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RESEARCH ARTICLE

A modern purification by accelerated solvent extraction and centrifugal partition chromatography and biological evaluation of capsaicin from *Capsicum chinense*

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Abstract

A special alkaloid compound known as capsaicin, which can only be found in the fruit of the capsicum plant, was isolated and tested for its anti-inflammatory activity. The purpose of this work is to establish a simple and quick approach for capsaicin purification utilizing centrifugal partition chromatography (CPC) as well as an effective method - accelerated solvent extraction (ASE), for extracting capsaicin from *Capsicum chinense*. After purification, capsaicin was validated by HPLC-DAD at 281 nm to be > 90% purity. The in vivo anti-inflammatory activity of the isolated capsaicin was also investigated, and the IC_{50} value of the capsaicin was determined to be 57.61 µg/mL. The current work emphasizes how an ASE and CPC system may combine to extract high-purity capsaicin from *Capsicum chinense*, which have the anti-inflammatory activity, as we evaluated in the experiment.

Keywords

Capsicum chinense; capsaicin; accelerated solvent extraction; centrifugal partition chromatography; anti-inflammatory

Introduction

Capsicum chinense, also known as habanero pepper, is a member of the *Capsicum* genus and the *Solanaceae* family. *Capsicum chinense* Jacq. has been paid attention to as a specialty agricultural crop in the Lai Chau (Vietnam) region due to its distinct spicy and aromatic flavor (1). It was also announced as the hottest chili pepper in the world (2). Besides being widely used in the food industry as food additives (3), capsicum extracts are used as a customary medication for stomachaches, disgorgement, and inflammation (4).

Chili peppers, especially the habanero (*Capsicum chinese*), are possible source of useful phytochemicals such as phenolic compounds, carotenoids, and capsaicinoids. Capsaicin, which is most abundant (46%) amongst other capsaicinoids, accounts for approximately 90% of the pungency (5, 6). Other capsaicinoid components including homocapsaicin II, nonivamide, and homodihydrocapsaicin II have also been discovered (7). For millennia, capsaicin has been applied therapeutically since it can affect lipid and sugar levels in the blood, and cholesterol. Besides that, anti-oxidative, anti-

obesity, and analgesic properties of capsaicin have also been reported in various studies related to capsaicin (8-10).

With a wealth of potential biological activities and their prevalence in traditional remedies, the extraction of capsaicin as well as other bioactive compounds from raw materials is the first and most crucial step. The convenient solid-liquid extraction employing organic solvents is the most often used and favored approach for extracting compounds from plant matrices. Vacuum filtration, percolation, the Soxhlet technique, and pressured liquid extraction are all methods for the solid-liquid extraction of capsaicin (11, 12). Pressurized solvent extraction (PLE) typically functions at elevated pressure and temperatures exceeding the boiling point of the extraction solvents. Excessive temperature and pressure can noticeably modify the properties of the solvents, allowing for better solvent permeation into the plant matrices (13). The use of PLE reduces the time and solvent for extraction and enhances extraction yield by adjusting factors like temperature, type of solvent, solvent volume, and time. Furthermore, PLE extraction employs highly automated tools, resulting in much more accurate results (14).

Centrifugal partition chromatography (CPC) is a variation of counter-current chromatography (CCC). CPC is an all-liquid chromatographic method that does not use a solid stationary phase and functions specifically on the partition of compounds between two immiscible liquid phases for separation (15). Adsorption losses are minimized and sample recovery is guaranteed to be 100% due to the lack of any solid stationary phase. CCC, in addition to having significantly higher sample capacity than HPLC, allows for the direct application of crude extracts with a mild separation condition (16, 17).

In addition to the benefits of the CPC approach, great selectivity for the targeted compounds may be attained using the large number of solvent combinations available to build a biphasic solvent system. Numerous solvent combinations can be employed to create the best biphasic system for the CPC process. The ARIZONA system, or its HEMWat (*n*- hexane/ethyl acetate/methanol/water) version, which consists of a combination in varying proportions of heptane (or n-hexane), ethyl acetate, methanol, and water, is the popular solvent in CPC for the separation of natural compounds (18). The process of selecting a suitable solvent system can be compound-dependent and laborious. To simplify this selection, a Thin Layer Chromatography (TLC)-based method called the Generally Useful Estimate of Solvent Systems (GUESS) was proposed for the n-hexane-ethyl acetate-methanol-water solvent system (19).

The present study is aimed at creating an automated extraction process for capsaicin from *Capsicum chinese* using PLE and devising a rapid and straightforward purification method employing CPC. The PLE extraction temperature, extraction time, and sample-solvent ratio are firstly optimized by a single factor experiment. We aimed to investigate the one-step CPC isolation after the PLE for the complete workflow. The *in vivo* anti-inflammatory activity of the isolated capsaicin was also investigated.

Materials and Methods

Materials

The fruits of *Capsicum chinense* Jacq. samples were gathered in August, 2020 in Muong Te (coordinates: 22.375023, 102.811225) (Lai Chau, Vietnam). These samples were verified at the Department of Medicinal Material Resources, NIMM (Vietnam). Before extraction, these samples were dried in an oven (50 ^{0}C) and milled into powder. Moisture content (< 10%) was measured before the experiment.

Chemical: Deionized water; acetonitrile (ACN), phosphoric acid for HPLC, methanol (MeOH), *n*-hexane, ethyl acetate (EtOAC) of analytical grade, sulfuric acid for analysis (Merck, Germany); ethanol absolute (Vietnam); capsaicin (Sigma Aldrich (CAS:404-86-4, Lot#BCC0162, ≥98.5%).

Extraction of plant materials

The PLE extraction was carried out utilizing an ASE 350 Accelerated Solvent Extractor (Dionex, Sunnyvale, CA, USA). In a 100 mL stainless extraction cell, two grams of material were combined with four grams of diatomaceous earth. A stainless-steel frit and a cellulose filter were placed at the bottom of the extraction cell to filter out any potential contaminants before collection. The extraction cells were arranged in a tray, and the samples were subjected to specific conditions during extraction. After extraction, the collecting bottles were stored at 4 °C for further analysis.

Optimization of extraction parameters

Before proceeding with the separation of the crude extract, a series of tests were conducted to determine the optimal values for the extraction parameters. These factors, which influence the capsaicin content, encompassed ethanol concentration (%), extraction time (min), extraction temperature (°C), and the volume of solvent used (mL). During the optimization process, one factor was altered at a time, while the other factors were kept constant at specified values.

Calculation of the extract result

The capsaicin contents in these samples were calculated using the following formula:

$$C_{capsaicin} = \frac{M_{capsaicin}}{M_{material}} \times 100\%$$

when $C_{capsaicin}$ (%) is the content of capsaicin in the sample; $M_{capsaicin}$ is the total mass of extracted capsaicin (g), $M_{material}$ is the mass of sample (g).

Thin-layer chromatography

Normal-phase (NP) TLC was executed on silica plates (Silica gel 60F_{254}, E. Merck, Germany) by using the solvent system hexane-ethyl acetate. The crude extract of

Capsicum chinense and standard capsaicin were observed on the TLC plate. Visualizations were performed under UV light λ1 = 254 nm and λ2 = 366 nm. After that, the plate was heated at 105°C for a minute and sprayed with TLC reagent (vanillin/sulfuric acid). The appearance of capsaicin spots in blue was observed.

CPC equipment

The separations were performed on a FCPC® A1000 system (Kromaton, France) with an internal volume of 1000 mL. This apparatus was able to rotate from 800 to 2000 rpm and support up to 80 bars (1160 psi). The FCPC A1000 system was coupled with the PuriFlash 5.250 system (Interchim, France) built in with a quaternary pump, a diode array detector, and a fraction collector. Interchim Intersoft X was used to control the instrumentation.

Solvent system screening

The TLC-based GUESS method was performed on silica gel TLC plates with three solvent systems based on *n*- hexane/ethyl acetate/methanol/water. The proportions of the mixture were 7/3/5/5 (v/v/v/v) for system solvent 1, 1/1/1/1 (v/v/v/v) for system solvent 2, and 3/7/5/5 (v/v/v/v) for system solvent 3, after consulting the previous data of the journal and report (4, 20). In the HEMWat method, the simplest approach is to utilize the n-hexane/ethyl acetate mixtures specified in Table 1 for the TLC analysis. The ideal HEMWat solvent system is determined by selecting the TLC solvent system that yields an Rf value closest to 0.5 for the target compound (19).

Table 1. Equivalence of HEMWat in CPC and n-hexane/ethyl acetate solvent systems for TLC

HEMWat	n-Hexane	EtOAc	MeOH	Water	TLC	n-Hexane	EtOac
1	7	3	5	5	***1***	7	3
2	5	5	5	5	***2***	5	5
3	3	7	5	5	***3***	3	7

CPC experimental conditions

For this study, the aqueous, heavier phase was chosen as the stationary phase, while the organic, lighter phase served as the mobile phase, running the CPC in ascending mode.

The CPC column was initially filled entirely with the aqueous stationary phase at a flow rate of 50 mL/min without rotation. Subsequently, the apparatus was rotated at 1600 rpm, and the upper mobile phase of the solvent mixture was pumped into the column (rotor) at a flow rate of 20 mL/min in the ascending mode. Once the mobile phase emerged from the column and hydrodynamic equilibrium was achieved, a total of 3 g of the crude extract to be purified was dissolved in 10 mL of the solvent system mixture. The sample was filtered and introduced to the 40 mL injection loop. Fractions were monitored at λ = 281 nm and collected in 30 mL tubes. The combined fractions were evaporated under vacuum until dryness and then dissolved in methanol for subsequent analysis using TLC and HPLC.

HPLC analysis

Capsicum chinense crude extract and CPC fraction peaks were analyzed by HPLC-DAD. The Shimadzu SPD-20A system (Shimadzu, Japan) used for the HPLC analysis consisted of a quaternary pump, a degasser, an autosampler, an injector with a 200-µL loop, a column oven, and a diode-array detector (DAD). Quantitative estimation was performed with LabSolutions software programs. The separation of compounds was performed on a 4.6 × 250 mm, 130Å pore size, 5 µm particle size XBridge BEH Phenyl Column (Waters Corporation) analytical column. The mobile phase was a mixture of acetonitrile and 0.1% aqueous phosphoric acid (40:60, v/v) pumped at a flow rate of 0.7 mL/min. The injection volume was 20 µL. Detection was set at a wavelength of 281 nm. The run time was 40 min.

Anti-inflammatory assay

The anti-inflammatory activity of the extracts and isolated compounds was evaluated by measuring the inhibition of nitrite (NO) production in lipopolysaccharide (LPS)-induced RAW264.7 cells (ATCC, Manassas, VA, USA). All data are expressed as the means of three replicates ± standard deviations.

Cell culture

A mouse macrophage cell line RAW 264.7 was purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS; HyClone, GE Healthcare, UT, USA) and 1% penicillin-streptomycin at 37°C in a 5% CO_2 incubator.

Cell viability assay

RAW 264.7 cells (2.5 x 10^5) were seeded into 96-well microtiter plates with different sample concentrations and cultured for 24 h at 37°C in an incubator with 5% CO_2. The MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at 0.5 mg/mL in PBS] was added to each well at the end of the treatment, and the plates were incubated for a further 4 h. Finally, absorbance was measured at 450 nm with a microtiter plate reader, the Infinite F50 (Tecan, Männedorf, Switzerland).

NO production detection

RAW 264.7 cells (1×10^5 cells/ml) were pre-treated with various concentrations of 4→256 µg/mL for 30 min and then stimulated for 24 h with or without 1 µg/ml LPS at 37°C, 5% CO_2. Cardamonin (Sigma-Aldrich, >98% HPLC) was used as a positive control. The nitrite (NO) concentration in the culture supernatants was measured using Griess reagents (Merck KgaA, Darmstadt, Germany). Subsequently, the absorbance of the mixture solution at 570 nm was measured. A standard curve was prepared using $NaNO_2$ as

a standard solution in the same manner and was used to calculate the concentration of NO.

Results

Single factor analysis

The effects of extraction time (min), extraction temperature (◦C), ethanol concentration (%), and solvent used (mL) on the extraction yield of capsaicin are shown in Fig. 1.

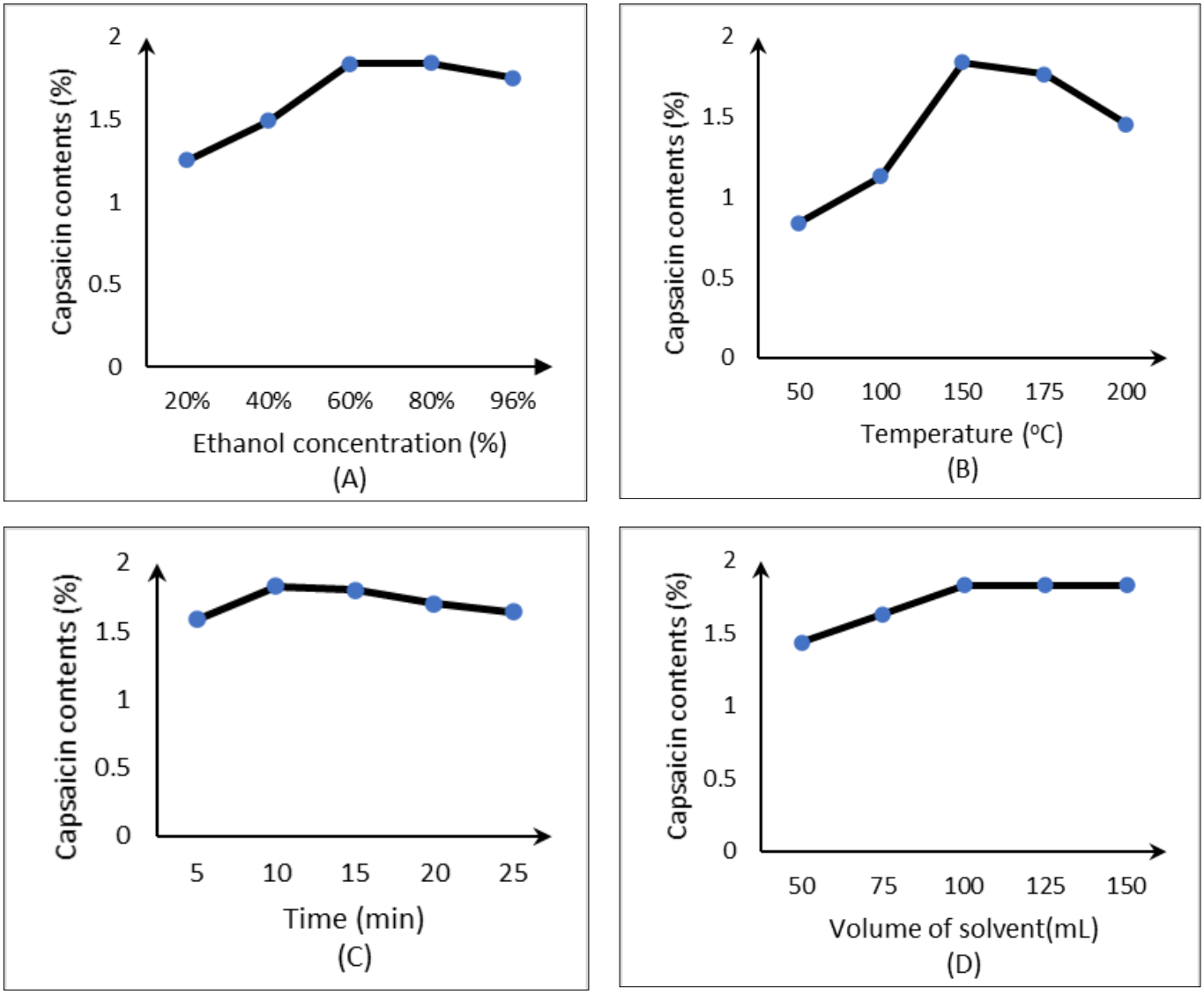

Fig. 1. Effects of extraction factors on yield of capsaicin extraction: (**A**) ethanol concentration; (**B**) temperature; (**C**) time and (**D**) volume of solvent

In Fig. 1A, extractions were performed with ethanol concentrations ranging from 20% to 96%, while other conditions were established as follows: extraction temperature at 150 ^{0}C, extraction time of 15 min, and volume of solvent used was 100 (mL). Results showed that the maximum capsaicin yield (1.8404%) was reached with an ethanol concentration of 60%, from which point yield began to stabilize as no significant changes were noted. As particularized in Fig. 1C (all other parameters are constant as described above), increased time had no noticeable effect on capsaicin content after 10-minute point. This indicated that an extraction time of 10 minutes was adequate to obtain a high capsaicin content (1.8324%). As shown in Fig. 1B and D, capsaicin yields increased with increasing temperature and sample/solvent ratio, reaching their maximum yields when the temperature was 150^0 C (1.8423%) and solvent volume was 100 mL (1.8326%). However, when the temperature was increased, degradation could be observed, which further reduced the extraction efficiency. These results were in agreement with the Wang experiment, in which capsaicin degraded quickly at high temperatures (>190°C) (21). Otherwise, such a change in the capsaicin content was not spotted when increasing the solvent volume in the present study. This might be because the capsaicin was almost exhausted in the material, and adding new solvents was redundant and difficult to extract more. In accordance with the results of the single-factor study, a solvent of 60% ethanol concentration, an extraction time of 15 min, a temperature of 150 °C and a volume of solvent of 100 mL were chosen to obtain the crude capsaicin extract for further purification.

Analytical TLC for solvent selection

The Rf values gained from the HEMWat-based TLC solvent systems 1, 2, and 3, as per the GUESS method, are presented in Table 2. An Rf value close to 0.5 is deemed ideal and is presumed to match the K value of the sample. According to the TLC experiment, the CPC conditions of 1:1:1:1 were chosen for the purification of capsaicin.

Table 2. Corresponding pairs of selected HEMWat CPC solvent systems and n-hexane-ethyl acetate TLC solvent systems, along with TLC Rf values.

CPC conditions (*n*-hexane-ethyl acetate-methanol-water)	TLC Condition (*n*-hexane-ethyl acetate)	TLC Rf values
7:3:5:5	7:3	0.183
5:5:5:5	5:5	0.400
3:7:5:5	3:7	0.667

CPC purification results

The purification of capsaicin using CPC was performed. After running for 1 hour, all the fractions of separation were collected and analysed by HPLC. The CPC separation result of components in the crude extract is shown in Fig. 2.

The analysis of CPC fractions showed a successful purification of capsaicin in one run, leading to the isolation of the major compounds in pure form or in enriched fractions. Centrifugal partition chromatography can be a useful tool for the handling of these challenging mixtures and the effective recovery of high-value compounds since it provides the benefit of excellent scaling-up from analytical to preparative and pilot procedures (22-24).

Nitric oxide inhibition and cell viability test

The NO inhibition activity of the isolated capsaicin was assessed against LPS-stimulated RAW 264.7 cells using the Griess assay (Table 3). The cells were incubated with LPS along with the sample, with concentrations ranging from 4 µg/ml to 256 µg/ml. The isolated capsaicin showed the most potent inhibition (75.18%) at a concentration of 256 µg/ mL. However, at this concentration, capsaicin also exhibited a high cytotoxicity against RAW 264.7 cells (cell viability was only approx.11,61%). At much lower concentrations of 64 µg/mL, capsaicin still showed a more than

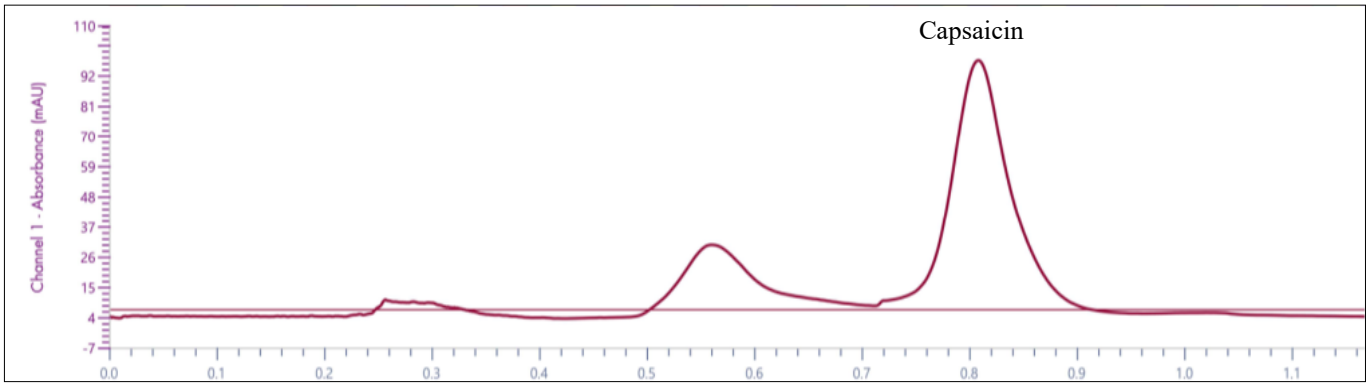

Fig. 2. The chromatogram of the capsaicin purification method

The HPLC analyses of the crude extract and fractions from CPC are shown in Fig.3. After one-step HSCCC separation, 101.8 mg of capsaicin with a purity of no less than 90% was obtained.

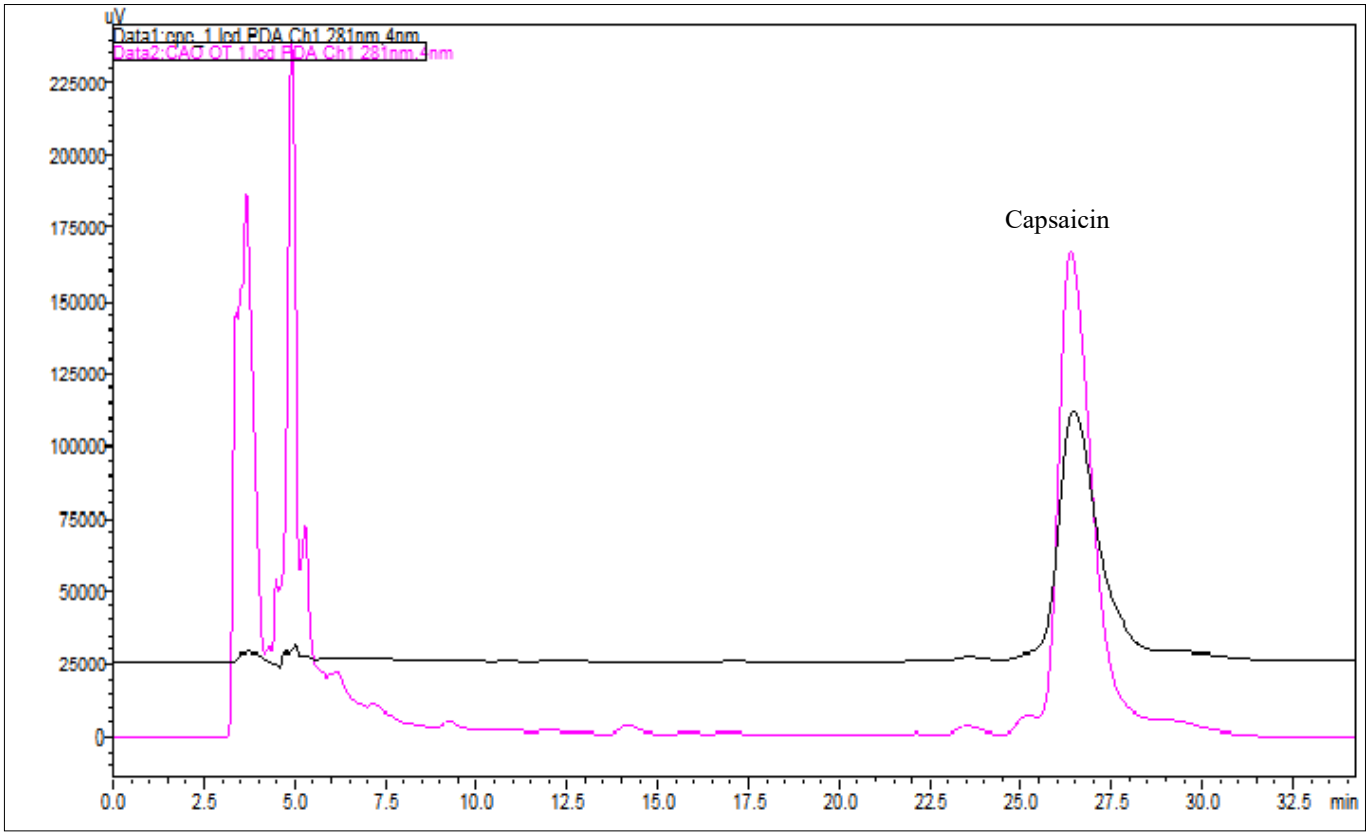

Fig. 3. HPLC-DAD overlaid chromatogram of *Capsicum chinense* extract (**pink chromatogram**) and the purified capsaicin (**black chromatogram**) at 281 nm.

50% inhibition of NO production by RAW 264.7 cells (cell viability >80%), which obviously indicated anti-inflammatory activity *in vitro*. Thus, the IC_{50} value of the capsaicin was determined to be 57.61 µg/mL.

Table 3. Result of nitric oxide inhibition and cell viability test for isolated capsaicin

Sample	Concentration	Percentage of inhibition of NO production (%)	Percentage of cell viability (%)
Negative control	-	100.0±1.3	104.76±0.15
Positive control (Cardamonin)	0.3 µM	45.85±2.12	86.47±0.21
	3.0 µM	86.93±0.96	71.8±0.51
LPS	-	0.0±0.9	100.0±0.13
Isolated capsaicin	256 µg/mL	75.18 ± 0.41	11.61 ± 0.25
	64 µg/mL	51.50 ± 0.11	85.87 ± 0.14
	16 µg/mL	40.14 ± 0.14	90.17 ± 0.10
	4 µg/mL	36.35 ± 0.07	93.92 ± 0.21

Discussion

*Capsicum chinens*e, also called "habanero-type pepper", is well renowned for its distinctive tastes and exceptionally high heat levels in several varieties. The major chemical component, which adds to the spice as well as the bioactive property of *Capsicum chinense,* is capsaicin. Many different biological effects of capsaicin have been studied in the past. The benefits of capsaicin on metabolic problems, such as weight loss, blood pressure decrease, and insulin reduction, are essential. By causing apoptosis and reducing the multiplication of tumour cells, capsaicin has also been shown to be beneficial in avoiding human malignancies such as lung, stomach, colon, and breast cancer. Previous studies have shown that capsaicin has therapeutic properties to treat pain (25).

One of the most widely used techniques for differentiating and purifying bioactive compounds from plants is extraction. The several extraction techniques for capsaicinoids from fresh and dried pepper fruits were evaluated by Muwen Lu in 2017, including maceration, microwave assisted extraction (MAE), ultrasound-assisted extraction (UAE), Soxhlet extraction, extraction with liquids under pressure (PLE), supercritical fluid extraction (SFE) (26). According to the results, extraction efficiency will be the highest (98.31%) with PLE technology. The PLE technology is also the most practical in terms of the range of utilized solvents, the parameters, and the available resources. The extraction procedure is faster, and the total energy consumption is lower. High solvent penetration into the sample matrix is achieved by allowing for high chemical solubility while keeping the solvent below its boiling point since the PLE technique is performed at high temperatures and pressures.

There have been several reports for separating and purifying capsaicinoids, especially capsaicin, which involves column chromatography (27). According to Fan Y, capsaicin was isolated from the capsaicinoid extract through a two-step process involving macroporous adsorption resin (MAR). Following the initial purification with the non-polar MAR ADS-17, the capsaicin exhibited a recovery rate of 83.7% and a purity of 50.3%. Subsequent purification using the weakly polar MAR AB-8 resulted in a capsaicin recovery rate of 88.0% and a purity level of 85.1% (28). But instead of using the solid material as the stationary phase, which needs to be replaced after some uses, the CPC techniques apply the liquid solvent for both phases: the liquid stationary phase is held within the column by a centrifugal field, while the liquid mobile phase is driven through the stationary phase. The liquid stationary phase brings numerous advantages: no irreversible adsorption occurs due to the solid stationary phase; the wide range of polarities (NP/RP in one column) depends on your choice of solvent combinations; and the technique can quite easily scale-up (29). CPC had been applied for the purification and separation of capsaicinoids in the past, especially capsaicin (4, 20), but this is the first time the PLE extraction and the CPC purification have been combined into a one-step workflow from the natural plant material *Capsicum chinense*, resulting in the production of capsaicin with a purity exceeding 90% and a remarkable recovery rate of 99%. This procedure can easily be reproduced and upgraded to a large scale for industry production.

Inflammation is a fundamental physiological response that the body employs to protect itself from harmful stimuli, such as pathogens and tissue damage. However, when inflammation becomes chronic or excessive, it can contribute to various diseases, including arthritis, gastritis, and neuroinflammatory disorders. Numerous studies aim to investigate the anti-inflammatory activity of herbal plants, including both crude extracts and their active compounds (30, 31). In this study, the isolated capsaicin was proven to have potential anti-inflammatory properties with the evaluation of the production of nitric oxide in RAW 264.7 cells. These results were in agreement with numerous studies, but the cytoprotective or cytotoxic action of capsaicin was dependent on cell types and control concentrations utilized by various researchers (32-34). The mechanism of anti-inflammatory properties has also been studied by some researchers. Capsaicin's primary mode of action is its interaction with Transient Receptor Potential Vanilloid 1 (TRPV1) receptors (35). TRPV1 receptors are predominantly located on sensory nerve endings and are responsible for detecting sensations of heat and pain. The remarkable

aspect of capsaicin's effect on TRPV1 receptors is its biphasic nature. Initially, capsaicin activates these receptors, leading to a heightened sensation of pain and inflammation. However, with repeated exposure, the opposite phenomenon occurs—TRPV1 receptor desensitization. This desensitization process results in a reduction in the transmission of pain signals and the release of substance P, a neuropeptide involved in pain and inflammation (33). Capsaicin's impact extends beyond its interaction with TRPV1 receptors. It has been demonstrated to modulate the production and release of pro-inflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) (36). These cytokines play a central role in the inflammatory response. Capsaicin's effect on cytokine modulation helps reduce the overall intensity of the inflammatory response. While more research is needed to fully understand the extent of capsaicin's potential in clinical applications, its unique properties hold promise for the management of various inflammatory conditions and pain relief. With further investigation and testing, capsaicin or even *Capsicum chinense* extract may be a promising drug candidate against inflammatory diseases.

Conclusion

The purpose of the present article was to emphasize the potential of combining pressurized liquid extraction and centrifugal partition chromatography when applied in the field of an autonomous workflow process. In a single run, the high purity of isolated capsaicin was acquired. Our findings revealed that ASE was preferable, providing higher extraction efficiency and repeatability while being significantly faster than traditional approaches. Pure compounds are created on a preparative scale in conjunction with an appropriate extraction and cleaning technique prior to CPC separation and may be utilized as standards for HPLC analysis as well as for additional bioactivity research. Capsaicin exhibits anti-inflammatory activity by inhibiting NO in vitro, thereby endorsing its therapeutic latent in attenuating inflammatory progressions.

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Authors contributions

HTD carried out the CPC isolation and drafted the manuscript. DTTL, TTH carried out the HPLC analysis. LXD participated in the extraction of plant materials. NCC, PVT, NMK. LQT participated in the design of the study and performed the statistical analysis. DHN carried out the anti-inflammatory assay. NTH conceived and designed the study. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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