitory power and antioxidant activity values of UAE differed significantly ($P<0.05$) from those of the standard maceration procedure. This demonstrates that the UAE method is more effective than conventional methods for extracting sappan wood.

Conclusion

The research findings indicate that various extraction procedures can impact the extraction yield, total phenolic content (TPC), and antioxidant activity (IC_{50}) of sappan wood. The ultrasound-assisted extraction (UAE) approach demonstrated a higher total phenolic content (TPC) of 206.70±6.56 mgGAE/g DE and IC_{50} value of 20.68±0.29 µg/mL compared to other extraction methods. It can be inferred that the UAE is an efficient method for extracting sappan wood quickly and easily compared to the traditional maceration process. Ultrasonic waves used in extraction induce cavitation, resulting in the generation of fracture power and mechanical disruption of cell walls. This enables ultrasonic technology to minimize the need for chemical usage in the pretreatment process. This process promotes the formation of spontaneous bubbles and enhances the permeability of cell walls. The ultrasonic method offers several advantages, including obtaining more concentrated extracts, more active substances, and a shorter processing time.

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