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A comparative study of sequential and simultaneous enzymatic and ultrasound-assisted aqueous two-phase extraction for anticholesterol compounds from *Strobilanthes crispus* leaves

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Abstract: An innovative ultrasonic-assisted enzymatic aqueous two-phase extraction (UAE-ATPE) method was applied to enhance the yield from *Strobilanthes crispus* leaves, exploring both sequential and simultaneous approaches. Comparative analysis included assessing total phenolic content (TPC), total flavonoid content (TFC), partition coefficient (k) and recovery (R). Liquid chromatography-mass spectrometry and scanning electron microscopy evaluated extracts from both techniques. Simultaneous UAE-ATPE demonstrated significantly higher TPC (5.7±0.1 mg GAE/g dry leaves) and TFC (3.3±0.1 mg QE/g dry leaves) compared to sequential extraction, where TPC and TFC measured 4.5±0.3 mg GAE/g dry leaves and 1.7±0.1 mg QE/g dry leaves. Additionally, simultaneous UAE-ATPE yielded higher k and R values for phenolic and flavonoid compounds. Notably, it identified 32.4% of the area corresponding to 6 compounds, surpassing the 25.3% area identified sequentially with 13 compounds. A collaborative effect of enzymatic hydrolysis and ultrasonic extraction was observed in simultaneous UAE-ATPE. In the inhibition test on the HMG-CoA reductase enzyme, simultaneous UAE-ATPE extract (200 µg/mL) exhibited exceptional results, achieving superior inhibition of 66.1% compared to the sequential method's inhibition of 39.4%. This underscores the efficacy of simultaneous UAE-ATPE in producing concentrated anti-cholesterol compounds. The study strongly emphasizes the superiority of simultaneous UAE-ATPE over the sequential approach.

Keywords: Enzymatic, ultrasound, aqueous two-phase, extraction, sequential, simultaneous.

INTRODUCTION

With the increasing global incidence of cardiovascular diseases, there is a growing demand for effective anticholesterol medication. Although statins are currently effective in reducing cholesterol, long-term consumption carries the potential for serious side effects (Baskaran *et al*., 2015; Betowski *et al*., 2009). Research has demonstrated that phenolic and flavonoid compounds could serve as alternative substitutes for statins, showing potential as inhibitors of HMG-CoA reductase (Seenivasan *et al*., 2011).

Strobilanthes crispus (*S. crispus*), commonly known as pecah beling' and yellow strobilanthus, is native to Madagascar, Indonesia and Malaysia (Zakaria *et al*., 2023). Classified under the Acanthaceae family, this plant is characterized by lance-shaped leaves with a rough, small-hair-covered surface (Baraya *et al*., 2021; Zakaria *et al*., 2023). With a long history of traditional use in Indonesia, Malaysia and other countries, Strobilanthes crispus has been employed to address a spectrum of health conditions, including cancer, gastrointestinal and kidney diseases, diabetes mellitus and hypertension (Chen *et al*., 2023).

Acknowledged for its abundant polyphenolic and flavonoid compounds, such as rutin, (+)-catechin, (-)-epicatechin, luteolin, myricetin, naringenin, apigenin and kaempferol, alongside alkaloids, caffeine and tannins (Ramadhani *et al*., 2021; Zakaria *et al*., 2023), this plant exhibits not only anti-hypercholesterolemic activity but also various therapeutic properties both *in vivo* and *in vitro*. These include antidiabetic, laxative, anticancer, diuretic, anti-inflammatory, antiobesity and immune-boosting effects (Baraya *et al*., 2021; Norfarizan-Hanoon *et al*., 2009; Ramadhani *et al*., 2021; Yankuzo *et al*., 2018; Zakaria *et al*., 2023), positioning it as a noteworthy subject for comprehensive scientific investigation.

Ultrasound-assisted aqueous two-phase extraction (UA-ATPE) and enzyme-assisted extraction (EAE) represent sustainable techniques for obtaining bioactive compounds. UA-ATPE offers efficiency, eco-friendliness and promising extraction bioactive compounds (Devgan *et al*., 2013; Dong *et al*., 2015; Guo *et al*., 2015; Ji *et al*., 2018; Zhang *et al*., 2018; Zhou *et al*., 2019; Zhu *et al*., 2022). EAE, an eco-friendly method, gains attention for its efficiency and ease of operation (Gligor *et al*., 2019; Nadar *et al*., 2018). Numerous studies have shown that different extraction methods have adverse effect on the pharmacological activities of the plant extract (Devgan *et al*., 2013; Khalili *et al*., 2016; Samejo *et al*., 2013). In the pursuit of developing anti-cholesterol drugs from *S.*

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crispus, combining UA-ATPE with enzymatic hydrolysis is intriguing.

Previous studies have reported the efficacy of ultrasound-assisted enzymatic aqueous two-phase extraction (UAE-ATPE) in maximizing the production of bioactive compound from *S. crispus* leaves (Arbianti *et al*., 2022). This study investigates the effect of sequential and simultaneous enzymatic hydrolysis on UAE-ATPE of *S. crispus* leaves. The aim is to assess the extraction strategy in terms of yield, selectivity and bioactive compound composition, exploring potential anti-cholesterol properties.

To comprehensively characterize extracted compounds, advanced analytical techniques, including Liquid Chromatography-Mass Spectrometry/Mass Spectrometry with Quadrupole Time-of-Flight (LC-MS/MS-Q-TOF) and Scanning Electron Microscopy (SEM), are employed.

MATERIALS AND METHODS

Material

S. crispus leaves, sourced from Kulon Progo, Daerah Istimewa Yogyakarta, serve as simplicia for extraction. The extraction process utilized ethanol (CH_3CH_2OH) and ammonium sulfate ($(NH_4)_2SO_4$) by Merck as the solvent. The hydrolysis reaction employed Cellulase enzyme from *Aspergillus niger*, obtained from Sigma Aldrich, as a biocatalyst. To create a phosphate buffer with a pH of 6.5, basic material such as dipotassium phosphate (K_2HPO_4) and monopotassium phosphate (KH_2PO_4) from Merck were used. Total phenolic content (TPC) test utilized Folin-Ciocalteu reagen from Merck, total flavonoid content (TFC) test involved the use of CH_3COOK and $AlCl_3$ from Merck. Gallic acid and quercetin served as standard parameters in test for TPC and TFC.

S. crispus leaves preparation

S. crispus leaves are obtained in powder form, eliminating the need for size reduction. The preparation initiates with sifting using a sieve analyzer (Sieve Shaker Electric Timer-Zia, Indonesia) with an 80-mesh size. The sifted simplicial is then stored in a desiccator before the extraction process begins.

Enzymatic hydrolysis

The enzymatic hydrolysis reaction in the sequencial extraction method followed the procedure outlined by Angelina (2019). The cellulase enzyme was applied at an enzyme-solid ratio of 70 mg/g dried leaves. A phosphate buffer with a pH of 6.5, prepared from K_2HPO_4 and KH_2PO_4 was utilized. The reaction was conducted for 2 hours at a temperature of 30°C with stirring at 700 rpm.

S. crispus leaves extraction

S. crispus leaves underwent extraction using the UAE-ATPE method, employing either sequential or simultaneous enzymatic hydrolysis reactions. In the sequential enzymatic hydrolysis approach of the UAE-ATPE method, the procedure commenced with enzymatic hydrolysis, as explained previously. Subsequently, the extraction process ensued by introducing 50 mL CH_3CH_2OH and 16.74 g $(NH_4)_2SO_4$, until the ratio CH_3CH_2OH:$(NH_4)_2SO_4$ at 33:14 % w/w. The ultrasonic bath (Elmasonic) facilitated the extraction process at a temperature of 27°C for 1 hour, with a dried leaves powder-solvent ratio of 1:20g/mL. Separation of the solid component from the extract was executed using a vacuum filter. The resulting filtrate was transferred to a separating funnel, allowing for the formation of two phases. The upper phase was isolated and subjected to drying via a rotary vacuum evaporator (IKA) at 55°C.

In the UAE-ATPE method with simultaneous enzymatic hydrolysis, the process occurred currently in an ultrasonic bath at a temperature of 27°C for 1 hour. The identical treatment was applied to the simultaneous UAE-ATPE extract as in the sequential UAE-ATPE process.

For comparison purposes, the UA-ATPE process unfolded under the same conditions as UAE-ATPE, excluding the addition of an enzyme. Table 1 below outlines the operational conditions for both methods and control for comparison.

Determination of TPC and TFC

The determination of TPC and TFC was conducted following the method outlined by Sonar & Rathod (2020) with slight modifications. Ethanol served as the solvent, gallic acid was employed as the standard for TPC and quercetin was utilized as the standard for TFC.

HMGR inhibition test of extract

HMG-CoA reductase inhibition was investigated using a kit from Sigma-Aldrich. The assay involved an extraction procedure outlined in the assay's technical bulletin, where NADPH quantification was carried out every 20 seconds over a 10-minute period. The percentage of HMGR inhibition was calculated by comparing the absorbance of the sample with that of the control.

Analysis of extract of S. crispus leaves with LC-MS/MS-Q-TOF

The chemical composition of *S. crispus* leaf extracts were analyzed using the LC-MS/MS-Q-TOF instrument. The LC system employed was the ACQUITY UPLC®H-Class System (Waters, USA), featuring an ACQUITY UPLC® HSS C18 (1.8µm 2.1x100mm) (Waters, USA) column maintained at a temperature of 50°C. The mass spectrometer used was a Xevo G2-S QTof (Waters, USA). The mobile phase comprised solvent A (water + 5 mM Ammonium Formate) and solvent B (Acetonitrile + 0.05 % Formic Acid). The water flow rate was set at 0.2 mL per minute (step gradient) over 23 minutes, with an

injection volume of 5 µL. The ESI probe operated in positive mode and both source temperature and desolvation temperature were maintained at 100 and 350°C, respectively. The cone gas flow and desolvation gas flow were set at 0 and 793 L/hour, respectively. The collision energy for low energy was set at 4 volts and the mass analysis range spanned from 50 to 1200 m/z. All data obtained were analyzed using Masslynx software to retrieve information on time retention, peak area and detected formulas. Subsequently, the data was interpreted through ChemSpider, Massbank *and* HMDB websites to identify compound names and their associated activities.

Morphology Analysis of S. crispus leaves with SEM

The morphology of *S. crispus* leaf samples were examined before the treatment, after hydrolysis and extraction process using UAE-ATPE sequence or simultaneous. The analysis was conducted using SEM (FEI QUANTA 450).

STATISTICAL ANALYSIS

The study utilized a duplicate methodology for data collection and conducted a triplicate analysis to ensure precision. Mean values with standard deviations were used to present the data, and IBM SPSS Statistics 26 was employed for processing. A significance level of $p<0.05$ was considered statistically significant.

RESULTS

The extraction of *S. crispus* leaves was conducted using a modified ultrasound extraction method that integrates the EAE and ATPE techniques. This modification is designed to enhance both the yield and quality of the extract compounds. The results, encompassing TPC, TFC, recovery and partition coefficient for TPC and TFC, derived from the combined ultrasonic/enzymatic/aqueous two-phase system (UAE-ATPE) method, are depicted in fig. 1. To elucidate the impact of the hydrolysis reaction, the UA-ATPE method was employed as a control for extraction.

To evaluate the impact of the sequential UAE-ATPE and simultaneous UAE-ATPE methods on the content of active compounds in the ethanol extract of *S. crispus* leaves, it is crucial to identify the active compounds generated by each extraction method using the LC-MS/MS-Q-TOF instrument. Chromatograms and spectra obtained from the LC-MS/MS-Q-TOF instrument were processed using Masslynx software to acquire retention times, peak areas and formulas of the detected peaks. Data interpretation was then conducted through the ChemSpider, MassBank and The Human Metabolome Database (HMDB) websites to retrieve the names, chemical structures and activities of the identified compounds, which can be seen in table 2.

SEM observed the leaf cell wall surface structure. The observation was performed with dried leaves powder and leaves powder after extraction, with sequence and simultaneous UAE-ATPE methods. Fig. 2 depicts the surface structure of dried leaves powder in its untreated state, as well as leaves subjected to extraction using both the UAE-ATPE sequential and simultaneous methods.

Analysis of the anti-cholesterol activity, often referred to as hypolipidemic activity, of the ethanol extract of *S. crispus* leaves, was carried out through the HMG-CoA Reductase Inhibition Test. From the results of this test, the hypolipidemic ability of the ethanol extract of *S. crispus* leaves to inhibit the action of the HMG-CoA reductase enzyme can be evaluated. The test results in the form inhibition percentage can be seen in the following fig. 3 and table 3.

DISCUSSIONS

Effect of Enzymatic Hydrolysis Placement in UAE-ATPE

The analysis of extraction results, employing the ultrasonic/enzymatic/aqueous two-phase (UAE-ATPE) method, reveals significant findings. As illustrated in fig.1, the simultaneous application of the UAE-ATPE method yields markedly higher values for Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) ($p< 0.05$) compared to the sequential UAE-ATPE method. Additionally, the simultaneous UAE-ATPE method demonstrates elevated recovery values and partition coefficients. These results indicate a notable improvement in the extraction of bioactive compounds from the matrix, concurrently reducing the presence of compounds migrating to lower phases in the simultaneous method.

Table 1: Comparison of conditions in sequential and simultaneous UAE-ATPE processes

Operational conditions	UA-ATPE (Control)	Sequential UAE-ATPE	Simultaneous UAE-ATPE
Enzyme-solid ratio (mg/g)	-	70	70
Solid-solvent ratio (g/mL)	1:20	1:20	1:20
Enzyme concentration (% w/w)		7	7
Hydrolysis/Extraction temperature (°C)	30	30/27	27
Hydrolysis/Extraction time (h)	1	2/1	1
pH	-	6.5	6.5

Table 2: Identification of bioactive compounds in *S. crispus* leaf extract using LC-MS/MS-Q-TOF with the Sequential UAE-ATPE[a)] and Simultaneous UAE-ATPE[b)] methods

RT (min)	% Area	Compounds	Chemical formula	Chemical weight	Parent Ion (m/z): $[M+H]^+$	$[M+H]+ \rightarrow$ Daughter Ion (m/z)	Activity
1.211	12.03[a)] 7.94[b)]	Norvaline[a), b)]	$C_5H_{11}NO_2$	118	151.0366	97.9694, 96.9613	Antihyperglycemic (Javrushyan *et al*., 2022)
5.098	1.74[a)]	Isoyatein[a)]	$C_{22}H_{25}O_7$	401	401.1607	371, 219, 209	Anti-cancer, anti-inflammation, and anti-microba (Graidist *et al*., 2015)
5.760	1.09[a)] 0.34[b)]	Astragalin[a), b)] Kaempferol 3-O-glucoside	$C_{21}H_{21}O_{11}$	449	449.1068	287, 153, 85	Anti-inflammatory, antioxidant, antiobesity, anticancer, antiulcer, antidiabetic (Riaz *et al*., 2018)
5.872	1.09[a)]	Citreovirenone[a)]	$C_{14}H_{17}O_4$	249	249.1128	167.0715, 129.0694	Antioxidant, anti-inflammatory and antimicrobial (Koleva *et al.*, 2018)
6.534	0.76[a)]	Pratensein 3'-glucoside[a)]	$C_{22}H_{23}O_{11}$	463	463.1224	301, 445, 163	Anticancer, antioxidant, antiproliferative, antimitotic, and antidiabetic (Szeja *et al.*, 2017)
6.948	1.29[a)]	Vestitone 7-glucoside[a)]	$C_{22}H_{27}O_9$	435	435.1653	417, 401, 167, 137, 181, 239	Anticancer, antioxidant, antiproliferative, antimitotic, and antidiabetic (Szeja *et al*., 2017)
7.320	0.24[a)]	Scutellarin[a)]	$C_{22}H_{21}O_{12}$	477	463.0901	287, 271	Anticancer, antioxidant, antiproliferative, antimitotic, and antidiabetic (Szeja *et al*., 2017)
7.651	0.07[a)]	Baicalin[a)]	$C_{21}H_{19}O_{11}$	447	447.0927	271, 177	Antidiabetic, antibacterial, anticancer, anti-inflammatory, and antioxidant (*Fu et al.*, 2014)
7.911	0.3[a)]	Luteolin[a)]	$C_{15}H_{11}O_6$	287	287.0560	181.0504, 119,0.61	Used for hypertension, diabetes mellitus, and hypercholesterolemia (Havsteen, 2002)
8.664	1.13[a)]	Genistein[a)]	$C_{15}H_{11}O_5$	271	271.0607	153.0189, 204.0874	Reduce cholesterol synthesis (Notarnicola *et al*., 2008)
8.818	1.13[a)]	Diosmetin[a)]	$C_{16}H_{13}O_6$	301	301.0720	286.0487, 171.0662	Treatment of hypertension, rheumatism, diabetes mellitus, asthma, heart disease, gastric ulcers, hypercholesterolemia, and HIV (Havsteen, 2002)
10.112	1.64[b)]	4-Methoxyphenyl 4-butyl cyclohexane carboxylate [b)]	$C_{18}H_{27}O_3$	291	291.1975	105.0343, 204.0868	Antimicrobial, antibacterial, antitumor, anticancer, and anti-inflammatory agent (Doyle *et al*., 2019)
11.932	13.59[b)]	2,2'-(2,5-Furandiyldi-4,1-phenylene)bis (N'-isopropyl-1H-benzimidazole-6-carboximidamide)[b)]	$C_{38}H_{37}N_8O$	621	621.3058	561.2836; 204.0864	Anti-bacterial, anti-asthmatic and anti-diabetic (Vinodkumar *et al*., 2008)
14.020	0.18[a)]	TMS (2,3',4,5'-Tetramethoxystilbene)[a)]	$C_{18}H_{21}O_4$	301	301.1452	262.2568, 109.1032	Antimicrobial, anti-diabetic, anti-inflammatory (Akinwumi *et al*., 2018)
14.920	4.25[a)]	1-Isomangostin[a)]	$C_{24}H_{27}O_6$	411	411.1814	355.1173, 262.2534	Antioxidant, anticancer (Putri, 2015)
15.518	5.81[b)]	{3'-(4-Methylphenyl)-4-[4-(2-methyl-2-propanyl)phenyl]-2-phenyl-2H,3'H-spiro[phthalazine-1,2'-[1,3,4]thiadiazol]-5'-yl}(phenyl)methanone[b)]	$C_{39}H_{35}N_4OS$	607	607.2549	261,2222; 355,0699	Antimicrobial, anti-inflammatory, antituberculosis, antidiabetic, diuretic, anti depressant, radio-protective, anti-leishmanial and cytotoxic activity (Alam, 2018)
18.155	3.04[b)]	2,4,14-Eicosatrienoic acid isobutylamide[b)]	$C_{24}H_{44}NO$	362	362.3423	281.0515, 95.0864	Play a role in cell migration, inflammation, diabetes, cancer, neurodegenerative diseases(Paweł Waluk, 2012)

Table 3: Comparison of sequential and simultaneous UA-ATPE's TPC and TFC yields and their HMG-CoA reductase inhibition value.

Extraction Method	TPC (mg GAE/g dry leaves)	TFC (mg QE/g dry leaves)	HMG-CoA Reductase Inhibition (%)
Sequential UAE-ATPE	4.5±0.3	1.7±0.1	39.4
Simultaneous UAE-ATPE	5.7±0.1	3.3±0.1	66.1

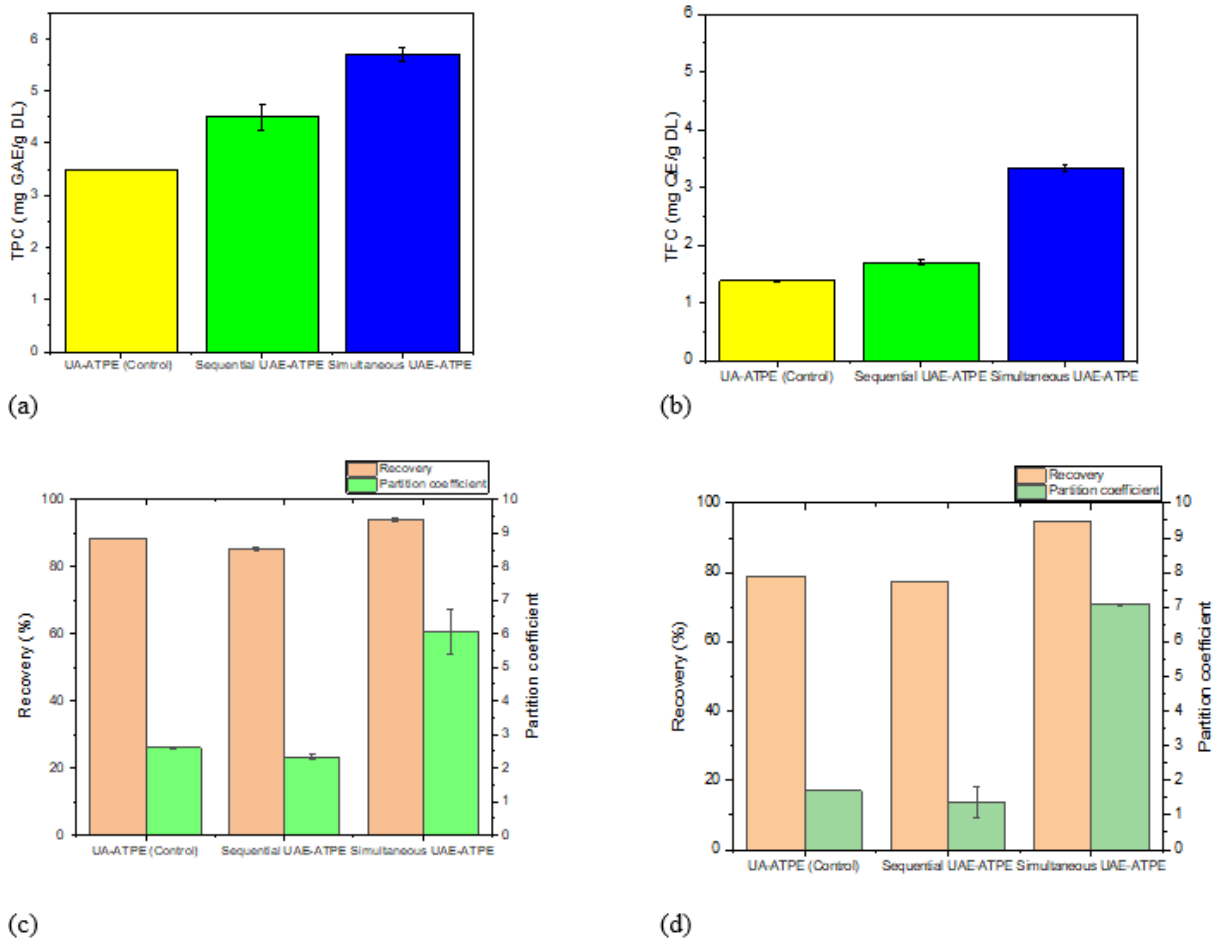

Fig. 1: The impact of ultrasound/aqueous two-phase extraction method with and without sequential and simultaneous hydrolysis reactions on (a) Total Phenolic Content (TPC), (b) Total Flavonoid Content (TFC), (c) recovery and partition coefficient of TPC *and* (d) recovery and partition coefficient of TFC.

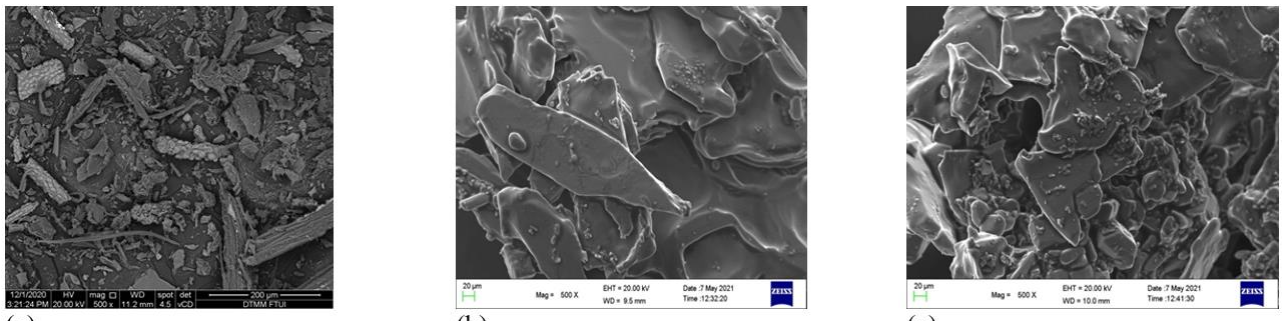

Fig. 2: Cell surface morphology of *S. crispus* leaves on (a) untreated leaves and leaves that have been extracted using (b) the UAE-ATPE sequential method and (c) the UAE-ATPE simultaneous method.

The utilization of enzymes in extraction may potentially compromise the cell wall matrix, resulting in an increased release of phenolic and flavonoid compounds, as illustrated by the findings of Li *et al.* (2017). The integration of ultrasonic waves into the UAE-ATPE method concurrently amplifies the enzyme's efficacy and ensures a uniform distribution of the enzyme (Yachmenev *et al*., 2004). In the sequential UAE-ATPE method, the initial treatment with cellulase enzyme requires 2 hours, followed by a 1-hour extraction process. Prolonging the hydrolysis duration may induce alterations in the molecular structure and bioactivity of phenolic compounds due to hydrolysis or oxidation (Ghafoor *et al*., 2009). In contrast, the simultaneous UAE-ATPE method necessitates only 1 hour for hydrolysis, extraction and the formation of the ATP system.

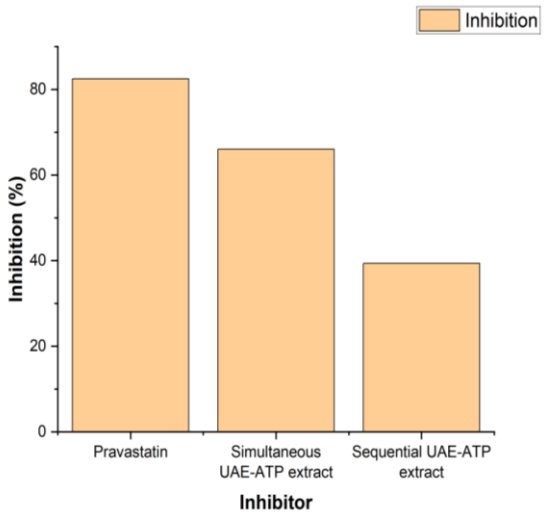

Fig. 3: Comparison of HMG-CoA reductase inhibition by *S. crispus* leaf extract expressed in %, for the sequential and simultaneous UAE-ATPE methods extract (200µg/mL). Pravastatin was used as a comparison of commercial drugs.

Extraction time stands out as a crucial parameter influencing extraction efficiency. Several studies have demonstrated that an extraction duration of around 60 minutes for the UAE extraction method can positively influence the production of phenolic compounds (Li *et al*., 2017). However, excessively prolonged extraction time can lead to changes in the molecular structure and bioactivity of phenolic compounds, as asserted by Ghafoor *et al*. (2009). Therefore, the simultaneous UAE-ATPE emerge as an approach capable of achieving optimal values for TPC and TFC, shorter processing times and reduced costs owing to its single-step process.

The utilization of a combined ultrasonic/ enzymatic/aqueous two-phase extraction approach was also implemented by Song *et al*. (2023) to enhance extraction yields. Song (2023) demonstrated a 26% improvement by incorporating a hydrolysis reaction step into UA-ATPE. In this study, a significant increase in TPC results was observed 22% for sequential UAE-ATPE and 39% for simultaneous UAE-ATPE in comparison to UA-ATPE. Similarly, TFC results exhibited a rise of 19% for sequential UAE-ATPE and 59% for simultaneous UAE-ATPE. These findings highlight the potential of combining ultrasonics and enzymatics to enhance extraction efficiency. Wang *et al*. (2023) employed ultrasonic techniques as a pretreatment for aqueous enzymatic extraction, resulting in a 24% increase in lipid yield compared to procedures without ultrasonics. Moreover, the synergy becomes more pronounced when the ultrasonic power aids in breaking down the outer part of the cell wall, facilitating enzymatic hydrolysis.

Chemical compound identification through LC-MS analysis results

Table 2 presents the phenolic and flavonoid compounds identified through LC-MS/MS analysis. From the identification results, several compounds exhibit activities that have been validated, both traditionally and through research findings. *S. crispus* is recognized for its various pharmacological effects, including antihyperglycemic, antidiabetic, anticancer and other pharmacological properties.

Table 2 reveals that the sequential UAE-ATPE method, utilizing ethanol extract, yielded 13 compounds (phenolic and flavonoid) with a chromatogram area of approximately 25.30%, while the simultaneous UAE-ATPE method showed 6 compounds in the same category, featuring a chromatogram area of about 32.36%. The disparity is attributed to the inclusion of the enzymatic hydrolysis reaction. Thus, the chromatogram area percentage for phenolic and flavonoid compounds in the simultaneous UAE-ATPE method surpasses that in the sequential UAE-ATPE method due to concurrent implementation. Yang *et al*. (2018) compared pectin yield, placing the EAE process before and after ultrasound extraction, showing a 16.5% increase when EAE preceded ultrasound. Latif and Anwar (2009) conducted a simultaneous enzymatic and extraction process (EAEE) for sunflower seed oil extraction, achieving a maximum oil yield of 87.5% with a TPC value of 1.5 mg GAE/200 g.

The increased yield observed in the simultaneous UAE-ATPE combination can be attributed to the ultrasonic effect, influencing molecular degradation through enzymatic hydrolysis reactions. Ultrasonic waves induce intense convection in liquid media, enhancing the probability and interaction energy between molecules undergoing enzymatic degradation (Delgado-Povedano & Luque de Castro, 2015). Integrating enzyme hydrolysis with ultrasound promotes better contact between the enzyme and substrate during the natural ingredient

extraction process, accelerating cell damage and facilitating the mass movement of

Several studies underscore the challenge of extracting most polyphenolic compounds using a single method due to their insoluble bonds conjugated with cell wall components through ester, ether, or glycosidic bonds. The integration of enzymes into the extraction process is crucial for the efficient release of polyphenolic compounds. Wu *et al.* (2015) observed that combining enzymatic and ultrasonic extraction methods resulted in elevated TPC, TFC *and* antioxidant capacity compared to using ultrasonic or microwave extraction techniques alone (Santos & Santana, 2022).

Moreover, various methods independently contribute to cell wall rupture, including enzymatic processes, fragmentation, erosion, cavitation, sonoporation, sono-capillary effects, local shear stress and detexturization (Patra *et al*., 2022). Simultaneous utilization of enzymes and ultrasonics exerts an additional impact on cell breakdown, influencing the profile of bioactive compounds compared to the sequential approach. The existence of a two-phase water system introduces selectivity processes based on physicochemical properties, with varying partitioning abilities of bioactive compounds between the phases, contingent on the compound's affinity for each phase. The presence of excess enzyme in one phase may influence extraction outcomes due to the impact of enzyme activity by the ATP system. These factors might have caused different extract composition obtained from sequential and simultaneous UAE-ATPE.

SEM analysis of leaf extract cell wall surface

From the figure, it can be seen that the dried leaves' cell surfaces were relatively intact, compact and smooth, with a small amount of tear that may occur during leaf size reduction in a blender machine. Blending was a physical treatment done to achieve a size 80 mesh in the preparation process. The physical treatment with correct size reduction can improve the contact area and the material porosity. Thus, the impact effectivity can be increased when the enzymatic hydrolysis process and the active material extraction rate increase. Meanwhile, the opposite results could happen if the size reduction is excessive (Huang *et al*., 2016).

Fig. 2. (b) and (c) show the leaf's cell wall surface structure after sequential and simultaneous UAE-ATPE processes. That figure shows the cell surface structure from simultaneous UAE-ATPE seen to be more disintegrated and perforated than the structure from sequential UAE-ATPE. The difference has occurred because enzymatic hydrolysis and ultrasonic wave extraction were carried out simultaneously, thus providing a synergistic effect between the two treatments and causing greater disintegration of the leaf cell walls. The leaf cell walls are tissue organized by cellulose microfibril, hemicellulose, pectin, lignin and dissolved protein composed of three main layers. The three main layers are primary cell walls, middle lamella and secondary cell walls (Sticklen, 2010). The phenolic and flavonoid compounds are trapped in polysaccharide cell walls connected with hydrophobic interaction, hydrogen bond, or glycosidic bond and some form ether or ester bond with polysaccharide (Nadar *et al*., 2018). The cell wall breakdown is the main step to extract the bioactive compound inside the cell walls (Cheng *et al*., 2015). Enzyme-assisted extraction relies on the ability of enzymes to hydrolyze cell wall components and weaken the structural integrity and will make the extraction and release of bioactive compounds efficient (Gardossi *et al*., 2010). However, the enzyme activity when hydrolyzing cellulose is inhibited by the cellulose matrix and other polysaccharides that are compacted inside the cell wall. With the addition of ultrasound which causes mechanical damage to the cell wall, the hydrolysis process is much easier and produces more porosity, as shown in fig.2.(c) (Yang, *et al*., 2018). This is by the extract yield, TPC and TFC values, which were higher in the extract obtained by the simultaneous UAE-ATPE method than in the sequential UAE-ATPE.

Analyzing HMG reductase inhibition: Assessing anti-cholesterol potential

The results of the HMG-CoA reductase inhibition test of *S. crispus* leaf extract showed the hypolipidemic activity of the leaf extract. The hypolipidemic activity might be caused by the phenolic compounds contained in the extract. An *in vivo* study proved that *Medicago sativa* L., which most of its identified compounds are phenolics, has antihyperlipidemic activity (Seida *et al*., 2015). Phenolic compound has hydroxyl groups that can scavenge free radicals, thus making it an antioxidant that has a potential to lower lipid profile (Gul *et al*., 2023). Based on the curve formed, the inhibition value of the leaf extracts from simultaneous UAE-ATPE is 66.05%, higher than the sequential UAE-ATPE of 39.38%. This shows that the ability to extract from the simultaneous method is better than the sequential method. This is probably due to the greater content of phenolic/flavonoid compounds from the simultaneous extracts, as shown in fig. 1 and table 2. Gebhardt (2003) reported that kaempferol and myricetin could stimulate cholesterol biosynthesis, especially in the low concentration range (between 0.1 and 10 µM), while they showed variable levels of inhibition at higher concentrations. The high content of phenolic and flavonoid compounds in the crude ethanol extract from the stem bark of *F. rukam* also shows higher anti-cholesterol activity than the isolated fraction, which has a lower content of phenolic and flavonoid compounds (Muharni *et al*., 2021). However, both leaf extracts had not been able to compete with pravastatin (catalog

number I5909, Sigma-Aldrich), a commercial drug commonly used for cholesterol treatment.

Although the activity demonstrated by the ethanol extract of *S. crispus* remains lower than that of pravastatin for both simultaneous and sequential extracts, the results obtained are consistent with those reported by Fadzelly *et al*. (2006), who conducted an *in vitro* study on the cholesterol-lowering abilities of fermented and non-fermented teas. Hence, the study concludes that the ethanol extract of S. crispus, especially when obtained through the simultaneous method, has the potential to serve as an alternative treatment for cholesterol due to its higher content of phenolic compounds and flavonoids, as indicated in table 3. Consequently, optimizing the UAE-ATPE method simultaneously in future research is crucial for achieving higher concentrations of phenolic and flavonoid bioactive compounds.

Overall pharmacological activity of a plant extract can result from synergistic, additive, or antagonistic activity of the compounds extracted (Caesar & Cech, 2019). Therefore, there might be a chance that a better HMG-CoA inhibition could be achieved by further isolating the active compound.

CONCLUSION

Strobilanthes crispus contains numerous bioactive compounds belonging to the phenolic and flavonoid classes, which exhibit anti-cholesterol activities. The combination of enzymatic hydrolysis with ultrasound extraction (simultaneous UAE-ATPE) resulted in higher extract yields, as well as elevated TPC and TFC values, as confirmed through LC-MS/MS-Q-TOF and SEM analysis. In conclusion, this method proves notably more efficient than the sequential UAE-ATPE approach. This integrated technique holds the potential for further refinement toward the commercial production of higher-quality anticholesterol products. Subsequent research should identify optimal variables and operational conditions to enhance production.

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