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Supercritical carbon dioxide extraction optimization of *Brassica oleracea* var. *capitata* f. *rubra* leaf extracts for cholinesterase and tyrosinase inhibitory activity

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ABSTRACT: Supercritical carbondioxide extraction has been an advanced system as the regulation of extraction parameters enables the control of the solvating power, resulting in a more selective process. While the efficient use of natural resources is critical for sustainability goals, the discovery of feasible plant sources for medicinal purposes is a valuable research objective. In this study, supercritical carbondioxide extraction of red cabbage (*Brassica oleracea* var. *capitata* f. *rubra*) was investigated using response surface methodology. A statistical design was used for the evaluation of the effect of extraction parameters as temperature (40, 60, 80°C), pressure (120, 160, 200 bar) and co-solvent ratio (0, 6, 12%). Spectrophotometric and UPLC-DAD, ESI-MS/MS analysis were performed to investigate total phenol, flavonoid, anthocyanin and dominant anthocyanin contents. The optimization models were determined as significant for all bioactive content analysis and enzyme inhibitory activities. 40°C, 160 bar, 12% co-solvent conditions were determined with highest total flavonoid content (47.11 mg QE/g). None of the supercritical carbondioxide extracts were able to extract red cabbage anthocyanins within the investigated parameter ranges. 60°C, 120 bar, 12% co-solvent extract showed the strongest tyrosinase inhibitory activity (IC_{50}: 1.71 mg/ml). Cholinesterase inhibitory activity of supercritical carbon dioxide extracts was found to be comparable to that of solvent extracts considering the tested concentrations. Optimum supercritical carbondioxide extract can be considered as a potential source with tyrosinase inhibitory activity, while identification of active molecules, especially flavonoids, can be targeted in further isolation studies.

KEYWORDS: Acetylcholinesterase; *Brassica oleracea* var. *capitata* f. *rubra*; butyrylcholinesterase; extraction; LC-MS/MS; red cabbage; supercritical carbondioxide.

1. INTRODUCTION

Brassica oleracea L. var. *capitata* f. *rubra* (Brassicaceae), red cabbage, originating from the Mediterranean region, has been a worldwide cultivated agricultural variety. Red cabbage belongs to Brassicaceae (Cruciferae) family, which contains various species that has nutritious properties. The rich composition of red cabbage has also been benefited for medicinal purposes. Red cabbage was recorded for use against peptic ulcer, diarrhea and gout. The studies with red cabbage extracts demonstrated antioxidant, antiinflammatory, immunomodulatory and antiatherosclerotic activities, which were mainly attributed to its phenolic content. Among red cabbage phenolics, anthocyanins are a large group consisting over 30 identified compounds that are mainly cyanidin glycosides [1-4].

Among plant bioactive metabolites, phenolic compounds have been valuable for their broad-spectrum activity potential. Plant phenolics and extracts have been shown to possess enzyme inhibitory activity [4, 5]. As red cabbage has been identified as rich in phenolic compounds [1], targeting acetylcholinesterase, butyrylcholinesterase and tyrosinase activity which were linked to cognitive impairment in Alzheimer's, Parkinson's diseases, and hyperpigmentation, melanoma [5-7], is an important research objective. High production capacity makes red cabbage also favourable for sustainable management whereas total cabbage production was reported as 106.80 million tonnes in 150 countries around the world [8].

Being a multifactorial neurodegenerative disease, several factors such as age, genetics and trauma have been defined as contributing to the development of Alzheimer's disease. With regard to the cholinergic hypothesis, the cognitive impairment has been linked to the degeneration of cholinergic neurons in cortex and hippocampus, resulting in cholinergic transmission deficits and reduced acetylcholine levels. While

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symptomatic treatment has focused on the restoring cholinergic function, inhibition of acetylcholinesterase activity has been one of the strategies employed. Although butyrylcholinesterase primarily exists in plasma and may not be directly involved in cholinergic disruption, significant activity has been reported in neuronal lesions of Alzheimer's disease patients. Therefore, inhibiting butyrylcholinesterase activity was also considered to be beneficial against disease progress [9]. A similar situation has been observed in Parkinson's disease. A Cochrane review reported that statistically significant improvement in cognitive impairment in Parkinson's disease patients were obtained with the treatment of cholinesterase inhibitors in six trials [10]. Tyrosinase plays a key role in melanogenesis by catalyzing the oxidation of tyrosine to dopaquinone in the first step. Although melanin serves to protect the skin from photodamage, excessive accumulation results in hyperpigmentation. Besides targeting hyperpigmentation, inhibition of tyrosinase activity was also reported to reduce melanogenesis in melanoma cells [5].

Supercritical carbondioxide (CO_2) has been an advanced extraction system for plant metabolites by the help of the supercritical phase, which is superior over conventional solvent extraction due to gas-like diffusivity and liquid-like dissolving power [11]. Regulation of viscosity and density *via* extraction parameters in supercritical CO_2 extraction enables the control of solvating power [12], which in turn provides a more selective extraction for targeted bioactive content. In addition, elimination of toxic residues and disposal stages for organic solvents are of great benefit to both humans and nature. While studying the novel activity potential of medicinal plants, it is also important to identify the optimum extraction conditions to reduce the use of organic solvent, energy and time. As plant phenolics are typically extracted using organic solvents, advanced technologies such as supercritical CO_2 can be benefited to develop suitable systems to reduce/eliminate solvent consumption [11].

In this supercritical CO_2 extraction study, revealing the possibility of extracting phenolic content of red cabbage leaves using supercritical CO_2, optimizing extraction parameters and investigating the potential for enzyme inhibitory activity were aimed. According to the accessible literature, this is the first study for the optimization of supercritical CO_2 extraction of red cabbage leaves for acetylcholinesterase, butyrylcholinesterase and tyrosinase inhibitory activities.

2. RESULTS and DISCUSSION

2.1. Validation tests

Blank extraction (extraction without the plant material) and extraction repeatability (three parallel extractions) were performed for the validation of extraction process. Analysis of the blank extraction yielded no targeted bioactive content or anthocyanin peak. Relative standard deviation (RSD) values of 5.45% and 6.53% were determined for total phenol and flavonoid contents in three parallel extractions, respectively.

Linearity, repeatability, intra-day and inter-day stability were performed for the validation of UPLC analysis of anthocyanins. R_2 value of 0.9998 was determined with the calibration curve of reference standard cyanidin-3-glucoside. RSD values of 0.65%, 0.22% and 1.20% were determined for repeatability (ten consecutive analysis), intra-day (analysis at the start, middle and end of the sequence on the same day) and inter-day (analysis on three consecutive days) stability tests, respectively.

Validation of activity protocol parameters for previously developed enzyme inhibitory activity methods [13] was studied. Michaelis-Menten, Lineweaver-Burk and Eadie-Hofstee plots were utilized for the determination of inhibition kinetics for reference drugs as galantamine and kojic acid against acetylcholinesterase and tyrosinase as single inhibitor-single substrate, respectively [14]. The results were found in full compliance with literature data [5, 9] where competitive type and mixed type inhibition were determined with studied protocols for galantamine and kojic acid, respectively.

2.2. Optimization of supercritical CO_2 extraction

2.2.1. Optimization model

Optimization of supercritical CO_2 extraction was studied for temperature (40, 60, 80°C), pressure (120, 160, 200 bar) and co-solvent ethanol ratio (0, 6, 12%). Total phenol content (response 1), total flavonoid content (response 2), acetylcholinesterase inhibitory (response 3), butyrylcholinesterase inhibitory (response 4) and tyrosinase inhibitory (response 5) activities were the selected responses for the optimization study (Table 1). As none of the supercritical CO_2 extracts possessed anthocyanin content with spectrophotometry and LC-MS/MS, total and dominant anthocyanin content analysis were excluded from the optimization design. In Fig. 1 and Fig. 2, plots for optimization model were presented which depicted the effect of extraction parameters on bioactive content and enzyme inhibitory activities, respectively. The analysis of variance (ANOVA) tables for all responses were presented as Supplementary Material. Optimization equations of the

significant models were presented below where extraction parameters as temperature, pressure and co-solvent ethanol ratio were resembled with A, B and C, respectively.

Total phenol content = 8.27- 0.45*A + 0.062*B + 4.48*C + 1.06*A*B - 1.00*A*C - 1.01*B*C - 0.44*A^2+ 0.72*B^2 - 1.80*C^2 + 1.07*A^2*B - 2.31*A^2*C + 1.84*A*B^2

Total flavonoid content = 28.25 - 11.48*A + 1.31*B + 2.88*C - 3.08*A*B - 6.08*A*C - 3.11*B*C + 1.94* A^2 + 4.30*B^2 - 0.80*C^2 - 5.74*A^2*B - 2.72*A^2*C + 14.69*A*B^2

AChE inhibitory activity = 10.33 + 0.21*A - 0.27*B - 1.95*C - 0.060*A*B + 1.89*A*C - 0.64*B*C + 1.37* A^2 + 2.10*B^2 + 0.42*C^2 - 0.31*A^2*B + 2.39*A^2*C

BChE inhibitory activity = 20.52 - 5.43*A + 1.29*B + 2.18*C - 0.24*A*B - 1.44*A*C - 0.025*B*C - 0.81* A^2 + 1.35*B^2 + 0.068*C^2 - 3.43*A^2*B - 5.09*A^2*C + 5.04*A*B^2

Tyrosinase inhibitory activity = 10.00 - 4.32*A - 13.24*B + 2.74*C - 0.77*A*B - 1.61*A*C - 3.72*B*C - 4.00 *A^2 + 4.77*B^2 + 8.46*C^2 + 14.01*A^2*B + 3.54*A*B^2

As shown in the results of optimization study (Supplementary Material), for all selected responses model fitness was achieved. In terms of bioactive content, correlation coefficients (R^2) of 0.9971 and 0.9932 were obtained for total phenol and flavonoid contents, respectively. For AChE, BChE and tyrosinase inhibitory activities, R^2 values were determined as 0.9882, 0.9939 and 0.9943, respectively. The statistically significant (p<0.05) model p-values for all selected responses (both for bioactive content and enzyme inhibitory activities) indicated the reliability of the optimization model for the extraction parameters. The significant extraction parameters (p<0.05) were found to be co-solvent ratio for total phenol content, and temperature and co-solvent ratio for total flavonoid content. Regarding the enzyme inhibitory activity, the effect of co-solvent was found to be significant for AChE inhibitory activity, while temperature and co-solvent ratio for BChE inhibition. All extraction parameters as temperature, pressure and co-solvent ratio had significant effect on tyrosinase inhibitory activity (p>0.05).

2.2.2. Analysis of bioactive content

The highest total phenol content was determined to be 12.62 mg GAE/g extract at 60°C, 120 bar, 12% co-solvent ratio conditions (Table 1). The effect of co-solvent ratio (p: 0.0025) was found to be significant while temperature (p: 0.1871) and pressure (p: 0.8076) were found to be not significant by the model results (model p-value: 0.0172) for total phenol content (Supplementary Material Table S1). The model plots (Figure 1A) indicated that at the highest co-solvent ratio of 12%, reducing the pressure from 200 bar to 120 bar with increasing temperature to 60°C allowed the extraction with highest total phenol content. However, a further increase in temperature to 80°C resulted in a decrease. Therefore, 60°C at 120 bar was determined as the optimum conditions. In terms of supercritical CO_2 extraction, the required solvating power for a more selective extraction of phenolic content *via* regulation of viscosity and density could be interpreted as being achieved at 60°C and 120 bar conditions with a co-solvent ratio of 12%. At 120 bar conditions (Figure 1A), the increase in co-solvent ratio was directly proportional to the total phenol content at 60°C, while both lower and higher levels caused a reduction. Increasing the pressure from 120 bar to 200 bar (Figure 1C) had a negative effect as the pressure increased to 200 bar. The optimization model pointed out 60°C, 120 bar, 12% co-solvent (Optimum 1) as the optimum conditions for total phenol content with the experimental results.

Differing from the total phenol content, the highest total flavonoid content was determined at 40°C, 160 bar, 12% co-solvent extract as 47.11 mg QE/g extract (Table 1). The effect of temperature (p: 0.0064) was found to be significant while pressure (p: 0.2919) and co-solvent (p: 0.0891) were found to be not significant by the model results (model p-value: 0.0400) for total flavonoid content (Supplementary Material Table S2). Reducing the temperature to 40°C with 12% co-solvent provided the extraction with the highest total flavonoid content while maintaining the pressure at 160 bar. Higher and lower pressure levels seemed to have a negative effect on total flavonoid content with these extraction conditions (Figure 1B). Similar to the total phenol content, the highest total flavonoid content (47.11 mg QE/g) was obtained with 12% co-solvent ethanol usage. Decreasing the temperature from 80°C to 40°C at 160 bar increased the total flavonoid content with elevated co-solvent usage. At 40°C, 160 bar was identified as the optimum point for pressure, as both lower (120 bar) and higher levels (200 bar) did not produce better results with increasing co-solvent usage up to 12%. The optimization model indicated 40°C, 160 bar, 12% co-solvent (Optimum 2) as the optimum

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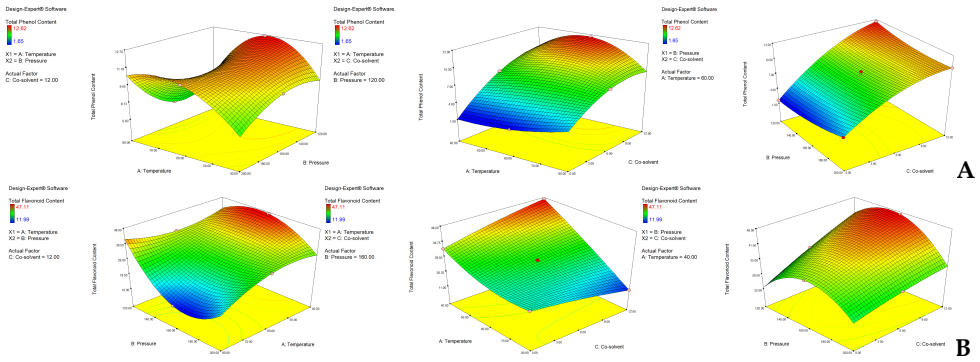

Figure 1. Optimization model graphs for the effect of extraction parameters on bioactive content; A: Total phenol content; B: Total flavonoid content

conditions for total flavonoid content with the experimental results. Compared to literature indicating 400 bar and 85% ethanol conditions for the extraction of flavonoids from citrus peels [15], determined optimized parameters for red cabbage extraction can be denoted as greener and more feasible. Comparison of conventional solvent extracts and optimum supercritical CO2 extracts was presented with Table 2. Among the solvent extracts, the highest total flavonoid content (30.45 mg QE/g) was found in the dichloromethane extract. As the Optimum 2 extract possessed 47.11 mg QE/g, optimized supercritical CO_2 could be denoted as more capable for a selective extraction of flavonoids (Table 2).

Table 1. Analysis of the bioactive content and enzyme inhibitory activity of supercritical CO_2 extracts

Supercritical CO2 Extracts	Temperature (°C)	Pressure (bar)	Co-solvent ratio (%)	TPC[a] (mg/g GAE)	TFC[a] (mg/g QE)	TAC[a] (mg/g Cya3 GluE)	DAC[a] (mg/g Cya3 GluE)	AChEInh (%inh[i])	BChEInh (%inh[i])	TyroInh (%inh[ii])
Run 1	60	200	12	10.73±0.65	32,82±1,59	ND	ND	10<	25,40±0,26	10<
Run 2	60	200	0	3.79±0.29	33,29±1,72	ND	ND	15.18±1.59	21,08±2,54	10<
Run 3	60	120	0	1.65±0.13	24,45±2,26	ND	ND	14.45±1.09	18,44±1,24	29.03±3.99
Run 4	80	160	0	4.43±0.35	23,83±2,09	ND	ND	10<	18,70±2,56	10<
Run 5	80	120	6	7.76±0.44	45,21±3,79	ND	ND	14.67±1.19	23,05±2,08	10<
Run 6	60	160	6	8.79±0.42	29,55±2,07	ND	ND	10<	21,35±2,11	10<
Run 7	60	120	12	12.62±0.46	36,43±1,59	ND	ND	11.81±1.07	22,86±1,52	43.91±1.67
Run 8	80	160	12	6.76±0.59	11,99±1,24	ND	ND	14.66±1.96	10<	10.30±1.02
Run 9	40	160	0	3.32±0.05	34,62±3,32	ND	ND	13.39±0.77	26,68±3,77	15.41±1.05
Run 10	40	120	6	7.09±0.08	32,62±2,83	ND	ND	14.10±1.44	23,35±2,27	10<
Run 11	40	200	6	7.23±0.19	29,91±1,37	ND	ND	13.07±0.91	19,56±0,49	13.09±1.19
Run 12	60	160	6	8.07±0.75	29,06±2,90	ND	ND	10<	20,49±2,25	10<
Run 13	40	160	12	9.65±0.64	47,11±2,56	ND	ND	10.48±1.46	23,75±1,65	22.16±1.81
Run 14	80	200	6	12.15±1.05	30,19±2,41	ND	ND	13.40±1.45	18,31±1,82	10<
Run 15	60	160	6	7.96±0.38	26.14±2,76	ND	ND	11.06±0.97	19,73±0,75	10<

[a]: mg/g dry extract ± SD (results of triplicate analysis); ND: Not determined; % inh: Inhibition percentage at highest soluble concentration ± SD; [i]:0.3125 mg/ml; [ii]: 1.25 mg/ml; TPC: Total phenol content; TFC: Total flavonoid content; TAC: Total anthocyanin content (spectrophotometry); DAC: Dominant anthocyanin content (LC-MS/MS); GAE: Gallic acid equivalent; QE: Quercetin equivalent; Cya3GluE: Cyanidin-3-glucoside equivalent; AChEInh: Acetylcholinesterase inhibitory activity; BChEInh: Butyrylcholinesterase inhibitory activity; TyroInh: Tyrosinase inhibitory activity; ANOVA (analysis of variance) results for all responses are presented as Supplementary Material

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Table 2. Comparison of optimum supercritical CO_2 and solvent extracts

Optimum Supercritical CO_2 and Solvent Extracts	Temperature (°C)	Pressure (bar)	Co-solvent ratio (%)	Time (min)	TPC[a] (mg/g GAE)	TFC[a] (mg/g QE)	TAC[a] (mg/g Cya3 GluE)	DAC[a] (mg/g Cya3 GluE)	AChEInh (%inh-IC_{50})	BChEInh (%inh-IC_{50})	TyroInh (%inh-IC_{50})
Optimum 1 60°C – 120 bar – 12%	60	120	12	60	12.62 ±0.46	36,43 ±1,59	ND	ND	12%[ii]	23%[ii]	IC_{50}: 1.71 mg/ml
Optimum 2 40°C – 160 bar – 12%	40	160	12	60	9.65 ±0.64	47,11 ±2,56	ND	ND	10%[ii]	24%[ii]	22%[iv]
Optimum 3 60°C – 200 bar – 0%	60	200	0	60	3.79 ±0.29	33,29 ±1,72	ND	ND	15%[ii]	21%[ii]	10<%[iv]
Optimum 4 40°C – 160 bar – 0%	40	160	0	60	3.32 ±0.05	34,62 ±3,32	ND	ND	13%[ii]	27%[ii]	15%[iv]
Dichloromethane	25	N/A	N/A	360	14.94 ±0.82	30.45 ±0.69	ND	ND	<10%[i]	<10%[i]	<10%[iii]
Ethanol (0.1% TFA)	25	N/A	N/A	360	25.88 ±0.04	1.94 ±0.17	5.77 ±0.23	2.11 ±0.03	41.87%[v]	<10%[v]	IC_{50}: 6.22 mg/ml
70% Ethanol (0.1% TFA)	25	N/A	N/A	360	35.10 ±1.61	4.69 ±0.30	8.82 ±0.48	5.50 ±0.01	30.50%[v]	IC_{50}: 2.44 mg/ml	IC_{50}: 7.32 mg/ml

Data of solvent extracts was previously published.
[a]: mg/g dry extract ± SD (results of triplicate analysis)
N/A: Not applied
ND: Not determined
%inh: Inhibition percentage at highest soluble concentration
[i]:0.125 mg/ml; [ii]:0.3125 mg/ml; [iii]:0.625 mg/ml; [iv]:1.25 mg/ml; [v]:6.25 mg/ml concentration
TPC: Total phenol content; TFC: Total flavonoid content; TAC: Total anthocyanin content (spectrophotometry); DAC: Dominant anthocyanin content (LC-MS/MS)
GAE: Gallic acid equivalent; QE: Quercetin equivalent; Cya3GluE: Cyanidin-3-glucoside equivalent
AChEInh: Acetylcholinesterase inhibitory activity; BChEInh: Butyrylcholinesterase inhibitory activity; TyroInh: Tyrosinase inhibitory activity

The anthocyanins were not detected in supercritical CO_2 extracts with total anthocyanin and dominant anthocyanin content analysis by spectrophotometer and UPLC-DAD, ESI-MS/MS, respectively. The dominant anthocyanin content of solvent extracts was quantified on the basis of reference standard, cyanidin-3-glucoside, where the major anthocyanins of red cabbage were determined as cyanidin-3-sophoroside-5-glucoside and cyanidin-3-(sinapoyl)-sophoroside-5-glucoside. Quantitative analysis was performed using diode array detector and qualitative confirmation was completed with mass detector results *via* selected reaction monitoring. The mass fragmentation pattern and elution order of the anthocyanin peaks were found to be consistent with literature data [1, 3, 4, 16]. DAD and MS chromatograms were shown with Figure 3. Among the solvent extracts (data was previously published), the highest total anthocyanin content was determined in 70% ethanol (0.1% TFA, pH 2.20) extract as 8.82 mg Cya3GluE/g, while the dominant anthocyanin content was determined as 5.50 mg Cya3GluE/g extract (Table 2). The ethanol (0.1% TFA) extract had a total anthocyanin content of 5.77 mg Cya3GluE/g, whereas no anthocyanin content was observed in the dichloromethane extract. Improved extraction of anthocyanins with solvents such as acidic aqueous ethanol (pH<3) was correlated well with the literature data [17].

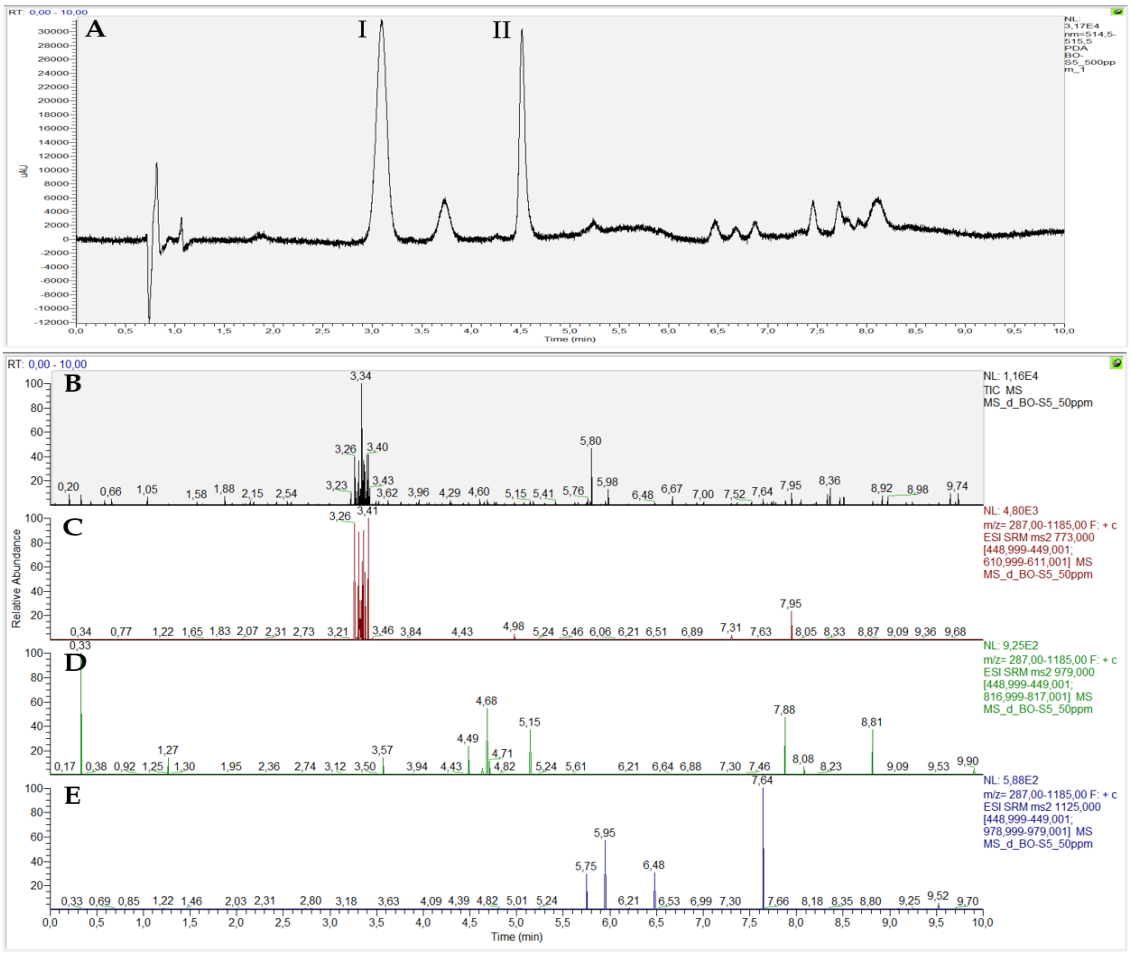

Figure 3. DAD and MS chromatograms of red cabbage 70% ethanol extract;
A: DAD chromatogram at 515 nm; cyanidin-3-sophoroside-5-glucoside (I); cyanidin-3-(sinapoyl)-sophoroside-5-glucoside (II)
B: MS chromatogram of red cabbage anthocyanins with Selected Reaction Monitoring
C: cyanidin-3-sophoroside-5-glucoside ($[M]^+$ 773, m/z 611-449)
D: cyanidin-3-(sinapoyl)-sophoroside-5-glucoside ($[M]^+$ 979, m/z 817-449)
E: cyanidin-3-(sinapoyl)(*p*-coumaroyl)-sophoroside-5-glucoside ($[M]^+$ 1125, m/z 979-449)

2.2.3. Enzyme inhibitory activity

The optimization model for AChE inhibitory activity was determined as significant (model *p*-value: 0.0129) whereas highest activity was found as 15% inhibition at 0.3125 mg/ml conc. Solubility was the main limiting factor for the study of enzyme inhibitory activity of supercritical CO_2 extracts whereas DMSO:water (1:9 for cholinesterase and 2:3 for tyrosinase) was used for sample preparation. Therefore, the highest soluble concentration as 0.3125 mg/ml was studied for the optimization study extracts. As the 50% inhibition were not able to be reached at studied doses, IC_{50} values could not be calculated. Despite the low values for AChE inhibitory activity, the optimization model and effect of co-solvent (*p:* 0.0037) was found as significant (Supplementary Material Table S3). Optimum conditions for AChE inhibition were determined as 60°C, 200 bar, 0% co-cosolvent conditions. Contrary to total phenol and flavonoid content, highest activity at studied dose was determined without use of any ethanol in the optimization study. At 0% co-solvent, decrease of temperature to 60°C with increasing pressure up to 200 bar provided the extract with highest AChE inhibitory activity. As the trend was observed, lowering the temperature below 40°C and elevating the pressure above 200 bar can be targeted in future optimization designs to maximize AChE inhibitory activity. Increasing co-solvent with decrease of temperature at 200 bar was identified as inversely proportional with activity. Other than the targeted content, plant bioactives present in supercritical CO_2 extracts with less polar character could be denoted as carrying a potential for AChE inhibition as without the addition of co-solvent ethanol, supercritical CO_2 mainly extracts apolar content. Similarly, highest total phenol and flavonoid content bearing solvent extract of 70% ethanol did not possess strongest activity for AChE inhibition (Table 2). Compared to

reference drug galantamine (IC_{50}: 0.21 µg/ml), both supercritical CO_2 and solvent extracts were found with remarkably weaker activity. Among the traditional solvent extracts, the ethanol extract (with an inhibition rate of 42%) exhibited the highest AChE inhibitory activity at a concentration of 6.25 mg/ml, while the 70% ethanol extract showed 31% inhibition. At its highest soluble concentration of 0.3125 mg/ml, the optimum supercritical CO_2 extract (Optimum 3) possessed 15% inhibition which could be described as more potent considering the doses studied. However, poor solubility of supercritical CO2 extracts in assay buffer could be identified as the main limiting factor for an accurate comparison for *in vitro* AChE inhibitory activity tests.

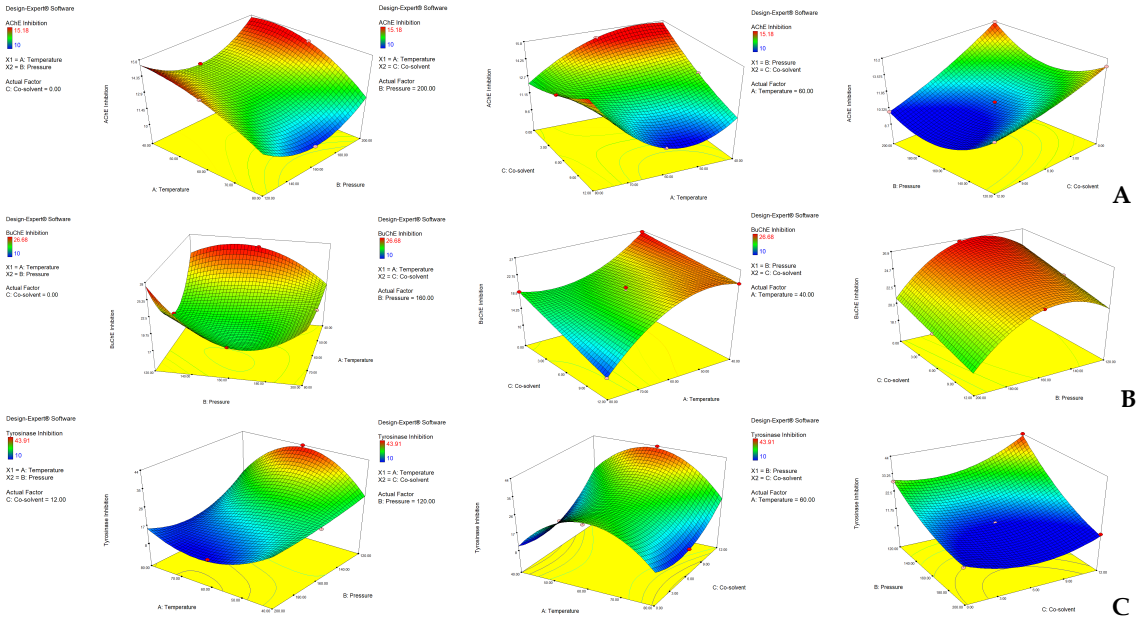

Figure 2. Optimization model graphs for the effect of extraction parameters on enzyme inhibitory activities; A: AChE inhibition; B: BChE inhibition; C: Tyrosinase inhibition

BChE inhibitory activity was highest determined as 27% inhibition at 0.3125 mg/ml conc where 40°C, 160 bar, 0% co-cosolvent conditions were identified as optimum (Optimum 4). The optimization model was determined as significant (model *p*-value: 0.0359) whereas the effect of temperature (*p*: 0.0055) and co-solvent ratio (*p*: 0.0327) were found as significant for BChE inhibitory activity (Supplementary Material Table S4). Decreasing the temperature to 40°C with 160 bar pressure, highest BChE inhibitory activity was obtained with 0% co-solvent ethanol usage. Achieving to extract bioactives with enzyme inhibitory potential without use of organic solvents could be denoted as a superiority of supercritical CO2 extraction. Parallel to AChE, solubility was the main limiting factor for BChE inhibitory studies. With the highest soluble concentration as 0.3125 mg/ml, IC_{50} values could not be calculated. However, 27% inhibition at 0.3125 mg/ml conc. by optimum extract could be practically comparable (even better) with solvent extract of 70% ethanol which possessed an IC_{50} of 2.44 mg/ml (Table 2). The total phenol and anthocyanin rich 70% ethanol extract was found with strongest BChE inhibitory activity which conformed well with literature data where increased phenolic content [18, 19] was associated with higher activity. Compared to reference drug galantamine (IC_{50}: 2.5 µg/ml), supercritical CO_2 and solvent extracts were found with remarkably weaker activity.

The highest tyrosinase inhibitory activity (IC_{50}: 1.71 mg/ml) was determined at 60°C, 120 bar, 12% co-solvent (Optimum 1) conditions. The optimization model was found to be significant (model *p*-value: 0.0044) along with all extraction parameters as temperature (*p*: 0.0124), pressure (*p*: 0.0005) and co-solvent ratio (*p*: 0.0167) (Supplementary Material Table S5). 60°C was determined as optimum at 12% co-solvent ratio conditions where reducing the pressure from 200 bar to 120 bar had a promoting effect on obtaining extracts with stronger tyrosinase inhibitory activity. Higher and lower temperatures than 60°C possessed extracts with weaker activity. At 120 bar, increase of co-solvent ratio provided better activity which could be a result of higher phenolic content. Optimum 1 extract was found with highest total phenol content and tyrosinase inhibitory activity. Similarly in solvent extracts, higher total phenol content containing ethanol and 70% ethanol extracts were found with stronger enzyme inhibitory activity (Table 2). Optimum supercritical CO_2 extract (IC_{50}: 1.71 mg/ml) was found to possess considerably more potent tyrosinase inhibitory activity than conventional solvent extracts, where ethanol and 70% ethanol extracts exhibited IC_{50} values of 6.22 mg/ml

and 7.32 mg/ml, respectively. Previous studies had also indicated increased total phenol and flavonoid contents with stronger activity [20, 21]. However, as the extracted major molecules could vary among the systems, a direct content-activity relationship might not be indicated on the basis of total phenol, flavonoid and anthocyanin contents. Compared to reference drug kojic acid (IC_{50}: 5.21 µg/ml), supercritical CO_2 and solvent extracts were found with remarkably weaker activity.

Supercritical CO_2 extraction of red cabbage was capable of obtaining 4.43 mg GAE/g and 33.29 mg QE/g total phenol and flavonoid content without the use of any ethanol as co-solvent. 80°C, 200 bar, 6% co-solvent conditions extracted the second highest total phenol content (12.15 mg GAE/g). Compared to the optimum conditions (Optimum 1), 6% co-solvent usage were seemed to provide the extract with a close total phenol amount by elevating temperature and pressure. However, on the side of the enzyme inhibitory activity, 6% co-solvent extract showed remarkably weaker tyrosinase inhibitory activity whereas both extracts (Run 7 and 14) possessed identical AChE and BChE inhibitory activity. The extraction of phenolic content was achieved with a relatively low ethanol consumption using supercritical CO_2, whereas literature data primarily indicates the use of organic solvents for the extraction of phenolic compounds [22].

3. CONCLUSION

To the best of knowledge, this is the first study for the optimization of supercritical CO_2 extraction of red cabbage leaves for AChE, BChE and tyrosinase inhibitory activity. The optimization study included the parameters as temperature (40, 60, 80°C), pressure (120, 160, 200 bar) and co-solvent ethanol ratio (0, 6, 12%). The optimization models of Box-Behnken Design were found to be significant for all bioactive content analysis and enzyme inhibitory activities. The effect of all parameters on tyrosinase inhibitory activity was determined to be significant whereas temperature and co-solvent ratio were significant parameters for BChE inhibitory activity. None of the anthocyanins were detected in supercritical CO_2 extracts of the studied parameters. Compared to solvent extracts, optimum supercritical CO_2 extracts possessed higher total flavonoid content and enzyme inhibitory activity. For the extraction of red cabbage, supercritical CO_2 could be identified as a useful and greener system by drastically reducing/removing organic solvent needs with the optimized parameters. Future studies can be designed to isolate major flavonoids that can be selectively extracted with supercritical CO_2 which contain enzyme inhibitory potential.

4. MATERIALS AND METHODS

4.1. Plant material

Brassica oleracea var. *capitata* f. *rubra* (red cabbage) fresh leaves were collected from an agricultural production site located in Bayindir, İzmir, Türkiye, in December 2018. The leaves were cleaned with distilled water and outermost leaves were discarded. Drying was carried out in air flow controlled drying cabinets. The dried plant material was stored at -24°C in airtight storage boxes until the experiments. Prior to the extraction studies, the dried leaves were ground and used.

4.2. Chemicals

Gallic acid, quercetin, Folin-Ciocalteu reagent, kojic acid, tyrosinase, acetylcholinesterase (AChE), butyrlcholinesterase (BChE), acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BUTCI) were purchased from Sigma-Aldrich (Steinheim, Germany). Galantamine, levodopa, DTNB were purchased from Alfa Aesar (MA, USA). DMSO, trifluoroacetic acid, aluminum chloride, potassium phosphate dibasic, potassium phosphate monobasic, potassium acetate were purchased from Carlo Erba (Emmendingen, Germany). Ethanol, sodium carbonate, sodium acetate, sodium phosphate monobasic, sodium phosphate dibasic, potassium chloride were purchased from Merck (Darmstadt, Germany). Cyanidin-3-glucoside was purchased from Extrasynthese (Genay, France). All of the chemicals were of analytical grade and water was obtained with Stakpure Omnia Type I Ultrapure Water Systems (Niederahr, Germany).

4.3. Solvent extractions

Before the extractions, grinding and homogenization of dried leaves were carried out with a blender (Isolab blender). In glass flasks, 10 g of ground leaves were mixed with 200 ml of extraction solvents as dichloromethane, ethanol (0.1% TFA) and 70% ethanol (0.1% TFA). For the extraction of anthocyanins, 0.1% TFA was added to obtain a pH of under 3. The solvent extractions were carried out at 25°C in the dark for 1 h per round with an ultrasonic bath (Isolab 13lt). The residues were extracted twice more after separation of the filtrates. The combined filtrates were then concentrated at 40°C using a rotary evaporator (Buchi R100) [23].

The vacuum concentrator system (Thermo Scientific Speedvac) and lyophiliser (Labconco Freezone) were used to maintain total dryness. All extracts were stored at -24°C during the experiments.

4.4. Supercritical CO_2 extraction

A laboratory scale supercritical CO_2 extractor system with automated temperature and pressure controls was used for the extractions (Thar Designs SFE-100, U.K.). For each run, 20 g of dried leaves were placed in the extraction chamber and the extractions were performed according to the optimization design. Dynamic extractions were performed for 60 min at a CO_2 flow rate of 15 g/min. Extraction parameters as independent variables were temperature (40, 60, 80°C), pressure (120, 160, 180 bar) and co-solvent ethanol ratio (0, 6, 12%). Box-Behnken Design was utilized for the optimization study with the selected parameters (Design Expert Software 7.0). After the extraction process, the extracts were filtered and concentrated at 40°C using a rotary evaporator (Buchi R100). The vacuum concentrator system (Thermo Scientific Speedvac) and lyophiliser (Labconco Freezone) were used to maintain total dryness. All extracts were stored at -24°C during the experiments.

4.5. Statistical design

Response Surface Methodology (RSM) have been used as a mathematical and statistical technique for modelling and analyzing problems. The steps of process for RSM were defined as selection of independent variables with appropriate experimental design, execution of experiments according to the design, evaluation of the obtained data with mathematical-statistical models, and analysis of the model fitness for finding the optimal conditions [24]. In this study, a three-level model was used with three independent variables for supercritical CO_2 extraction as temperature, pressure and co-solvent ratio. Extraction time and CO_2 flow rate were held constant. The stability of the process and the intrinsic variability were measured by three center points in the experimental design (Design Expert Software 7.0).

4.6. Total phenol content

Total phenol content was determined with a 96 well microplate adapted method (based on the Folin-Ciocalteu method) [25]. In each well, 100 µl of distilled water, 20 µl of sample and 10 µl of Folin-Ciocalteu reagent were mixed. The microplate was kept in dark for 4 min after shaking at 200 rpm (Jeio Tech Vortex). Finally, 100 µl of saturated sodium carbonate solution was added and kept in dark for 2 h at room temperature. Absorbance was measured at 760 nm against blank (70% ethanol) with microplate reader (BMG Labtech Clariostar). The calibration curve of gallic acid as the reference standard was constructed in the range of 1-250 µg/ml with seven data points. All analyses were performed in triplicate. The results were expressed as mg gallic acid equivalent (GAE)/g extract.

4.7. Total flavonoid content

Total flavonoid content was determined with a 96 well microplate adapted method (based on the aluminum chloride method) [26]. In each well, 70 µl ethanol, 130 µl distilled water, 25 µl of sample, 10 µl of 10% aluminum chloride and 10 µl of 1 M potassium acetate were mixed. The microplate was kept in dark for 40 min after shaking at 200 rpm. Absorbance was measured at 415 nm against blank (70% ethanol) with microplate reader. The calibration curve of quercetin as the reference standard was constructed in the range of 1-250 µg/ml with seven data points. All analyses were performed in triplicate. The results were expressed as mg quercetin equivalent (QE)/g extract.

4.8. Total anthocyanin content

Total anthocyanin content was determined with a 96 well microplate adapted method (based on pH change method) [27, 28]. In each well, samples were mixed with 0.025 M potassium chloride (pH 1) and 0.4 M sodium acetate (pH 4.5) buffers which were adjusted with HCl. The microplate was kept in dark for 20 min at room temperature. Absorbance was measured at 520 nm and 700 nm against blank (70% ethanol) with microplate reader. All analyses were performed in triplicate. The results were expressed as mg cyanidin-3-glucoside equivalent (Cya3GluE)/g extract. The following formula was used to determine the results;

total anthocyanin content=(A x MW x DF x 1000)/(e x L)

where;

A= [(Absorbance 520nm – Abs 700nm) pH1] – [(Abs 520nm – Abs 700nm) pH4.5]

MW (molecular weight) = 449.2 g/mole for cyanidin-3-glucoside

DF: Dilution factor (ratio of buffer/sample volume that gives absorbance below 1.4 with pH 1 buffer)

e (molar absorption coefficient): 26.900 Lxmole-1xcm-1 for cyanidin-3-glucoside

L: Pathlength as cm (calculated *via* pH 1 buffer absorbance with reaction volume at 900 and 975 nm in 96 well microplate and 1 cm pathlength glass cuvettes)
microplate well pathlength=1cm x ((Abs$_{975nm}$-Abs$_{900nm}$)well with pH1 buffer)/((Abs$_{975nm}$-Abs$_{900nm}$)cuvette with pH1 buffer) [29].

4.9. Dominant anthocyanin content

The dominant anthocyanins in 70% ethanol (0.1% TFA) extract were quantified with UPLC-DAD, ESI-MS/MS [13]. The system (Thermo Scientific Accela 1250) consisted of diode array and mass detectors (Thermo Scientific TSQ Quantum Access Maxx triple quadrupole). The column size was 150 x 2.1 mm, 3 µm (Thermo Syncronis C18) with a guard column of 10 x 2.1 mm, 5 µm particle size (Thermo Syncronis C18). The flow rate was 500 µl/min and the injection volume was 5 µl. The column oven was set to 35°C. All samples were filtered through 0.45 µm PTFE filters (Sartorius) prior to injection. The gradient system for the mobile phase was as; at 0.1 min 90A:10B; at 1 min 90A:10B; at 7 min 70A:30B; at 9 min 90A:10B and at 10 min 90A:10B, where the mobile phase A is water:formic acid (90:10) and B is water:formic acid:acetonitrile (40:10:50). Mass spectra were acquired in selected reaction monitoring (SRM) mode and electrospray ionization (ESI) was performed in positive ion mode [30]. The ionization conditions were 380°C for capillary temperature, 200°C for evaporator temperature, 3000 V for spray voltage, 60 arbitrary units for sheath gas flow rate and 20 arbitrary units for auxiliary gas flow rate. The calibration curve of cyanidin-3-glucoside as the reference standard was constructed in the range of 0.5-15 µg/ml with five data points. All analyses were performed in triplicate. The results were expressed as mg cyanidin-3-glucoside equivalent/g extract by calculating the peak areas of the dominant anthocyanins as cyanidin derivatives [1, 4, 23, 31]. The quantitative and qualitative determination by Diode Array Detector (at 515 nm) and MASS Detector, respectively, ensured the reliability of anthocyanin analysis.

4.10. Cholinesterase inhibitory activity

Cholinesterase inhibitory activity was determined using a 96 well microplate method based on based on Ellman's method [32, 33]. ATCI and BUTCI were used as substrates for the determination of AChE and BChE inhibitory activity, respectively, where galantamine was used as the reference drug. 30 µl extracts (in DMSO:water 1:9) were mixed with 150 µl 0.1 M sodium phosphate buffer (pH 8), 25 µl 0.01 M DTNB (in buffer) and 25 µl AChE or BChE enzymes (0.2 Unit/ml and 0.1 Unit/ml in buffer containing 1 mg/ml bovine serum albumin). The microplate was then pre-incubated for 5 min on 200 rpm shaker at 25°C in the dark. Finally, 25 µl substrate as 3 mM ATCI or BUTCI (in buffer) was added to initiate the reaction. During the 10 min incubation at 25°C, kinetic readings were taken at 412 nm with 30 s intervals using the microplate reader to determine the linear change in absorbance. The following formula was used to determine the results;

$$\% \text{ inhibition} = [(\Delta A_{blank} - \Delta A_{sample})/\Delta A_{blank}] \times 100$$

where;
ΔAblank and ΔAsample represent the rate of absorbance change at 412 nm during 10 min incubation period with the blank (DMSO:water 1:9) and the extract, respectively. All analyses were performed in triplicate.

4.11. Tyrosinase inhibitory activity

Tyrosinase inhibitory activity was determined with a 96 well microplate assay where kojic acid was used as the reference drug [13]. 25 µl of extracts (in DMSO:water 2:3) were mixed with 150 µl 2 mM L-dopa (in 50 mM pH 6.8 phosphate buffer). The microplate was pre-incubated for 5 min on 200 rpm shaker at 25°C in the dark. Finally, 25 µl of tyrosinase enzyme (50 Unit/ml in phosphate buffer) was added to initiate the reaction. The microplate was incubated during 5 min at 25°C while kinetic readings with 30 s intervals were taken at 475 nm. The following formula was used to determine the results;

$$\% \text{ inhibition} = [(\Delta A_{blank} - \Delta A_{sample})/\Delta A_{blank}] \times 100$$

where;
ΔAblank and ΔAsample represent the rate of absorbance change at 475 nm during 5 min incubation period with the blank (DMSO:water 2:3) and the extracts, respectively. All analyses were performed in triplicate.

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