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by tytyshafiqa 1

Utilization of Triethylammonium Hydrogen Sulphate-Mediated Solvent for Optimization of Asiaticoside Extraction and Antioxidant Capacity of *Centella asiatica (L.)*

ABSTRACT

Asiaticoside, a pentacyclic triterpene of *Centella asiatica* with broad pharmacological actions. Higher asiaticoside content of Centella extracts in food products increases their nutritional and medicinal values. Protic ionic liquids (PIL) were utilized as bioactive extraction additives. The research focuses on obtaining the optimum extraction parameters for higher asiaticoside yield, and Centella extract antioxidant capacity. Optimization of all responses (asiaticoside yield, TPC, TEAC) achieved through faced-centered composite design (FCCD) involving three factors (temperature, extraction time, and triethylammonium hydrogen sulfate, [TEA][HSO_4] %). The optimal conditions were 66°C, 12 hours duration, and 20% [TEA][HSO_4], which resulted in 4.44 ± 0.05 % (w/w) asiaticoside, TPC of 114.11 ± 12.58 mg GAE /g and TEAC of 70.01 ± 5.74 μmol TE/g respectively. All responses fit the quadratic model with proximity between predicted and experimented values. Procurement of higher asiaticoside yield, TPC, and TEAC verified the pertinent of the optimal conditions. In addition, the outcomes give an overview of PILs potential for higher bioactive extractions. Research expansion by utilizing other PILs for plant extraction in addition to solute-solvent interaction study will be beneficial for the designation of an efficient plant extraction process that maximizes the plant-based product market.

Keywords: Triethylammonium hydrogen sulfate; asiaticoside; phenolic compounds; antioxidant; optimization

1. Introduction

Centella asiatica (L.) or locally known as “Pegaga” in Malaysia, is a prominent therapeutic herb with multiple pharmacological actions (Jhansi & Kola 2019 ; Wong & Ramli 2021) such as anti-inflammatory, antioxidant, wound ameliorating, neuroprotective (Yadav 2021), antimicrobial, anti-diabetic, antifungal, and anticancer properties (Tripathy & Srivastav 2023; Tripathy et al. 2022). These benefits contributed by flavonoids and terpenoids content such as asiatic acid, madecassoside, and asiaticoside (Fig. 1). Commercially, there were more than 100 Centella-based formulations in the market with at least 2 % asiaticoside and madecoside content required for the product benefits (Idris & Mohd Nadzir 2021; Prasad et al. 2019).

Therefore, the extraction processes parameters such as solvent concentration, pH, temperature, and extraction duration are crucial in obtaining the bioactive compounds (Kumar et al. 2021 ; Sridhar et al. 2021) . Ethanolic extraction has been widely employed for *C. asiatica* leaves extraction (Idris & Mohd Nadzir 2021 ; Monton et al. 2019 ; Thong-On et al. 2021 ; Yingngam et al. 2020). The asiaticoside yield through ethanol-based extraction reportedly ranged from 0.09% to 0.193% (Monton et al. 2019). Other studies reported the optimum conditions for polyphenols extraction from Centella to be 37% ethanol concentration at 70.2°C and 110.5 minutes (Mohapatra et al. 2021). Even at optimum conditions reported, the asiaticoside yield was still low than the minimum requirement.

Ionic liquids (ILs) efficiency as green solvents for bioactive extraction attracts attention (Choi & Verpoorte 2019 ; Ferreira et al. 2022 ; Lim et al. 2022 ; Yansheng et al. 2011). Protic ionic liquids (PILs) are a subgroup of IL that are non-volatile, non-flammable, and more stable at higher temperatures than conventional organic molecular solvents (Clough et al. 2015; Greaves & Drummond 2015; Nasirpour et al. 2020). Triethylammonium hydrogen sulfate, [TEA][HSO_4] (Fig. 1) is one of the PILs that has received increasing attention due to its ultra-low-cost that can be made at bulk scale for \$1.24 kg^{-1}, favorably comparable to acetone (Chen et al. 2014). [TEA][HSO_4] effectively deconstruct various types of biomass by providing dual functions: (1) a Brønsted acid catalyst that disrupts chemical linkages in biomass complex structure and; (2) a delignifier that dissolves lignin (Khan et al. 2020 ; Welton 2013 ; Zahari et al. 2018). Due to this, it is plausible to destruct the plant tissues and cell walls of *C. asiatica*, increasing their permeability and consequent molecular diffusion, playing a crucial role in higher extraction yield (Zhao et al. 2014). Moreover, asiaticoside was reportedly more stable in acidic pH (Puttarak et al. 2016).

There has yet to be the utilization of [TEA][HSO_4] reported for Centella extraction, and using previous research optimum parameters at different operational conditions is not plausible. Hence, this study investigated the optimum extraction condition for asiaticoside yield and antioxidant capacity by utilizing [TEA][HSO_4] as a co-solvent in the *C. asiatica* leaves extraction, aiding by response surface methodology (RSM).

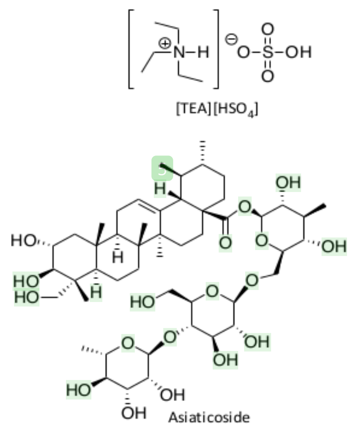

Fig. 1 Chemical structure of [TEA][HSO_4] and asiaticoside structure

2. Methodology

2.1 Preparation of Triethylammonium Hydrogen Sulfate, [TEA][HSO_4] IL

The synthesis followed a method published elsewhere (Salahi et al. 2016; Wang et al. 2006; Zahari et al. 2018). 2.5 M of H_2SO_4 (98 g, 1 mol) was added dropwise to triethylamine, N_{222} (101 g, 1 mol) over 1 h at 60°C. The mixture continued to be stirred at 70°C for 1 h. Water traces were removed by heating the resultant liquid under vacuum at 80°C. The as-synthesized triethylammonium hydrogen sulfate, [TEA][HSO_4], obtained as a colorless solid, was characterized by 1D-NMR (see Fig. S1 in ESI† for ^{1}H and ^{13}C-NMR spectra) (ppm): ^{1}H-NMR (DMSO-d6): 1.16 (t, 9H, 7.4 Hz), 3.06 (q, 6H, 7.4 Hz), 9.26 (s, 1H); ^{13}C-NMR (DMSO-d6): 9.02 (CH_3) and 46.05 (CH_2).

2.2 Asiaticoside extraction from *Centella asiatica*

Centella asiatica leaves were collected in Negeri Sembilan, Tampin, Malaysia. They were cleaned, dried in an oven for 24 h at 30 °C, and ground into a particle size of 0.5 mm. A binary solvent system was first prepared by mixing [TEA][HSO_4] and EtOH-40% according to the desired ratio Table 1. The mixture at 10 ml/g ratio was incubated at a specific period. The asiaticoside yield in the collected extract was quantified using reverse phase-high performance liquid chromatography (RP-HPLC).

Table 1 Physical properties of [TEA][H_2SO_4]: EtOH binary solvent systems

[TEA][HSO_4] /EtOH ratio (g/ml)	%[TEA] [HSO_4]	Viscosity	pH
0.25	20	0.0037 ± 0.0001	1.20 ± 0.012
0.66	40	0.0081 ± 0.0006	1.27 ± 0.006
1.0	50	0.0116 ± 0.0007	1.37 ± 0.042
1.5	60	0.0189 ± 0.0000	1.39 ± 0.010

2.3 Quantification of asiaticoside yield (AY)

The extract was diluted with deionized water at a 1:1 ratio, filtered using a 0.22 mm nylon syringe filter, and analyzed on a Shimadzu LC-20 with a photodiode array detector (PDA) at 220 nm and a C-18 column. The following conditions were used: 0.8 ml/min flow rate, 20 μl injection volume, methanol, and deionized water (70/30 (vol/vol)) as mobile phase and column temperature of 30°C. Standards asiaticoside solution prepared in deionized water at concentrations ranging from 10 to 100 ppm (see Fig. S2 in ESI† for the calibration curve)

2.4 Measurement of total phenolic content (TPC)

Diluted extracts (1 mg/ml, 20 μl of each sample) were placed in microplate wells. Subsequently, the wells were left for 10 min in the dark at room temperature after the addition of 10 v/v%, 100 μl Folin Ciocalteu reagent, followed by Na_2CO_3 (7.5%, 80 μl) addition to each sample. After 2 h left in the dark, the mixture absorbance was read at 765 nm. Gallic acid calibration curve plot (see Fig S2 in ESI†) aids TPC quantification in mg gallic acid equivalents (GAE) unit per g of dried extract.

2.5 Trolox equivalent antioxidant capacity (TEAC) quantification

DPPH scavenging ability measures the DPPH radicals quantity scavenged by phenolic compounds (ArOH) in the extract. Neutralization of DPPH radical in the assay occurred by accepting hydrogen atom or electron from antioxidant species, resulting in reduced DPPH (DPPH-H) (Bibi Sadeer et al. 2020), as shown in Equation 1. The extracts (100 µL) and 0.2 mM of DPPH solution (100 µL) were pipetted into a 96-well plate. The absorbance was read at 517 nm after 30 min dark incubation. Equation 2 was used to determine DPPH radical scavenging %. TEAC represented the antioxidant activity in μmol TE/g and was calculated using DPPH scavenging activity of trolox (%) against the log series concentration calibration curve (see Fig. S2 in ESI†).

DPPH* + ArOH à DPPH-H + ArO* Equation 1

$$\text{DPPH scavenging } \% = [(A_{DPPH} - A_{extract})/A_{DPPH}] \times 100 \qquad \text{Equation 2}$$

2.6 Optimization through response surface methodology (RSM)

RSM allowed the analysis of multiple factor effects and their interactions towards response variables (Pais-Chanfrau et al. 2021). Thus, more information can be obtained from a limited number of experiments (Goren et al. 2022). Twenty experimental trials were performed per Face-centered composite design (FCCD) with temperature, X_1 (30°C,55°C, 80°C), extraction time, X_2 (12 hours,18 hours, 24 hours), and [TEA][HSO_4] %, X_3 (20%, 40%, 60%) as variables, while, AY, TPC, and TEAC as responses. Design Expert 13 was used as a statistical tool for the experimental design and analysis. The FCCD consists of six axial points, eight factorial points, and one center with six replications.

3. Discussion

3.1 Time course extraction and experimental output

We monitored the asiaticoside yield (AY) in designing the conditions for RSM. As a control, the ground leaves were soaked in EtOH-40% at 65°C for 1 h, yielding 0.28 ± 0.02 %w/w of asiaticoside. [TEA][HSO_4]/EtOH (1g/ml) addition significantly enhanced the AY by 9 times (2.5±0.27 %w/w). Hence, time course extraction under the same conditions was performed. Fig. 2 shows that the AY increases significantly up to 12 h and then plateaus thereafter, which can be explained by Fick's second law of diffusion (Benchikh et al. 2021).

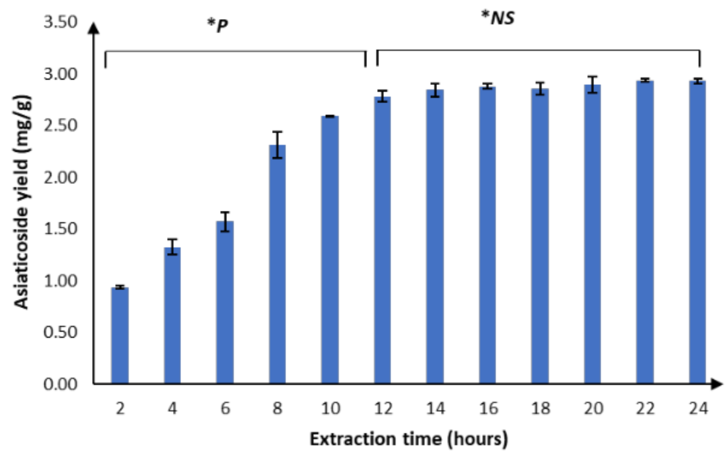

Fig. 2. Asiaticoside yields extracted from *Centella asiatica* (L.) by [TEA][HSO_4] /EtOH=1g/ml at 65°C. (*NS (non-significant): $p > 0.05$) and *$p < 0.05$(significant))

Overall, the twenty experimental trials gave the following responses: AY ranging from 2.75.% to 4.75% (w/w); TPC of 18.98 to 112.58 mg GAE/g, and; TEAC of 29.28 to 72.05 μmol TE/g. All responses adequately fitted quadratic polynomial equations, as indicated by a significant model, non-significant Lack-of-fit test, and R^2 values higher than 0.75 (Table 3).

Table 2 Experimental design parameters and output

Order	Variables			Responses		
	X_1: Temperature (°C)	X_2: Extraction time (Hours)	X_3: $[TEA][HSO_4]$ %	Y_1: AY (%,w/w)	Y_2: TPC (mg GAE /g)	Y_3: TEAC (μmolTE/g)
1	55	24	40	3.55 ± 0.030	60.84 ± 0.00	40.04 ± 1.69
2	30	24	20	3.52 ± 0.040	66.57 ± 16.24	61.88 ± 2.28
3	55	12	40	4.44 ± 0.030	68.98 ± 0.00	56.36 ± 0.00
4	30	24	60	2.75 ± 0.015	32.47 ± 0.00	29.28 ± 0.00
5	80	12	60	4.27 ± 0.017	50.60 ± 1.64	48.67 ± 13.90
6	30	18	40	3.96 ± 0.013	57.00 ± 2.47	50.07 ± 2.63
7	55	18	40	4.43 ± 0.005	75.95 ± 0.00	63.76 ± 3.02
8	55	18	40	4.29 ± 0.009	71.88 ± 6.74	53.50 ± 0.00
9	55	18	40	4.27 ± 0.002	88.28 ± 0.99	53.50 ± 0.00
10	80	24	60	3.57 ± 0.032	34.56 ± 0.00	36.60 ± 0.00
11	30	12	20	3.87 ± 0.010	111.30 ± 21.05	66.64 ± 0.35
12	55	18	20	4.00 ± 0.033	112.58 ± 26.80	72.05 ± 0.00
13	80	24	20	3.92 ± 0.001	18.98 ± 1.97	50.41 ± 0.00
14	55	18	40	4.34 ± 0.046	91.19 ± 0.16	59.38 ± 0.00
15	55	18	40	4.04 ± 0.011	82.35 ± 1.81	65.41 ± 0.00
16	80	12	20	4.75 ± 0.030	89.44 ± 0.00	66.97 ± 9.49
17	30	12	60	3.01 ± 0.019	25.72 ± 0.00	31.28 ± 0.00
18	55	18	40	4.32 ± 0.020	77.23 ± 3.12	58.43 ± 5.83
19	80	18	40	4.54 ± 0.003	55.02 ± 0.00	50.26 ± 2.91
20	55	18	60	2.97 ± 0.023	57.58 ± 8.22	36.33 ± 0.38

Table 3 Multiple regression analysis and model equations fitted for all responses

Responses	Model Equation	Model Significant	Lack-of-fit Test	R^2
AY	$+4.20 + 0.3931 X_1 - 0.3031 X_2 - 0.3486 X_3 + 0.1676 X_1^2 - 0.0901 X_2^2 - 0.5981 X_3^2 - 0.1155 X_1X_2 + 0.0999 X_1X_3 + 0.0276 X_2 X_3$	<0.0001 (Significant)	0.1118(not significant)	0.9384 Adjusted 0.8830 Predicted 0.6940
TPC	$+79.69 - 4.45 X_1 - 13.26 X_2 - 19.80 X_3 - 21.50 X_1^2 - 12.61 X_2^2 + 7.57 X_3^2 - 6.06 X_1 X_2 + 12.05 X_1 X_3 + 13.25 X_2 X_3$	< 0.0001 (Significant)	0.3229 (not significant)	0.9447 Adjusted 0.8948 Predicted 0.7221
TEAC	$+56.90 + 1.38 X_1 - 5.17 X_2 - 13.58 X_3 - 3.59 X_1^2 - 5.56 X_2^2 + 0.43 X_3^2 - 2.73 X_1 X_2 + 4.48 X_1 X_3 + 0.91 X_2 X_3$	0.0006 (Significant)	0.3876 (not significant)	0.9028 Adjusted 0.8153 Predicted 0.6258

3.2 Independent variable effects on responses

According to the analysis of variance (ANOVA) in Table 4 (Entry 1-3), all independent variables were significant towards AY. For TPC and TEAC, extraction temperature had a non-significant individual effect, while the other variables were significant.

Temperature showed a positive coefficient towards AY (Table 4, Entry 1), as depicted by Run 4 *versus* Run 10 (Table 2). Generally, secondary metabolites are secluded within the cell wall. Higher temperature aid in cell wall destruction, releasing abundant bioactive compounds (Gómez-Maqueo et al. 2020). Meanwhile, extraction time and [TEA][HSO_4]% exhibited a negative coefficient toward all responses (Table 4, Entry 2, Entry 3). This indicates that longer extraction time (Table 2, Run 1 *versus* Run 7) and an increase in [TEA][HSO_4] % (Table 2, Run 2 *versus* Run 4) led to a decrement in responses. Polyphenols degradation at extended extraction duration at high temperatures (Kim et al. 2018) and high viscosity of the binary solvent (Table 1), leading to mass transfer limitations (Fuad & Nadzir, 2023), explained this occurrence.

3.3 Independent variables interactions

AY does not significantly influence by all independent variables interactions (Table 4, Entry 4-6). In contrast, TPC was positively impacted by the interaction of [TEA][HSO_4]% with temperature and extraction time (X_1X_3; X_2X_3) (Table 4, Entry 5 and 6). Extended extraction time aid in leaching the bioactive compound out into the solvent system, resulting in higher TPC (More & Arya 2021; Sharma & Dash 2021). Meanwhile, only positive interaction of temperature-[TEA][HSO_4] % (X_1X_3) was observed for TEAC (Table 4, Entry 5). This occurrence is plausibly due to lower solvent viscosity with temperature increment, enhancing the bioactive mass transfer into the solvent system (Yusoff et al. 2022).

The variable interactions on TPC and TEAC were further visualized in 3D-surface plots, as shown in Fig. 3. In Fig. 3 (a), at 55 °C, the highest TPC was given when variables X_2 and X_3 had the smallest value. This suggests that lower [TEA][HSO_4] % and shorter extraction time favor higher TPC value.

Fig. 3 (b) shows a TPC response of X_1 *versus* X_3 at 18 h. The plot proposes that lower [TEA][HSO_4] % and moderate temperature leads to higher TPC value. Similar patterns were observed for the TEAC response of X_1 versus X_3 at 18 h (Fig. 3 (c)).

Table 4 Analysis of variance (ANOVA) for all responses

	AY (%, w/w)			**TPC (mg GAE/g)**			**TEAC (μmol TE/g)**		
Variables	**Coefficient**	**F**	**Prob>F**	**Coefficient**	**F**	**Prob>F**	**Coefficient**	**F**	**Prob>F**
X_1-temperature	0.3931	43.03	< 0.0001	-4.45	2.77	0.1269	1.38	0.66	0.4361
X_2-extraction time	-0.3031	25.58	0.0005	-13.26	24.58	0.0006	-5.17	9.29	0.0123
X_3-[TEA][HSO_4] %	-0.3486	33.83	0.0002	-19.80	54.83	< 0.0001	-13.58	64.05	< 0.0001
Interaction									
X_1X_2	-0.1155	2.97	0.1155	-6.06	4.10	0.0703	-2.73	2.08	0.1801
X_1X_3	0.0999	2.22	0.1668	12.05	16.23	0.0024	4.48	5.58	0.0398
X_2X_3	0.0276	0.17	0.6885	13.25	19.63	0.0013	0.91	0.23	0.6431

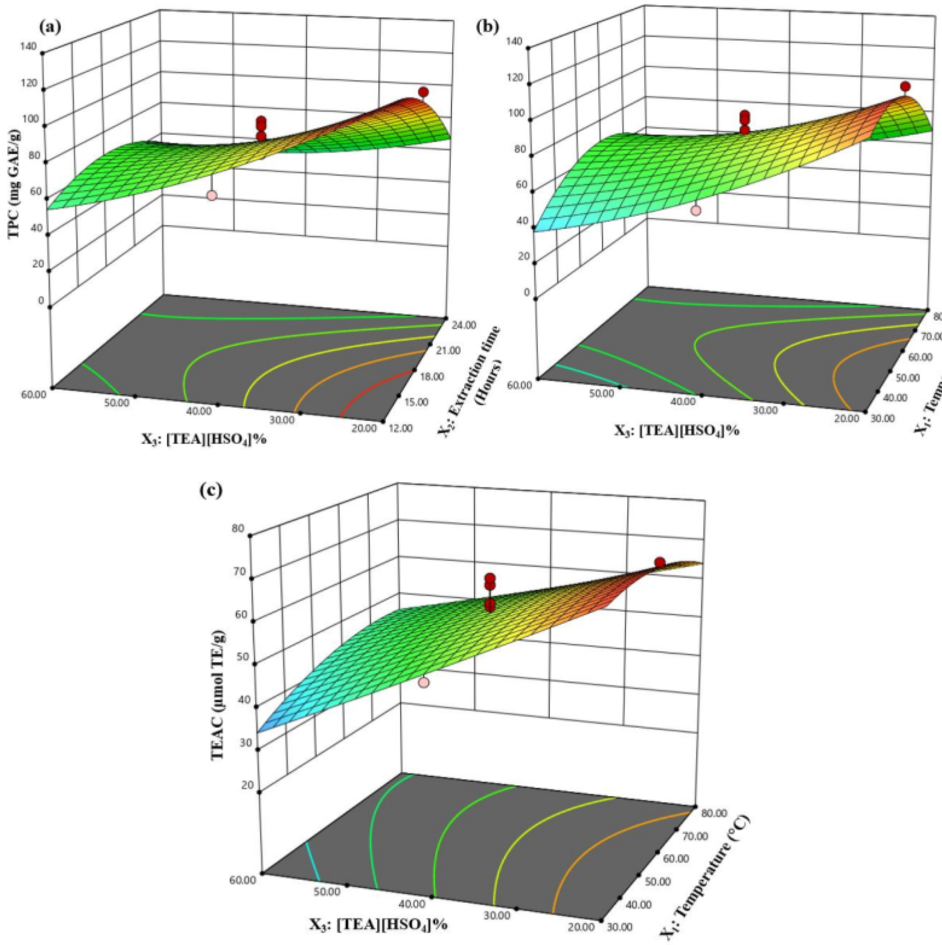

Fig. 3. Three-dimensional surface of (a) TPC: extraction time - [TEA][HSO_4]%; (b) TPC: temperature - [TEA][HSO4]%, and; (c) TEAC: temperature -[TEA][HSO_4]%.

3.4 Role of [TEA][HSO_4]

Generally, bioactive compounds are secluded in rigid, thick cell walls containing polysaccharides as the major components. Hence, any extraction techniques should be able to make the cell walls permeable, permitting the bioactive emission from the cells.

We obtained low AY when we first performed ethanolic extraction (EtOH-40%) at 65°C for 1 h. These suggest minimal destruction of cell walls by ethanolic extraction. Interestingly, adding [TEA][HSO_4] as the co-solvent enhanced asiaticoside yield by nine times. We associate this with the intensified destruction of cell walls caused by $[H_3O]^+$ ions, which arose from H_2O molecules protonation by the acidic protons of $[HSO_4]^-$ ions during the extraction process (Roy et al. 2020).

Regarding TPC and subsequent TEAC, the release of phenolic compounds appeared to increase with the temperature at a fixed $[TEA][HSO_4]$%. Higher temperatures lead to H_2O molecules protonation increment, generating more $[H_3O]^+$ ions that further intensify the destruction of cell walls.

3.5 Validation of the predictive model

The optimal C. asiatica extraction was 66°C, 12 hours, and 20% $[TEA][HSO_4]$ at predicted asiaticoside yield of 4.39 % (w/w), TPC of 112.58 mg GAE /g, and TEAC of 70.62 μmol TE/g respectively. At the same time, the experimental data at optimum conditions were 4.44 ± 0.05 % (w/w), 114.11 ± 12.58 mg GAE/g, and 70.01 ± 5.74 μmol TE/g. The experimental and predicted value proximity confirms the practicability of optimum conditions.

Interestingly, the results above were far higher than those reported in previous studies. The asiaticoside yield obtained was ca. 3.40%, 3.29%, and 4.27% higher than reported (Table 5). Similarly, TPC and TEAC were markedly increased by 89% and 72%, respectively, compared to previous studies (Table 5).

Table 5 Asiaticoside yield, TPC, and TEAC in previously reported studies

Extraction conditions	**AY (%,w/w)**	**TPC (mg GAE/g)**	**TEAC (μmol TE/g)**
MAE:40% EtOH, 153W, 10 min (Thong-On et al. 2021)	1.031	-	-
UAE: 40% EtOH, 55°C, 90 min(Thong-On et al. 2021)	1.155	-	-
95% EtOH, 60°C, 120 min (Monton et al. 2019)	0.174	-	-
40% EtOH, 65°C, 60 min (Chew et al. 2011)	-	12.03	19.48

4. Conclusion

Triethylammonium hydrogen sulfate, [TEA]HSO_4] mediated co-solvent able to enhance asiaticoside extraction. At optimal conditions of 66°C, 12 h, and 20% [TEA]HSO_4], the yield of asiaticoside, TPC, and TEAC were 4.44 ± 0.05% (w/w), 114.11 ± 12.58 mg GAE /g, and 70.01 ± 5.74 μmol TE/g respectively. All responses fit the quadratic model, and the optimal conditions can be applied practically for efficient C. asiatica extraction. The outcomes of this research, give an overview of PILs potential as bioactive extractants, besides widening the application of other PILs toward plant extraction. The extract's high antioxidant capacity will be beneficial in plant-based product development. Research expansion comprising the solute-solvent interaction will be beneficial in designing more efficient plant extraction and broadening the plant-based product market.

Conflicts of interest

There are no conflicts to declare.

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