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Combined enzymatic and ultrasound-assisted aqueous two-phase extraction of antidiabetic flavonoid compounds from *Strobilanthes crispus* leaves

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Abstract

A novel green extraction method combining enzymatic and ultrasound-assisted aqueous two-phase extractions was employed to enhance and purify the flavonoid extract from *Strobilanthes crispus* leaves. Cellulase was used in the pretreatment, and ethanol-$(NH_4)_2SO_4$ was selected as the solvent. A concentration of 7% (w/w) cellulase and a duration of 2 h were the optimal conditions for pretreatment. The optimal conditions for ultrasound-assisted aqueous two-phase extraction were 33% (w/w) ethanol and 14% (w/w) $(NH_4)_2SO_4$ as they produced a yield (77.81%), partition coefficient (31.17), extraction efficiency (98.04%), and a high total flavonoid content (0.3666 mg QE/g dry leaf powder). Six compounds from the leaf extract were identified through liquid chromatography-mass spectrometry/mass spectrometry-quadrupole-time of flight (LCMS/MS-Q-TOF) analysis. The crude extract and three compounds in it (kaempferol, graveobioside A, and genistein) showed an antidiabetic activity with IC_{50} values of 390.35, 201.87, 292.73, and 431.82 mg/mL, respectively. These values are comparable to the standard drug acarbose.

Keywords: Ultrasound; enzymatic; aqueous two-phase, *S. crispus*; antidiabetic

1. Introduction

Strobilanthes crispus (*S. crispus*), a species that belongs to the *Acanthaceae* family, is an herbaceous plant that grows from Madagascar to Indonesia. It is also known as Keji Beling in Indonesia [1]. Traditionally, *S. crispus* is used as a remedy for kidney stones, gallstones, and tuberculosis [1]. Some studies have also shown that this plant is rich in bioactive compounds with some antioxidant properties [2], anticancer [3], antiurease [4], gastroprotective [5], wound healing, and hypolipidemic effects [6]. Moreover, traditional knowledge and some studies have suggested that *S. crispus* can lower blood sugar (antidiabetic activity) [1,6,7]. However, no studies, so far, have identified the chemical compounds responsible for the antidiabetic activity of *S. crispus* leaves. Given that the number of people suffering from diabetes increases every year and the World Health Organization estimates that 21.3 million Indonesians will become diabetics by 2030 (number 4 in the world) [8], and to get higher results from compounds that have antidiabetic, the extraction method should be evaluated.

S. crispus leaf extract is generally obtained through conventional methods, such as maceration [2,3,9,10], Soxhletation, or reflux [9,11,12,13]. However, these methods have many drawbacks, such as being time-consuming, costly, and degrading bioactive compounds [14]. To address these shortcomings, many researchers have been attempting to develop a number of extraction methods that satisfy the concept of green chemistry.

One of the proposed alternative extraction methods is ultrasound-assisted extraction (UAE), a green extraction method that has several benefits, such as improving the aqueous extraction process without using organic solvents, increasing extraction yields, and improving extraction performance with a shorter time and less solvent needed [15, 16]. UAE increases the surface area by forming cavitation through ultrasonic waves although with less intensity compared to microwave-assisted extraction (MAE) [17]. MAE uses microwave energy, which can lead to more effective heating and faster energy transfer [18]. However, due to the heat generated, it can damage the bioactive compound [17].

To increase its efficiency, the UAE should be combined with other extraction techniques. Ultrasound-assisted enzymatic extraction (UAEE) is a combination of ultrasound and enzymatic extraction techniques, where the enzymatic reaction enhances the effect of the ultrasound extraction [16]. UAEE is simple and can shorten extraction time, reduce operating costs, and increase efficiency [19, 20]. It also provides higher yields compared to maceration, UAE, and enzyme-assisted extraction (EAE) [21,22]. Meanwhile, EAE is an extraction technique using enzymes and can be another alternative to conventional extraction techniques [23]. However, enzymes cannot totally hydrolyze bonds in plant cell walls because of their selectivity, so only some active

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compounds are released [16,21]. Therefore, UAE and EAE should be combined simultaneously or serially to achieve an optimal extraction [16].

Although UAEE can increase the release of bioactive compounds into the solvent, the level of purity of the extract must still be optimized [24]. The extract should be purified by applying techniques such as aqueous two-phase extraction (ATPE). ATPE is based on the formation of a two-phase system, which commonly involves short-chain alcohols together with inorganic salts, causing bioactive compounds to be partitioned into the alcoholic phase, while impurities are partitioned into the salt phase [25]. To simplify the simultaneous extraction and purification process, UAE should be combined with ATPE (UA-ATPE), which not only improves efficiency but also shortens the purification stage [26]. UA-ATPE is an efficient, eco-friendly, and promising technique for extracting and enriching the plant's bioactive compounds in extracts [27].

Concerning the benefits of the flavonoid compounds from *S. crispus*, recent studies have confirmed that a combined extraction method can overcome the shortcomings of a single extraction method. Therefore, in the current study, we combined EAE and UA-ATPE, referred to as ultrasound-assisted enzymatic aqueous two-phase extraction (UAE-ATPE) to increase the yield and purity of the flavonoid compounds and develop an efficient extraction technique. Moreover, the UAE-ATPE technique, to the best of our knowledge, has never been used in previous studies to extract active compounds from *S. crispus*. The compounds were characterized by liquid chromatography-mass spectrometry/mass spectrometry-quadrupole-time of flight (LC-MS/MS-Q-TOF) and scanning electron microscopy (SEM), and the antidiabetic activities of the extract and the compounds isolated from it were also studied.

2. Materials and Methods

2.1. Chemicals and Reagents

Ammonium sulfate, absolute ethanol, gallic acid, Folin-Ciocalteu reagent, sodium carbonate, aluminum chloride, and potassium methyl carboxylate were purchased from Merck. Meanwhile, cellulase enzyme from Aspergillus Niger (0.8 U/mg) and Quercetin were obtained from Sigma Aldrich.

2.2. Sample preparation

The samples of *S. crispus* leaves were obtained from the Kulon Progo region, Indonesia. The leaves were washed, dried, and pulverized to produce dry leaf powder (DLP). The *S. crispus* DLP subsequently was passed through an 80-mesh sieve analyzer (Sieve Shaker Electric Timer-Zia, Indonesia). The powdered leaves were then stored in a desiccator until being used.

2.3. Ultrasound-assisted enzymatic extraction (UAEE)

As a pretreatment step, the *S. crispus* leaves were subjected to enzyme-assisted extraction. The enzyme used was cellulase from *Aspergillus Niger* (0.8 U/mg, Sigma-Aldrich, Japan). 30 mg of cellulase was dissolved in 100 mL of distilled water and a buffer was added to maintain the pH level. Next, 1 g of *S. crispus* DLP was added to the mixture. Enzymatic hydrolysis was carried out by stirring the mixture using a magnetic stirrer at 27ºC for 2 h. All parameters remained constant, while the hydrolysis time (1, 1.5, 2, 2.5, and 3 h) and enzyme loading (0, 3, 5, 7, and 9% (w/w) DLP) were varied.

After the hydrolysis process was completed, 100 mL of 96% ethanol (Merck) was added to the mixture. The extraction process was carried out in a sonicator bath (OVAN, Type BUS 6, Spain) at 27ºC for 1 h. After filtering with a Buchner funnel and evaporating with a rotary evaporator (IKA type RV 8, Germany), the obtained extract was weighed and the yield was determined.

Other similar conditions were applied in the ultrasound-assisted extraction method without any prior hydrolysis pretreatment.

2.4. Phase diagrams

The phase diagram for the aqueous two-phase system (ATPS) was produced based upon to the procedure as outlined by Cheng (2017) with some modifications, wherein absolute ethanol was gradually added together with a salt solution ($(NH_4)_2SO_4$) and homogenized by vortexing (Thermo Scientific) [28]. The mixture was left to stand, and the solutions continued to be added until two phases were formed [26]. The amount of ethanol and $(NH_4)_2SO_4$ solution added were recorded. This procedure was repeated several times to construct a phase diagram curve.

2.5. Ultrasound-assisted enzymatic aqueous two-phase extraction (UAE-ATPE)

The ultrasound-assisted enzymatic aqueous two-phase extraction (UAE-ATPE) of *S. crispus* leaves was carried out in an ultrasound bath with enzymatic hydrolysis as a pretreatment. Having conducted the enzymatic hydrolysis, we determined the optimal concentration of ethanol and salt ($(NH_4)_2SO_4$). Ethanol was added to the hydrolyzed leaf samples, followed by saline solution, and extraction was carried out at 27ºC for 1 h. After the extraction process was completed, the upper and lower phases were separated using a separatory funnel. Subsequently, the total flavonoid contents (TFC) of both phases were analyzed. The partition coefficient (k), referred to as the distribution coefficient by Wijayanti (2023), compares flavonoid compounds in the extract phase (upper phase) with those in the raffinate phase (lower phase), and it is calculated using equation [29]:

$$k = \frac{C_a}{C_b} \tag{1}$$

Meanwhile, the recovery or extraction efficiency (R) and volume ratio (Vr) are determined using the following equations [25]:

$$R = \frac{C_a V_a}{C_a V_a + C_b V_b} x100\% \tag{2}$$

$$V_r = \frac{V_a}{V_b} \tag{3}$$

where C_a and C_b are the flavonoid concentrations of the upper and lower phases, and V_a and V_b are the volumes of the upper and lower phases. To obtain the optimal ethanol and salt concentrations, experiments were first carried out using varying ethanol concentrations (29, 31, 33, and 35%, w/w) at a constant concentration of $(NH_4)_2SO_4$ (14%). Then, the concentration of $(NH_4)_2SO_4$ was varied (14, 15, 16, and 17%, w/w) at the optimum ethanol concentration. After filtering to separate the extract and solids, the resulting extract solution was dried and prepared for further testing.

2.6. Total flavonoid content (TFC) determination

The TFC value was determined based upon a published colorimetric method with some modifications [30]. The methanol solvent was replaced with ethanol with the wavelength of 434 nm. A Shimadzu UV1280 UV-Vis spectrophotometer was used for the analysis. The TFC value was expressed as mg quercetin equivalent/g DLP.

2.7. Isolation of bioactive compounds

The bioactive compounds were isolated from the ethanol extract of *S. crispus* by column chromatography, using 30 g of silica gel 60 (Merck, Germany) as the stationary phase and toluene-ethyl acetate (1:1, v/v) as the eluent. For each fraction, thin-layer chromatography was performed using a silica gel 60 F254 TLC plate (Merck, Germany). The TLC plate was visualized under UV light at a 254 nm wavelength.

2.8. Antidiabetic activity test

The inhibition of α-glucosidase was studied using p-nitrophenyl-α-D-glucopyranoside. Tests were carried out on the crude extract and all isolates. Other tests were also performed on the best isolate with variations in substrate concentration to determine the type of inhibition. p-nitrophenyl-α-D-glucopyranoside (150.65 mg) was dissolved in phosphate buffer (pH 7.0). 1.0 mg of α-glucosidase subsequently was dissolved into phosphate buffer (pH 7.0) containing 200 mg of bovine serum albumin. A 4 mg sample was dissolved into 100 µL of DMSO as the master solution before being diluted with DMSO to yield the concentrations of 100, 50, 25, and 10 µg/mL. Afterward, 250 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution and 490 µL of 0.1 M phosphate buffer (pH 7.0) were added into a test tube containing 5 µL (standard solution)/10 µL (sample solution) of DMSO with various sample concentrations. The mixture was preincubated at 37ºC for 5 min. The reaction was started by adding 250 µL of α-glucosidase solution (0.062 U), and allowed to take place for 15 min. The reaction was terminated by adding 1 mL of 0.2 M Na_2CO_3. The enzymatic activity was measured based on the absorption of the formed p-nitrophenol at λ 400 nm. The inhibitory activity was calculated using the equation below [31]:

$$Percent\ Inhibition = \frac{Control - Sample}{Control} \times 100\% \quad (4)$$

The type of inhibition was determined for the sample with the best IC_{50} value using the Lineweaver-Burk equation [32].

2.9. SEM characterization

The *S. crispus* surface morphology was observed before extraction, after enzymatic extraction, and after ultrasound extraction under a scanning electron microscope (SEM) (FEI QUANTA 450, USA).

2.10. LC-MS/MS-Q-TOF characterization

LC-MS/MS analyses of all samples were performed using an ACQUITY UPLC® H-Class high-performance liquid chromatography system (Waters, USA) coupled to a Xevo G2-S Q-TOF mass spectrometer (MS) (Waters, USA). The analysis was performed by means of electrospray ionization (ESI). Chromatographic separation was achieved using an ACQUITY UPLC® HSS C18 (1.8 m, 2.1 × 100 mm) column. The injection volume was set to 5 µL. Meanwhile, the flow rate was set to 0.2 mL/min and the two mobile phases consisted of 100% water + 5 mM ammonium formate (A) and 100% acetonitrile + 0.05% formic acid (B). The following instrument parameters were applied in all cases: desolvation gas flow at 793 L/h, desolvation temperature at 350ºC, column oven at 50ºC, impact energy at 4 V, and ramp impact energy at 25–60 V.

The analysis of the crude extract and isolates was carried out in the positive ESI mode with a gradient elution system, as follows: 0–2 min, 5% B; 2–3 min, 25% B; 3–14 min, 25% B; 14–15 min, 100% B; 15–19 min, 100% B; 19–23 min, and 5% B.

The data obtained (chromatograms and mass spectra) were accessed and processed using MassLynx v.4.1. The processed mass spectra and compound formulas were then compared with databases such as ChemSpider, the Human Metabolome Database, and MassBank.

3. Results and Discussion

3.1. Determination of the optimal conditions for hydrolysis

The basic principle of the EAE method is the destruction of the plant's cell matrix, which has a stable structure and is resistant against the extraction of intracellular bioactive compounds through enzymatic hydrolysis [23, 33]. Plant cells consist of complex structural polysaccharides, such as cellulose, hemicellulose, pectin, and lignin, and can be hydrolyzed with specific enzymes [23]. The plant cell wall binds to the active site of the enzyme, which causes the enzyme to change its shape so that the substrate fits into the active site of the enzyme. This causes the breakage of the plant cell wall bonds and facilitates the escape of the active compounds [33]. Enzymes such as cellulase, xylanase, amylase, papain, pectinase, and hemicellulose are widely used for pretreatment before the actual extraction process is carried out [33]. In the current study, cellulase (an enzyme that catalyzes the hydrolysis of cellulose) was used.

In this study, hydrolysis was followed by ultrasound extraction to obtain polyphenolic extracts. Ultrasound treatment can increase the extract yields in a shorter extraction time compared to EAE and is preferred considering its energy consumption and productivity, which are important criteria in the industry [21,34]. Diverging from the approach of Rokhati

(2023), who initiated the cellulase hydrolysis process with ultrasound treatment, their findings suggest that initiating cellulase hydrolysis after ultrasound pretreatment yields more optimal results compared to scenarios without pretreatment or those involving microwave pretreatment [35]. Meanwhile, Yang's (2018) research underscores that commencing the ultrasound-assisted extraction process with the use of cellulase enzyme during hydrolysis can achieve significantly higher yields than processes initiated with ultrasound (UAE) and followed by hydrolysis (EAE) [36]. Another study by Gao (2022) revealed a substantial increase in the yield of polyphenols with UAEE compared to UAE alone or EAE alone. In UAEE, there is a synergistic effect between enzymes and ultrasound that increases the extract yields, wherein enzymes hydrolyze the cell walls and destroy cell structures to release polyphenols, while ultrasound generates a cavitation effect to increase the affinity between enzymes and substrates to generate extracts [22].

The hydrolysis time and enzyme concentration (expressed by enzyme loading, i.e. the ratio of enzyme weight to DLP weight) were varied according to one variable at a time (OVAT) method to obtain the optimal conditions. The optimal conditions were determined based on the extract yield obtained after the ultrasound extraction, which was carried out when the ethanol concentration, ultrasound frequency, and time and temperature of ultrasound extraction were set at a constant value. In general, an increase in the yield correlates with an increase in the TFC value. This was reported by Saaed (2022), showing that the optimal conditions for yield and TFC were the same as those used here (pH of 5.5, 5.5 mL of enzyme, 65ºC, and 150 min) [37]. Figure 1 shows the effect of the hydrolysis time and enzyme loading on the extract yield.

As stated earlier, the duration of hydrolysis also greatly influences the yield, as shown in Figure 1(a). The longer the duration of contact between the enzyme and substrate, the greater the solubilization of the cell wall components. A long incubation period lowers the quality of the extract and is inefficient regarding the amount of energy used. The yield obtained decreases after a certain incubation time because the enzyme reacts with all substrates, and only a few active sites are left for further enzymatic reactions [38]. The increase in extract yield was substantial from 1 h to 2 h, but after 2 h, the extract yield decreased. Increasing the incubation time is likely to increase the degree of hydrolysis, but can also result in the product inhibition of the enzyme; hence, the active site of the enzyme can no longer be occupied by the substrate [38].

Figure 1(b) shows that an increase in enzyme loading from 0 to 7% (w/w) DLP increases the extract yield, but at a concentration of 9% (w/w) DLP, the extract yield decreases. The higher the enzyme loading, the more interactions between the enzyme and the substrate, and this contributes to faster and more extensive hydrolysis of the cell walls [34,38]. This condition can boost the extract yield because increasingly more active compounds are extracted. This boost also occurs due to the contribution of ultrasound-generated cavitation bubbles, which increase the mass transfer rate and enhance the enzymatic kinetics [34]. Increasing the enzyme loading further results in a more viscous solution because of dynamic changes in the enzyme-substrate ratio. This condition is not conducive to enzymatic processes and inhibition of the final product may occur [34,39]. Luo (2019) reported that the use of cellulase at concentrations in the range of 0.5 g/L to 3.5 g/L as an extraction pretreatment substantially increased the yield of polyphenols, but increased the enzyme concentration to 4.0 g/L resulting in a decrease in the yield of polyphenols [40].

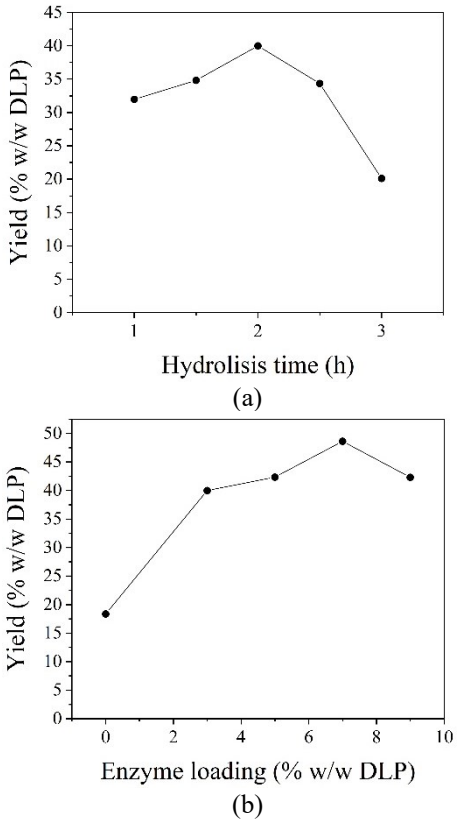


Fig. 1. Effect of (a) hydrolysis time and (b) enzyme loading on the leaf extract yield obtained with the UAEE method (ethanol concentration of 50% (v/v), ultrasound extraction time of 1 h, and extraction temperature of 27 ºC)

Hydrolysis by cellulase is a reaction that results in the formation of glucose monomers or cellobiose dimers from cellulose polysaccharides. Increasing the enzyme loading produces glucose dissolved in water in which it in turn results in a greater concentration of polar compounds in the solution. Therefore, when the ultrasound extraction is carried out, the expected ethanol concentration (50%, v/v) decreases and affects the extract yield. Moreover, this might affect the ultrasound extraction process, where the more viscous solution that results from the presence of more enzymes interferes with the propagation of ultrasound waves to reach the plant cells. In the current study, the optimum cellulase loading was 7% (w/w) DLP with an extract yield of 48.63% (w/w) DLP (Figure 1(b)).

3.2. Determination of the optimal concentration for the UAE-ATPE

An initial experiment was conducted to determine the ATP solvent system by observing the effects of several mineral salts on the partition coefficient and extraction efficiency of the quercetin used as a model compound (Table 1).

As shown in Table 1, $(NH_4)_2SO_4$ has a higher partition coefficient and extraction efficiency values than other mineral salts. A similar result was reported by Dong (2015), who compared $(NH_4)_2SO_4$, K_2HPO_4, and sodium citrate for

extracting phenylethanoid glycosides from the stems of *Cistanche deserticola* Y.C. Ma [27]. According to Dong (2015), the ethanol/$(NH_4)_2SO_4$ system showed an excellent partitioning behavior and could be recycled. The equilibrium curve of the ethanol-$(NH_4)_2SO_4$ system (Figure 2) shows that the solubility of ethanol-$(NH_4)_2SO_4$ is stable over a wide temperature range. Therefore, in this study, the ethanol-$(NH_4)_2SO_4$ system was chosen to separate the flavonoid compounds.

Table 1. Partition coefficient values and extraction efficiency of quercetin in the ethanol-salt system

No.	Mineral salts	Partition coefficient	Extraction efficiency (%, w/w)
1	$(NH_4)_2SO_4$	3.16	76.48
2	Na_2SO_4	3.11	45.94
3	Na_2CO_3	1.85	29.72
4	Na_2HPO_4	2.33	64.88

Figure 2 shows a binodal curve separating the monophasic system and biphasic system regions. The upper part of the curve is the biphase region and the bottom of the curve is the monophase region. Therefore, for the ATP system, the region above the binodal curve was chosen [41].

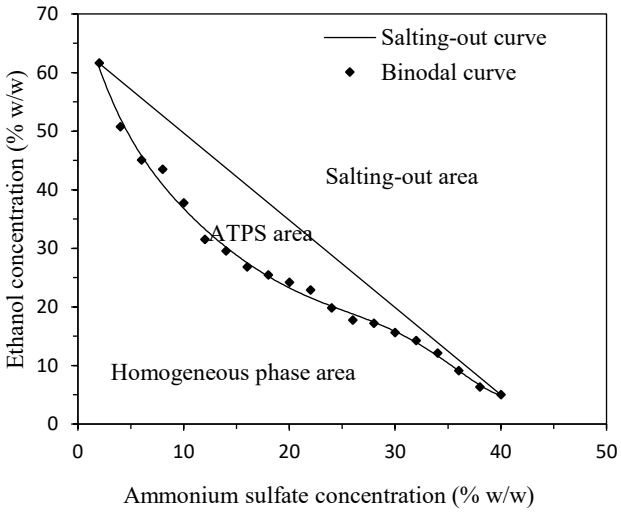


Fig. 2. Phase diagram of the ethanol-ammonium sulfate system

To determine the effectiveness of the ethanol-$(NH_4)_2SO_4$ system for the separation of polyphenolic compounds in crude extracts, the ethanol concentration was varied at a constant $(NH_4)_2SO_4$ concentration, and vice versa. The parameter measured was the yield of *S. crispus* leaf extract, expressed as quercetin equivalents. The effectiveness of quercetin separation was determined based on the partition coefficient and extraction efficiency. Figure 3 shows the effect of the ethanol concentration on the extract yield at a constant $(NH_4)_2SO_4$ concentration (15%, w/w). Figure 4 shows the effect of the $(NH_4)_2SO_4$ concentration on the extract yield at a constant ethanol concentration (33%, w/w).

As shown in Figure 3, as the ethanol concentration increases at a constant $(NH_4)_2SO_4$ concentration, the yield of the upper phase extract, expressed by the TFC, also increases. Likewise, the extraction efficiency increases when the ethanol concentration increases. The highest TFC (0.1249 mg QE/g DLP) and extraction efficiency (98.19%) were obtained at a 35% ethanol concentration. The similar thing did not occur to the partition coefficient, which increased when the ethanol concentration increased from 29% to 33%; however, the partition coefficient decreased at a 35% ethanol concentration. A decrease in the partition coefficient showed that many compounds have been transferred to the aqueous phase because of the greater concentration of ethanol. A decrease in the partition coefficient at a 35% ethanol concentration might also be associated with an excessive increase in the volume ratio of ethanol (Figure 5).

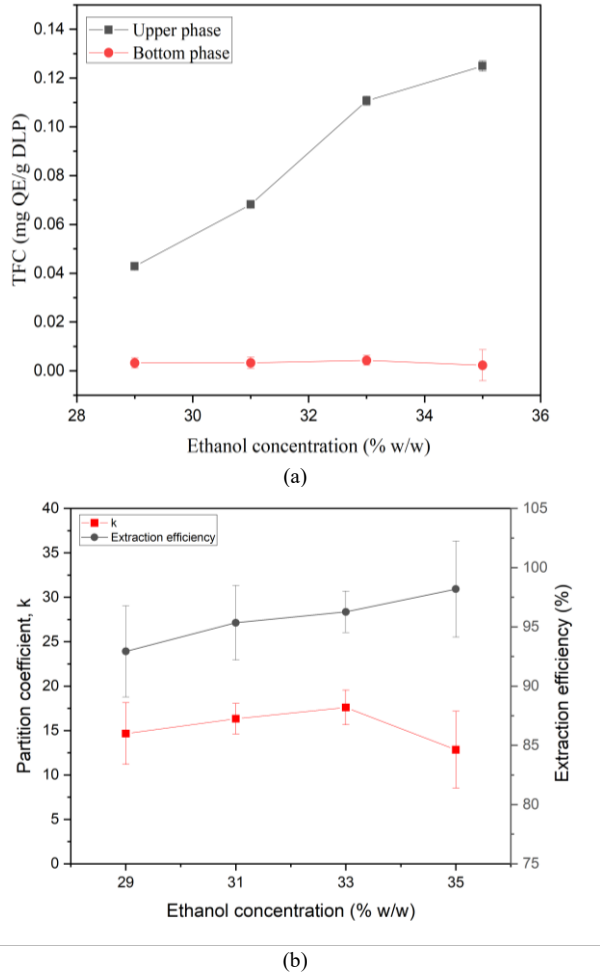
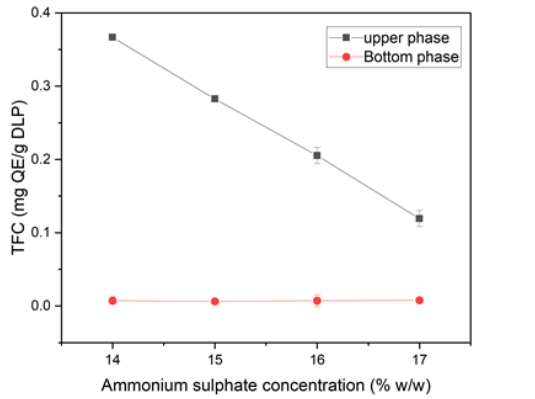


Fig. 3. Effect of the ethanol concentration on the ethanol-$(NH_4)_2SO_4$ system on the (a) TFC and (b) partition coefficient and extraction efficiency of *S. crispus* leaves at an $(NH_4)_2SO_4$ concentration of 15% (w/w)

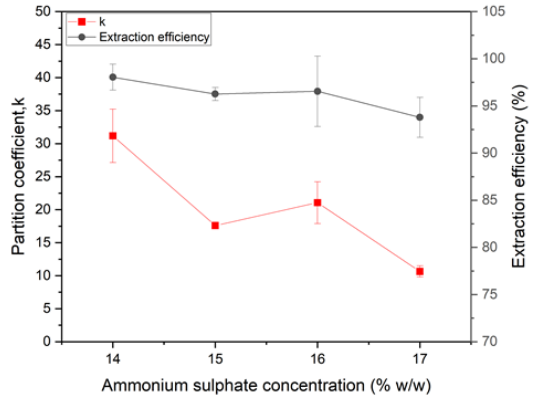
The same result was obtained by Dong (2015), who reported that the extract yield increased over a certain range of ethanol concentrations, but decreased when the concentrations went beyond this range. Dong (2015) argued that some compounds are stable in a certain range of ethanol concentrations. Therefore, in this study, a 33% ethanol concentration was chosen for further research.

Figure 4 shows that with an increasing $(NH_4)_2SO_4$ concentration at a constant ethanol concentration, the extract yield of the upper phase (expressed by the TFC value) decreases. The results obtained were corroborated by the extraction efficiency and partition coefficient, which also decreased as the $(NH_4)_2SO_4$ concentration increased. The highest TFC value (0.3666 mg QE/g DLP), partition coefficient

(98.04%), and extraction efficiency (31.17%) were obtained at a 14% $(NH_4)_2SO_4$ concentration.



(a)



(b)

Fig. 4. Effect of the $(NH_4)_2SO_4$ concentration on the ethanol-$(NH_4)_2SO_4$ system: (a) TFC value and (b) partition coefficient and extraction efficiency of *S. crispus* leaves at a 33% (w/w) ethanol concentration

When associating the volume ratio of the upper and lower phases, an increase in the concentration of $(NH_4)_2SO_4$ leads to a decrease in the volume ratio because the water mass is transferred to the lower phase, lowering the content of the compounds dissolved in the water (Figure 5) [27].

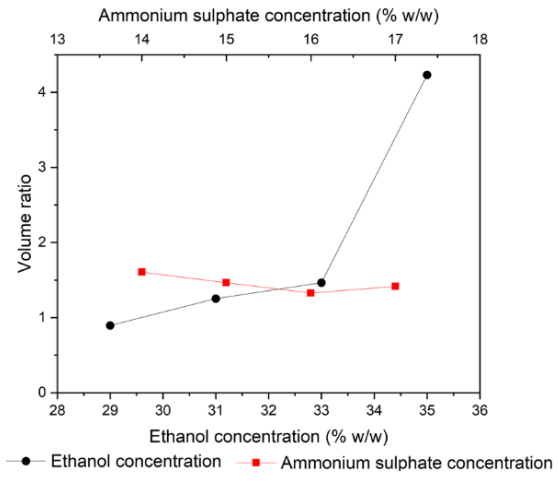


Fig. 5. Effect of the ethanol and $(NH_4)_2SO_4$ concentration on the volume ratio of the upper and lower phases

3.3. Comparison of UAE-ATPE with UAE and UAEE

The UAE-ATPE technique is a combination of the EAE, UAE, and ATPE techniques. In the current study, the combination of UAE-ATPE techniques was carried out simultaneously (i.e. the extraction, which began with EAE for 2 h was followed by the UAE and ATPE processes carried out simultaneously for 1 h). The same method was chosen by Yang (2018) who evaluated the combination of EAE and UAE, and found that the extraction technique, which started with EAE as a pretreatment followed by UAE, provided higher yields than EAE, UAE, or UAE followed by EAE. Table 2 shows the increasing yield obtained when using UAE-ATPE compared to UAE or UAEE.

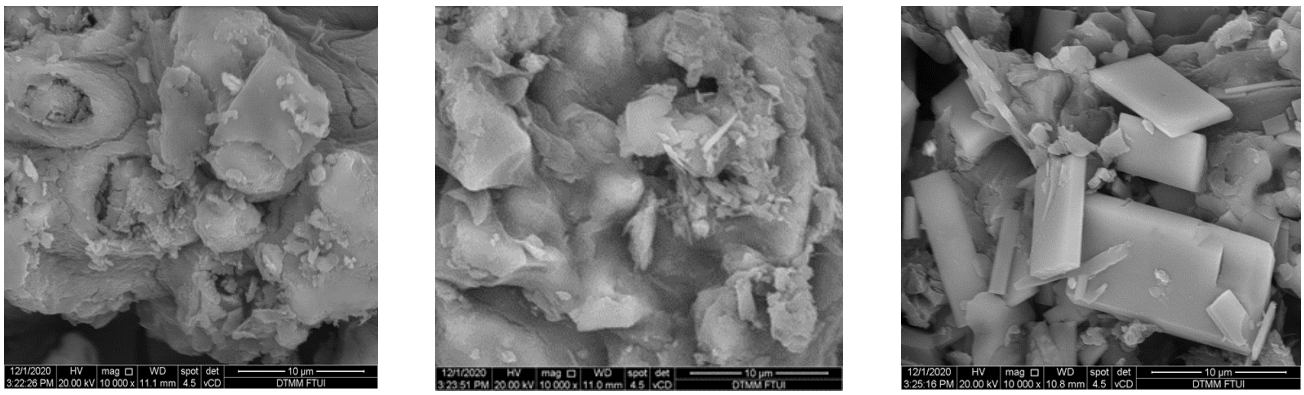
Table 2. Comparison of ultrasound extraction methods

Operating conditions	Methods		
	UAE	UAEE	UAE-ATPE
Solvent (%)	Ethanol = 50	Ethanol = 50	Ethanol/ $(NH_4)_2SO_4$ = 33/14
Extraction temperature (ºC)	27	27	27
Pretreatment stage	-	√	√
Extraction time (h)	1	1	1
Pretreatment time (h)	-	2	2
Yield (%)	25	48.63	77.81

In general, ultrasound-assisted extraction can provide higher yields compared to conventional techniques because the cavitation process can mechanically damage the plant cell walls and cause swelling to the plant material, thus facilitating solvent penetration into the plant cells and improving the extraction process [42]. The process of destroying the cell walls can also be carried out with the help of enzymes, which degrade the cell walls through hydrolysis. This allows a greater release of bioactive compounds compared to conventional processes. The rate of release of these bioactive compounds is also largely determined by the success of the hydrolysis, which depends on an interaction between the enzyme and substrate [20]. In UAEE and UAE-ATPE, the destruction of cell walls is carried out by both enzymes and ultrasound, so that they can have a greater effect on the solvent penetration and release of bioactive compounds. Incorporating ATP extraction to UAE-ATPE can increase the extract yield because of the decrease in the viscosity of the solution that results from the transfer of the hydrolysis products to the aqueous phase. This decrease in viscosity has a notable cavitation effect on solid materials, thereby increasing the yield of bioactive compounds.

SEM characterization was performed on three samples: DLP, DLP after enzymatic hydrolysis, and DLP after enzymatic hydrolysis and ultrasonic extraction processes. The goal was to evaluate the damage caused by hydrolysis and ultrasonic waves. After the hydrolysis with cellulase, ultrasonic waves enhanced the mechanical (mechanoacoustic) and chemical (sonochemical) processes. These two processes resulted in the separation, cracking, and degradation of the cellulose polymer [43]. The SEM characterization results are displayed in Figure 6.

The enzymatic hydrolysis of the DLP leads to fiber development, enlargement of the diameter of the cell lumen, and thinning of the cell walls as a result of the breakdown of cellulose in the cell walls of the leaves [39]. Figure 6 (b) shows that the cell walls of the leaves were increasingly damaged after



(a) (b) (c)

Fig. 6. SEM images (10,000× magnification) of (a) dry leaf powder (DLP), (b) DLP after enzymatic hydrolysis, and (c) DLP after enzymatic hydrolysis and ultrasonic extraction

enzymatic hydrolysis, compared to the cell walls of the untreated leaves (Figure 6 (a)). This result indicated the degradation of cellulose. In addition, the bonds between cellulose and hemicellulose were damaged.

The use of ultrasonic waves in the extraction process can further damage the cell wall structure [44], as shown in Figure 6 (c). The use of low-frequency ultrasonic waves (20–60 kHz) causes cavitation, which can damage the glycosidic bonds of cellulose that have been weakened by the action of cellulase [39]. Furthermore, in certain parts of the cell wall that are not too exposed to cellulase during hydrolysis, ultrasonic waves can cause acoustic cavitation that can cause frictional forces on the plant cell walls [39]. Thus, the use of ultrasonic waves leads to further damage to the cell walls.

3.4. Isolation of compounds from the ethanol extract of S. crispus leaves and antidiabetic activity test

The compounds in the ethanol extract of *S. crispus* leaves were isolated by column chromatography with toluene-ethyl acetate (1:1, v/v) as the mobile phase and silica gel 60 as the stationary phase. The collected eluate was tested for purity using thin-layer chromatography (TLC) with toluene-ethyl acetate (1:1, v/v) as the mobile phase and a G 60 F254 silica gel plate as the stationary phase. The isolated and identified fractions are shown in Table 3.

Six fractions were obtained by chromatographic separation. The six fractions, which showed a single TLC spot, were tested for antidiabetic activity and LC-MS/MS-Q-TOF characterization was performed.

Table 3. Monitoring results of the eluate fractions

Fraction	I	II	III	IV	V	VI
Retention factor (R_f)	0.96	0.91	0.68	0.58	0.51	0.18

The antidiabetic activities of the crude extract and isolated fractions from *S. crispus* leaves were studied *in vitro* by testing their ability to inhibit α-glucosidase. α-Glucosidase is a complex enzyme located in the small intestine that hydrolyzes disaccharides such as sucrose and maltose by cleaving the α-(1,4) bond to produce glucose and fructose monosaccharides, which can enter the bloodstream [45]. The inhibition of this enzyme can be applied as a therapy for type 2 diabetes because this inhibition delays or reduces the absorption of carbohydrates [45]. Acarbose is used as a reference compound as it is a medicine for treating high glucose levels in type 2 diabetes.

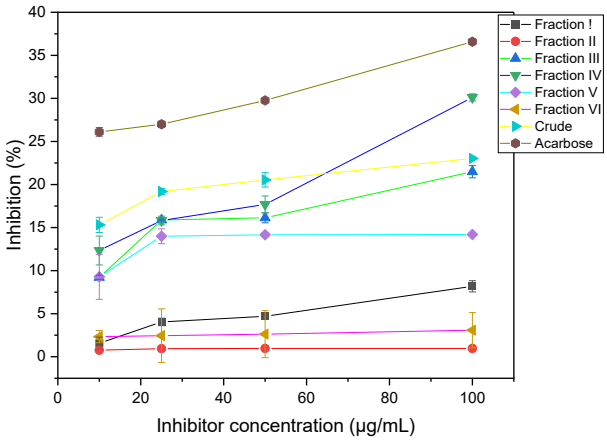
The antidiabetic activity can also be analyzed *in vitro* through the inhibition of α-amylase (an enzyme that breaks down starch and produces maltose) by bioactive compounds [46]. Joseph (2016) performed an *in vitro* analysis of the *S. cuspidate* leaf extract and found that the total saponins, lupeol, and stigmasterol isolated from the extract exhibited a high inhibition activity against α-amylase, indicating that they have the antidiabetic potential [46].

Joseph (2016) also carried out *in vivo* testing of the antidiabetic activity of the compounds in the *S. cuspidate* leaf extract. This author injected some rats with alloxan to induce diabetes and treated the animals with metformin and the extract. When the ethanol extract of *S. cuspidate* leaves was used at concentrations of 150 and 300 mg/kg, blood sugar levels were reduced by 38% and 41%, respectively [46]. Fadzelly (2006) conducted an *in vivo* test on some hyperglycemic rats induced by streptozotocin by administering fermented and unfermented *S. crispus* leaf extract. Glibenclamide was used for comparison. The high content of antioxidants and polyphenols in *S. crispus* leaves contributes to the antihyperglycemic properties of the leaves. Fadzelly (2006) also suspected the presence of epicatechin, a flavonoid with insulin-like properties, in the leaves of *S. crispus*.

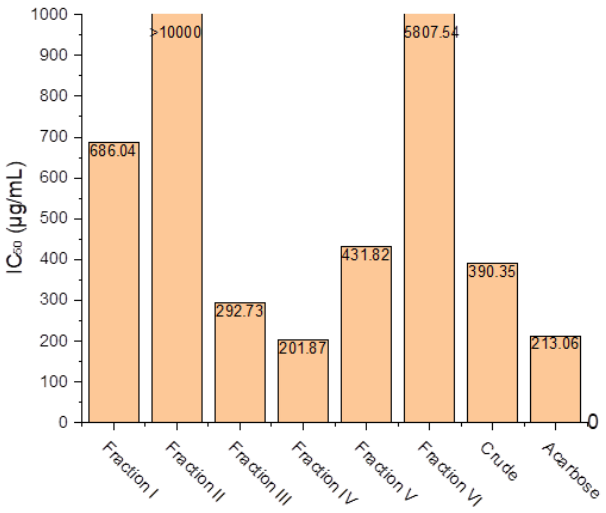
In the current study, the antidiabetic activity of the ethanol extract of *S. crispus* leaves and its fractions were expressed in IC_{50}, representing the concentration of a compound that inhibiting α-glucosidase activity by 50%. The lower the IC_{50} value of a compound, the higher its ability to inhibit α-glucosidase. The IC_{50} values of various extract concentrations and fractions are shown in Figures 7(a) and (b); acarbose was used for comparison.

Figure 7(a) shows the inhibitory effect of various concentrations of the extract and fractions on α-glucosidase. Acarbose exhibited a greater inhibitory effect than the *S. crispus* extract and its fractions for the concentrations tested. However, the curve revealed that the rate of increase in α-glucosidase inhibition of fraction IV was greater than that of acarbose so that at 50% inhibition, the concentration of fraction IV was lower than that of acarbose. Fraction III also showed a higher rate of increase in α-glucosidase inhibition than other

fractions, followed by the crude extract, fraction I, and fraction V. By contrast, fractions II and VI showed a low α-glucosidase inhibition ability as revealed by the flat shapes of the curves.



(a)



(b)

Fig. 7. Antidiabetic activities of the crude ethanol extract of *S. crispus* and its fractions expressed by (a) percent of inhibition, and (b) IC_{50} value; acarbose was used as a standard drug

To obtain the IC_{50} value, the curve in Fig. 7(a) was extrapolated to the 50% inhibition on the y-axis, and the IC_{50} became the corresponding concentration on the x-axis. In the α-amylase enzyme inhibition experiment carried out by Joseph (2016), for up to 50% enzyme inhibition, the data showed linearity and started sloping afterward. Therefore, Joseph (2016) determined the IC_{50} value of α-amylase inhibition using linear regression based on the inhibition percentage test conducted by Rao.

Figure 7(b) shows that the IC_{50} values of the crude extract of *S. crispus* leaves and its fractions were higher than that of acarbose (213.06 µg/mL), except for fraction IV, which had an IC_{50} value lower than that of acarbose (201.87 µg/mL). It is noteworthy that the IC_{50} value of acarbose obtained in our study was higher than the IC_{50} value of acarbose as reported by Wang (2018). The IC_{50} value for acarbose obtained by Wang was 101.12 micrograms/mL [47]. This difference may be attributed to variations in factors such as extraction methods, sample sources, or analytical conditions. The IC_{50} value of fraction III (292.73 µg/mL) was lower than that of the crude extract. Therefore, the compounds in this fraction had a better antidiabetic activity compared to the crude extract although the IC_{50} value was higher than that of acarbose. Fraction V also inhibited α-glucosidase, even though its IC_{50} value (431.82 µg/mL) was much higher than that of acarbose. The crude extract had an IC_{50} value of 390.35 µg/mL, which was much higher than that of acarbose, fraction III, and fraction IV. This outcome may be due to the presence of many bioactive compounds in the crude extract, which may be antagonistic to the fraction that exhibited a strong activity. Fractions II and VI showed very high IC_{50} values, suggesting that they had no potential as antidiabetic drugs. Figure 7(b) shows that the IC_{50} values of fractions III and IV were lower than those of the crude extract and fraction V. Hence, the compounds in these fractions had a better antidiabetic activity than those in other fractions. Moreover, this validated the idea that isolating the components of natural extracts can provide an increasing level of bioactivity [48].

The same results were obtained by Joseph (2016), who reported that the ethanol extract of *S. cuspidate* leaves, total saponins, stigmasterol, and lupeol, had IC_{50} values comparable to that of acarbose. Therefore, the isolation of compounds is crucial to obtain an enhanced bioactivity.

To determine the type of inhibition of the most active agent (i.e., fraction IV), a series of α-glucosidase inhibition tests were performed at two concentrations (0 and 10 µg/mL). Lineweaver-Burk plots that illustrate the $1/[v]$-$1/[S]$ curve were used to linearize the data.

The Lineweaver-Burk plot of fraction IV (Figure 8) revealed that the type of inhibition observed was consistent with mixed inhibition kinetics. This implied a change in the Michaelis-Menten constant and the maximum enzymatic reaction rate occurs when comparing the enzymatic reaction with the inhibitor (fraction IV) and without it [49]. The inhibitor's mechanism is illustrated in Figure 9.

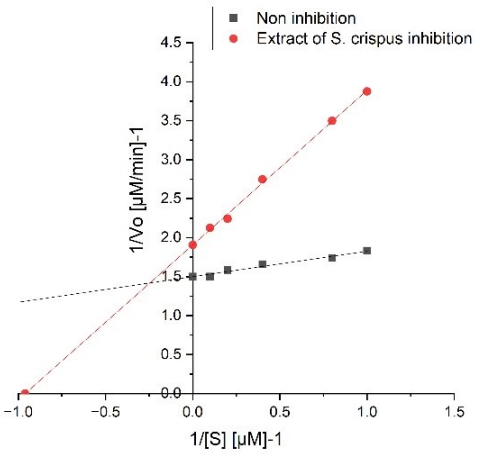


Fig. 8. Lineweaver-Burk plot for various concentrations of fraction IV

3.5. Elucidation of the molecular structure of the antidiabetic compound

The molecular structure of the antidiabetic compound was determined through LC-MS/MS-Q-TOF analysis. Table 4 presents the bioactive compounds identified in the isolated

crude extract using LC-MS/MS-Q-TOF. The structure of the bioactive compound is visualized in Figure 10.

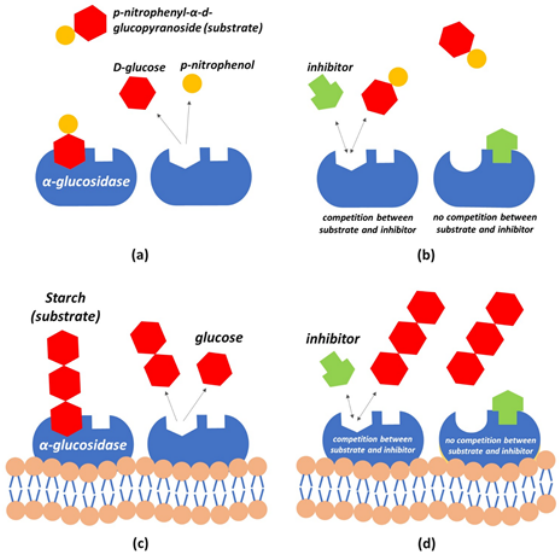


Fig. 9. Illustration of inhibition mechanism of α-Glucosidase by bioactive compounds: a) hydrolysis and (b) mixed inhibition mechanism of α-glucosidase with *S. crispus* isolate as inhibitor ; (c) hydrolysis and (d) the possible inbition mechanism of α-glucosidase by S. crispus isolate in human body

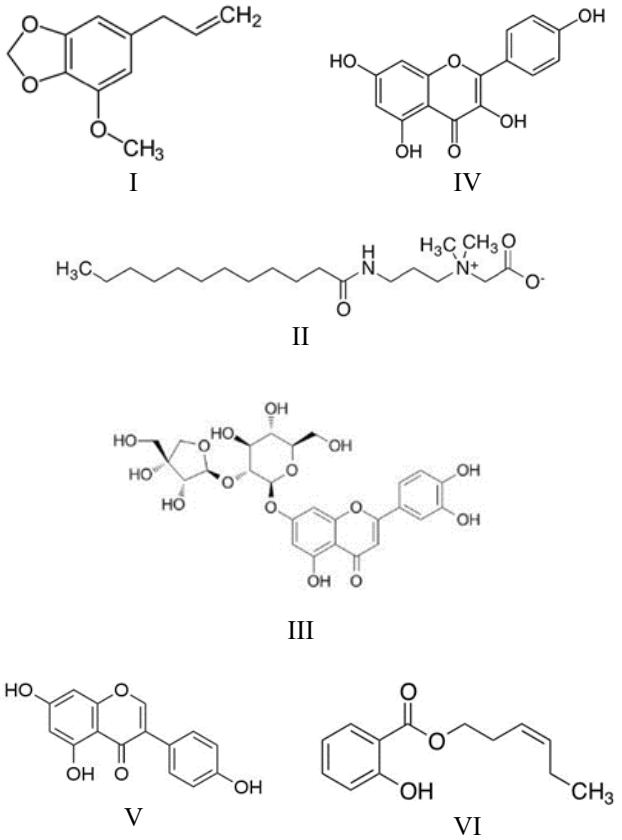


Fig. 10. The molecular structure of the bioactive compound isolated from *S. crispus* leaf extract

Based on LC-MS/MS-Q-TOF analysis, the compound in the most active fraction (fraction IV) was identified as kaempferol, a flavonol with a diphenylpropane structure synthesized through the condensation of p-coumaroyl-CoA (C_6-C_3) with three molecules of malonyl-CoA (C_6) catalyzed by chalcone synthase [49]. Kaempferol binds to α-glucosidase via hydrogen bonds and van der Waals forces with a fairly high affinity [32]. Many studies have reported the relationship between the intake of foods high in kaempferol (e.g. beans, apples, spinach, onions, strawberries, and broccoli) and a lowered risk of cancer [50]. Peng (2016) stated that kaempferol (analytical grade) exhibited an antidiabetic activity with an IC_{50} value of 4.580. µg/mL and showed potential as an antidiabetic drug. This IC_{50} value is much smaller than the one obtained in this study, which may be due to differences in the purity of the kaempferol used. Based on the LC-MS/MS-Q-TOF characterization, the kaempferol content in the ethanol extract of *S. crispus* leaves was 24.29%.

Table 4. Identified bioactive compounds from the *S. crispus* leaf extract

Retention time (t_R)	Area (%)	Identified compounds' formulas	Fraction
1.81	2.40	$C_{26}H_{28}O_{15}$	III
7.48	24.29	$C_{15}H_{10}O_6$	IV
10.60	3.62	$C_{15}H_{10}O_5$	V
13.12	3.75	$C_{13}H_{16}O_3$	VI
15.41	29.37	$C_{11}H_{12}O_3$	I
18.77	11.17	$C_{19}H_{38}N_2O_3$	II

Table 5. Comparison of key aspects between Joseph's (2016) study and this research

Aspects	Joseph (2016)	This research
Source	S. cuspidate	S. crispus
Extraction Technique	Soxhlet	Ultrasound-assisted enzymatic aqueous two-phase
Extraction Duration	24 h	2 h enzymatic hydrolysis and 1 h extraction
Testing Method	α-amylase enzyme inhibition	α-glucosidase enzyme inhibition
Bioactive compound	Saponin, triterpene (lupeol), sterol (stigmasterol)	Flavonoid (kaempferol, graveobioside A, genistein)

The compounds in fractions III and V, which showed potential as drugs for type 2 diabetes, were identified as graveobioside A and genistein. Both compounds are flavonoids. Graveobioside A obtained from the ethanol extract of celery was proven to have antiproliferative potential [51] and genistein from *Genista tinctoria* exhibited anticancer activity [52]. *S. crispus* leaves were found to contain a very small amount of graveobioside A and genistein. The most abundant compound was identified as myristicin, a phenylpropanoid isolated from fraction I. However, the IC_{50} value of this compound was quite high compared to that of acarbose.

According to Joseph (2016), the antidiabetic activity of the ethanol extract from S. cuspidate leaves is attributed to the presence of hypoglycemic saponins and triterpene compounds. In contrast, our research revealed that flavonoid compounds derived from the S. crispus extract also demonstrated an antidiabetic activity. Several differences exist between Joseph's (2016) study and our research, as evident in the following table.

Based on the table, it becomes evident that there are substantial disparities between Joseph's (2016) study and our own research. These differences highlight an intriguing area that warrants further investigation, potentially necessitating additional studies to elucidate the relative roles of flavonoids, saponins, and triterpenes in the antidiabetic activity of these plants. In general, it is well-established that plants contain bioactive components capable of enhancing insulin receptor sensitivity, stimulating Langerhans β-cells to release insulin, or boosting carbohydrate metabolism, all of which contribute to the reduction of blood sugar levels [46].

4. Conclusion

The proposed UAE-ATPE, which combines ultrasound, enzymatic, and aqueous two-phase extractions, is a promising and environmentally friendly method for natural product extraction. The yield obtained with the combined method was greater than that the one obtained with UAE and UAEE. This finding was corroborated by SEM characterization. Six compounds were isolated from the ethanol extract of *S. crispus*, using toluene-ethyl acetate (1:1, v/v) as the mobile phase. Kaempferol was the bioactive compound showing the highest antidiabetic activity with an IC50 value of 201.87 µg/mL and a mixed type of inhibition of α-glucosidase. Based on the LC-MS/MS-Q-TOF characterization, the kaempferol content in the ethanol extract of *S. crispus* leaves was 24.29%. To increase the yield of extracts with antidiabetic activity, optimizing the extraction process is deemed necessary for future studies.

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References

1. A. Agoes. *Tanaman Obat Indonesia*. Jakarta: Penerbit Salemba Medika, 2019.
2. M. Ismail, E. Manickam, A. M. Danial, A. Rahmat, and A. Yahaya, *Chemical composition and antioxidant activity of Strobilanthes crispus leaf extract*, J. Nutr. Biochem. 11 (2000) 536–542.
3. Y. S. Baraya, K. K. Wong, and N. S. Yaacob, *Strobilanthes crispus inhibits migration, invasion and metastasis in breast cancer*, J. Ethnopharmacolology 233 (2019) 13-21.
4. M. Aziz, S. Ahmad, M.N. Iqbal, U. Khurshid, H. Saleem, Kashif-ur-Rehman, A. Alamri, S. Anwar, A.S. Alamri, and T.A. Chohan, *Phytochemical, pharmacological, and In-silico molecular docking studies of Strobilanthes glutinosus Nees: An unexplored source of bioactive compounds*, S. Afr. J. Bot. 147 (2022) 618–627.
5. Mahmood A.A., A.A. Fard, Harita H., Zahra A. A., and Salmah I., *Evaluation of gastroprotective effects of Strobianthes crispus leaf extract on ethanol-induced gastric mucosal injury in rats*, Sci. Res. Essays. 6 (2011) 2306-2314.
6. N.A. Norfarizan-Hanoon, R. Asmah, M.Y. Rokiah, O. Fauziah, and H. Faridah, *Effects of Strobilanthes crispus juice on wound healing and antioxidant enzymes in normal and Streptozotocin-induced diabetic rats*, J. Biol. Sci. 9 (2009) 662–668.
7. A.B.M. Fadzelly, R. Asmah, and O. Fauziah, *Effects of Strobilanthes crispus tea aqueous extracts on glucose and lipid profile in normal and streptozotocin-induced hyperglycemic rats*, Plant Foods Hum. Nutr. 61 (2006) 7–12.
8. R. A. Aras, T. Lestari, H. A. Nugroho, I. Ardiyanto, *Segmentation of retinal blood vessels for detection of diabetic retinopathy: A review*, Commun. Sci. Technol. 1 (2016) 33-41
9. H. M. Yankuzo, Y. S. Baraya, Z. Mustapha, K. K. Wong, and N. S. Yaacob, *Immunomodulatory effects of a bioactive fraction of Strobilanthes crispus in NMU-induced rat mammary tumor model*, J. Ethnopharmacol. 213 (2018) 31–37.
10. Y-Y. Siew, H-C. Yew, S-Y. Neo, S-V. Seow, S-M. Lew,. S.W. Lim, CSE-S. Lim, Y-C. Ng, W-G. Seetoh, A. Ali, C.H. Tan, and H-L. Koh,. *Evaluation of anti-proliferative* S-W *activity of medicinal plants used in Asian Traditional Medicine to treat cancer*, J. Ethnopharmacol. 235 (2019) 75–87.
11. A. Ghasemzadeh, H. Z. E. Jaafar, and A. Rahmat, *Phytochemical constituents and biological activities of different extracts of Strobilanthes crispus (L.) Bremek leaves grown in different locations of Malaysia*, BMC Complement. Altern. Med. 15 (2015) 1-10.
12. W. Gu, W. Wang, X. Li, Y. Zhang, L. Wang, C. Yuan, L. Huang, and X. Hao, *A novel isocoumarin with anti-influenza virus activity from Strobilanthes cusia*, Fitoterapia 107 (2015) 60–62.
13. B. Singh, P.M. Sahu, and M.K. Sharma, *Anti-inflammatory and antimicrobial activities of triterpenoids from Strobilanthes callosus Nees*, Phytomedicine 9 (2002) 355-359.
14. J. Zhu, X. Kou, C. Wu, G. Fan, T. Li, J. Dou, and D. Shen, *Enhanced extraction of bioactive natural products using ultrasound-assisted aqueous two-phase system: Application to flavonoids extraction from jujube peels*, Food Chem. 395 (2022) 1-9.
15. B. K. Tiwari, *Ultrasound: A clean, green extraction technology*, Trends Anal. Chem. 71 (2015) 100–109.
16. M. Singla and N. Sit, *Application of ultrasound in combination with other technologies in food processing: A review*, Ultrason. Sonochem. 73 (2021) 1-13.
17. J. Q. Quiroz, A. M. N. Duran, M. S. Garcia, G. L. C. Gomez, and J. J. R. Camargo, *Ultrasound-assisted extraction of bioactive compounds from annatto seeds, evaluation of their antimicrobial and antioxidant activity, and identification of main compounds by LC/ESI-MS analysis*, Int. J. Food Sci. 2019 (2019) 1-9.
18. H.S. Kusuma, P.D. Amelia, C. Admiralia, and M. Mahfud, *Kinetics study of oil extraction from Citrus auranticum L. by solvent-free microwave extraction,* Commun. Sci. Technol. 1 (2016) 15-18.
19. J-B. Pu, B-H. Xia, Y-J. Hu, H-J. Zhang, J. Chen, J. Zhou, W-Q. Liang, and P. Xu, *Multi-optimization of ultrasonic-assisted enzymatic extraction of atratylodes macrocephala polysaccharides and antioxidants using response surface methodology and desirability function approach,* Molecules 20 (2015) 22220–22235.
20. S. J. Marathe, S. B. Jadhav, S. B. Bankar, K. K. Dubey, and R. S. Singhal, *Improvements in the extraction of bioactive compounds by enzymes*, Curr. Opin. Food Sci. 25 (2019) 62–72.
21. W. Tchabo, Y. Ma, F. N. Engmann, and H. Zhang, *Ultrasound-assisted enzymatic extraction (UAEE) of phytochemical compounds from mulberry (Morus nigra) must and optimization study using response surface methodology*, Ind. Crops Prod. 63 (2015) 214–225.
22. Y. Gao, Y. Shi, N. Miao, W. Xing, C. Yun, S. Wang, W. Wang, and H. Wang, H. *A green ultrasound-assisted enzymatic extraction method for efficient extraction of total polyphenols from Empetrum nigrum and determination of its bioactivities*, J. Ind. Eng. Chem. 109 (2022) 559–567.
23. O. Gligor, A. Mocan, C. Moldovan, M. Locatelli, G. Crişan, and I. C. F. R. Ferreira, *Enzyme-assisted extractions of polyphenols – A comprehensive review*, Trends Food Sci. Technol. 88 (2019) 302–315.
24. M. Puri, D. Sharma, and C. J. Barrow, *Enzyme-assisted extraction of bioactives from plants*, Trends Biotechnol. 30 (2012) 37–44.
25. X. Zhang, G. Teng, and J. Zhang, *Ethanol/salt aqueous two-phase system based ultrasonically assisted extraction of polysaccharides from Lilium davidiivar. unicolor Salisb: Physicochemical characterization and antiglycation properties*, J. Mol. Liq. 256 (2018) 497–506.
26. J. Zhu, X. Kou, C. Wu, G. Fan, T. Li, J. Dou, and D. Shen, *Enhanced extraction of bioactive natural products using ultrasound-assisted aqueous two-phase system: Application to flavonoids extraction from jujube peels*, Food Chem. 395 (2022) 1-9.
27. B. Dong, X. Yuan, Q. Zhao, Q. Feng, B. Liu, Y. Guo, and B. Zhao, *Ultrasound-assisted aqueous two-phase extraction of phenylethanoid*

glycosides from Cistanche deserticola Y. C. Ma stems, J. Sep. Sci. 38 (2015) 1194–1203.
28. Z. Cheng, H. Song, X. Cao, Q. Shen, D. Han, F. Zhong, H. Hu, and Y. Yang, *Simultaneous extraction and purification of polysaccharides from Gentiana scabra Bunge by microwave-assisted ethanol-salt aqueous two-phase system*, Ind. Crops Prod. 102 (2017) 75–87.
29. H. Wijayanti, P. Mardina, A. Tuhuloula, L. T. Ananda, Z. S. A. Rauf, A. Lutfi, S. F. Riyadi, *Evaluation of stirring rate and pH on phenolic compounds recovery from palm kernel shell heavy phase bio-oil*, Commun. Sci. Technol. 8 (2023) 25-30.
30. J. C. W. Ouédraogo, C. Dicko, F. B. Kini, Y. L. Bonzi-Coulibaly, and E.S. Dey, *Enhanced extraction of flavonoids from Odontonema strictum leaves with antioxidant activity using supercritical carbon dioxide fluid combined with ethanol*, J. Supercrit. Fluids. 131 (2018) 66–71.
31. A. Malik, H. Ardalani, S. Anam, L.M. McNair, K.J.K. Kromphardt, R.J.N. Frandsen, H. Franzyk, D. Staerk, and K.T. Kongstad, *Antidiabetic xanthones with α-glucosidase inhibitory activities from an endophytic Penicillium canescens*, Fitoterapia 142 (2020) 1-8.
32. X. Peng, G. Zhang, Y. Liao, and D. Gong, *Inhibitory kinetics and mechanism of kaempferol on α-glucosidase*, Food Chem. 190 (2016) 207–215.
33. S. S. Nadar, P. Rao, and V. K. Rathod, *Enzyme assisted extraction of biomolecules as an approach to novel extraction technology: A review*, Food Res. Int. 108 (2018) 309–330.
34. N. Liao, J. Zhong, X. Ye, S. Lu, W. Wang, R. Zhang, J. Xu, S. Chen, and D. Liu, *Ultrasonic-assisted enzymatic extraction of polysaccharide from Corbicula fluminea: Characterization and antioxidant activity*, LWT – Food Sci. and Tech. 60 (2015) 1113–1121.
35. N. Rokhati, Ratnawati, A. Prasetyaningrum, W. Anggraini, A. Nugroho, N. H. Novita, P. Andarani, and T. Riyanto, *Effect of microwave and ultrasonic irradiation on the enzymatic hydrolysis of water hyacinth biomass in the presence of surfactants*, Commun. Sci. Technol. 8 (2023) 57-65.
36. Y. Yang, Z. Wang, D. Hu, K. Xiao, and J. Y. Wu, *Efficient extraction of pectin from sisal waste by combined enzymatic and ultrasonic process*, Food Hydrocolloid. 79 (2018) 189–196.
37. R. Saeed, D. Ahmed, and M. Mushtaq, *Ultrasound-aided enzyme-assisted efficient extraction of bioactive compounds from Gymnema sylvestre and optimization as per response surface methodology*, Sustain. Chem. Pharm. 29 (2022) 1-15.
38. S. B. Zhang, Z. Wang, and S. Y. Xu, *Optimization of the aqueous enzymatic extraction of rapeseed oil and protein hydrolysates*, JAOCS 84 (2007) 97–105.
39. D. Huang, X. Zhou, J. Si, X. Gong, and S. Wang, *Studies on cellulase ultrasonic-assisted extraction technology for flavonoids from Illicium verum residues*, Chem. Cent. J. 10 (2016) 1-9.
40. X. Luo, R. Bai, D. Zhen, Z. Yang, D. Huang, H. Mao, X. Li, H. Zou, Y. Xiang, K. Liu, Z. Wen, and C. Fu, *Response surface optimization of the enzyme-based ultrasound-assisted extraction of acorn tannins and their corrosion inhibition properties*, Ind. Crops Prod. 129 (2019) 405–413.
41. L. Zhou, B. Jiang, T. Zhang, and S. Li, *Ultrasound-assisted aqueous two-phase extraction of resveratrol from the enzymatic hydrolysates of Polygonum cuspidatum*, Food Biosci. 31 (2019) 1-9.
42. H. Wijngaard, M. B. Hossain, D. K. Rai, and N. Brunton, *Techniques to extract bioactive compounds from food by-products of plant origin*, Food Res. Int. 46 (2012) 505–513.
43. Y. Sun and J. Cheng, *Hidrolysis of lignocellulosic materials for ethanol production: a review*, Bioresour. Technol. 83 (2002) 1-11.
44. T. Guo, D. Su, Y. Huang, Y. Wang, and Y. H. Li, *Ultrasound-assisted aqueous two-phase system for extraction and enrichment of zanthoxylum armatum lignans*, Molecules 20 (2015) 15273–15286.
45. F. Chávez-Silva, L. Ceron-Romero, L. Arias-Duran, G. Navarrete - Vázquez, J. Almanza-Pérez, R. Román-Ramos, G. Ramírez-Ávila, I. Perea-Arango, R. Villalobos-Molina, and S. Estrada-Soto, *Antidiabetic effect of Achillea millefollium through multitarget interactions: α-glucosidases inhibition, insulin sensitization and insulin secretagogue activities*, J. Ethnopharmacology 212 (2018) 1–7.
46. S. Joseph, L. Kumar, and V. N. Bai, *Evaluation of anti-diabetic activity of Strobilanthes cuspidata in alloxan induced diabetic rats and the effect of bioactive compounds on inhibition of α-amylase enzyme*, J. Pharmacogn. Phytochem. 5 (2016) 169–175.
47. C. Wang, W. Li, Z. Chen, X. Gao, G. Yuan, Y. Pan, and H. Chen, *Effect of simulated gastrointestinal digestion in vitro on the chemical properties, antioxidant activity, alpha-amilase and alpha glucosidase inhibitory activiti of polysaccharides from Innotus obliquus*, Food Res. Int. 103 (2018) 280-288.
48. R. J. Colegate, S. M., & Molyneux. *Bioactive Natural Products : Detection, Isolation, and Structural Determination*. Boca Raton: CRC Press, 2008.
49. M. L. Shuler and F. Kargi. *Bioprocess Engineering Basic Concepts*, 2nd ed. Upper Sadle River, NJ: Prentice Hall PTR, 2002.
50. M. Lin, J. Zhang, and X. Chen, *Bioactive flavonoids in Moringa oleifera and their health-promoting properties*, J. Funct. Foods. 47 (2018) 469–479.
51. S.S.T. Ahmed, J.R. Fahim, K.A. Youssif, M.N. Amin, H.M.H. Abdel-Aziz, I.A. Khadra, M.E. Rateb, U.R. Abdelmohsen, and A.N.E. Hamed, *Comparative study of the chemical composition and anti-proliferative activities of the aerial parts and roots of Apium graveolens L. (celery) and their biogenic nanoparticles*, S. Afr. J. Bot. 151 (2022) 34–45.
52. X. Chen, J. Gu, Y. Wu, P. Liang, M. Shen, J. Xi, and J. Qin, *Clinical characteristics of colorectal cancer patients and anti-neoplasm activity of genistein*, Biomed. Pharmacother. 124 (2020) 1-5.