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Ethyl Lactate as a Green Solvent for Aqueous Two-Phase Extraction of Bioactive Compounds from *Orthosiphon Stamineus*

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This study evaluates the utilization of ethyl lactate as an environmentally friendly solvent for extracting phenolic compounds from the *Orthosiphon stamineus* (*O. stamineus*) by employing aqueous two-phase system (ATPS). Ethyl lactate (EL) was selected as the primary component for the top phase due to its non-toxic attributes and cost-effectiveness. The impact of type of salt, phase compositions, sample loading, pH, equilibration time, and neutral salt's composition on the partition coefficient, *K*; yield, *Y*; and 2,2-diphenyl-1-pircrylhydrazyl (DPPH) scavenging activity of phenolic compounds was evaluated. The EL-salt ATPS contained 41.9% (w/w) EL and 14.6% (w/w) sulfate at 1.4% (w/w) of sample load, 1.5% (w/w) of potassium chloride, and pH 6.0, had the greatest *K* of 23.28 ± 0.12, *Y* of 99.03% ± 1.37%, and DPPH scavenging activity of 98.22% ± 0.40%. Therefore, the study underscores the efficiency of EL-based ATPS for extracting phenolic compounds from plants.

1. Introduction

Orthosiphon stamineus (*O. stamineus*) is perennial plant native to Malaysia. It contains more than 50 bioactive components such as terpenoids, sterols, polyphenols, sinensetin, and eupatorin that alleviate gastric discomfort, hypertension, and high blood pressure (Wang et al., 2022). Thus, effective extraction methods are crucial for extracting bioactive constituents from plants, enhancing extraction efficiency, and achieving a greater yield sustainably, while maintaining its bioactivity. Given the simplicity, biocompatibility and robustness of scaling-up, aqueous two-phase system (ATPS) is a viable option for extracting biomolecules (Mahaindran et al., 2023). Two distinct aqueous layers of ATPS are formed when two immiscible chemicals are combined above a threshold concentration, enabling the targeted compounds and impurities to separate into opposite phases.

Recently, ATPSs employing ethyl lactate (EL) as a green solvent have been introduced. EL is a monobasic ester which is non-toxic, biodegradable, and environmentally friendly (Velho et al., 2022). The US Food and Drug Administration (USFDA) has also approved its direct use in pharmaceutical and food products, thus proving its safety and versatility. EL has amphiphilic properties that enable it to extract various phenolics. EL contains both hydrophilic hydroxyl group and hydrophobic ethyl groups which can interact with polar components and the aromatic rings of phenolics to increase the extraction efficiency. Furthermore, EL improves mass transfer and reduces phase separation time due to its lower viscosity compared to conventional solvents. EL-based ATPS has been utilized for the extraction of rutin, quercetin, polyphenol, and vitamins (Velho et al., 2020; Velho et al., 2022). Nevertheless, studies on employing EL-based ATPS to extract of bioactive compounds from natural sources remain relatively scarce.

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Therefore, this work aims to evaluate the efficacy of ethyl lactate as a green solvent for extracting phenolic compounds from *O. stamineus* using the ATPS liquid-liquid extraction method. The impact of several EL-based ATPS parameters on the partitioning behaviour and extraction yield of phenolic compounds from natural source was evaluated comprehensively for the first time, providing valuable insights for the advancement of EL-based ATPS as a practical extraction method.

2. Methodology

2.1 Materials

Analytic-grade chemicals were used. Ammonium sulfate, di-potassium hydrogen phosphate, Folin-Ciocalteu phenol solution, sodium carbonate, sodium citrate, and potassium sodium tartrate tetrahydrate were sourced from Merck, Germany. Ascorbic acid, ethyl lactate (EL), and 2,2-diphenyl-1-pircylhydrazyl (DPPH) were obtained from Sigma-Aldrich Co, USA. 95% denatured ethanol was acquired from R&M Chemicals, Malaysia.

2.2 Sample Preparation

The leaves of *O. stamineus* plant were carefully rinsed and stored at -20°C for 48 hours. Then, the leaves were dried using the freeze dryer (CoolSafe Touch 110-4, Labogene, Denmark) at -103°C and 0.268 hPa for 24 h. Subsequently, the leaves were ground and sieved using a 500 μm sieve mesh. The samples were kept in a Schott bottle, sealed with parafilm, and stored in the dark at 4°C in the chiller.

2.3 Aqueous Two-Phase System Partitioning Experiment

EL-salt ATPSs were formed in 15 mL centrifuge tubes at room temperature, with a final mass of 5 g at various phase compositions and 0.2% (w/w) of *O. stamineus* leaves (unless otherwise stated) (Velho et al., 2020; Velho et al., 2022). The mixture was mixed using a vortex mixer (1500 rpm, 30 s) and centrifuged (4000 rpm, 10 min) to separate the mixture into two distinct aqueous layers (Sapone et al., 2022). The volumes of both phases were measured to estimate the volume ratio, V_R. Separate samples were taken from each phase to quantify the total phenolic content (TPC) and DPPH free radical scavenging activity. The impact of type of salt, sample loading (0.2 – 2.0% (w/w)), pH (4.5 – 8.0), potassium chloride's concentration (0 – 3% (w/w)), and equilibration time (5 – 15 min) on the phenolic compounds' extraction efficiency using was assessed using single-factor analysis. The pH of the EL-based ATPS was modified by adding sulfuric acid and sodium hydroxide. The phenolic compounds' extraction efficiency was assessed in terms of partition coefficient, K; yield, Y; and DPPH free radical scavenging activity as shown in Sections 2.4 to 2.6. Data were presented as mean ± standard deviation.

2.4 Quantification of Total Phenolic Content

The TPC in both phases was quantified using Folin-Ciocalteu assay, with gallic acid serving as the standard (Laina et al., 2021). Blank ATPS was prepared without *O. stamineus* leaves for each ATPS system to eliminate the absorbance contributed by EL and salt. The samples' absorbance reading was obtained using microplate reader (Spectrostar Nano, BMG Labtech, Germany) at 765 nm.

2.5 Determination of DPPH Free Radical Scavenging Activity

The decolouration of the DPPH reagent was utilised to quantify the antioxidant capacity of phenolic compounds of *O. stamineus* plant (Tran et al., 2023; Ng et al., 2017). The samples' absorbance was assessed using microplate reader at 517 nm, with ascorbic acid serving as the standard.

The samples' DPPH free radical scavenging activity was determined using Eq. (1). A_{sample} refers to the absorbance of the resulting mixture containing DPPH and sample. A_{blank} denotes the sample's absorbance, while A_{control} denotes the absorbance of DPPH without sample.

$$\text{DPPH free radical scavenging activity} = \frac{A_{control}-(A_{sample}-A_{blank})}{A_{control}} \times 100\% \quad (1)$$

2.6 Calculation of Partitioning Parameters

The volume ratio, V_R was determined by dividing the EL-rich top phase's volume, V_T by the salt-rich bottom phase's volume, V_B. The partition coefficient, K was calculated by dividing the top phase's TPC, C_T, by the TPC in the bottom phase, C_B. The yield, Y of phenolic compounds in the top phase was determined using Eq. (2).

$$Y = \frac{1}{1+\frac{1}{V_R K}} \times 100\% \quad (2)$$

3. Results and Discussion

3.1 Impact of types of salt and phase composition

Different salts exhibit distinctive salting-out abilities and have a great impact on the extraction efficiency of phenolic compounds in the ATPS (Pereira & Coutinho, 2019). Table 1 shows the partition coefficient (*K*), yield (*Y*), and DPPH free scavenging activity of *O. stamineus* leaves in the EL-based ATPSs composed using different types of salt and phase composition. The EL-based ATPS formed using dipotassium hydrogen phosphate exhibited the lowest extraction efficiency, with *K* values below 1, and *Y* ranging from 45% to 85%, indicating that the phenolic compounds were primarily separated to the bottom phase. Higher extraction efficiency (*K* > 1 and Y > 72%) was achieved using EL-citrate ATPS, with phenolic compounds primarily partitioning to the top phase. These findings revealed that the salts' salting-out ability increased in the following order: SO_4^{2}> citrate^{3-} > HPO_4^{2-}, which aligned with the Hofmeister series (Kang et al., 2020).

Table 1: Effect of types of salt and phase composition on the K, Y, and DPPH scavenging activity of O. stamineus leaves phenolic compounds in EL-based ATPS

Types of ATPS	EL concentration, [% (w/w)]	Salt concentration, [% (w/w)]	Partition Coefficient, *K*	Yield, *Y* [%]	DPPH scavenging activity [%]
Phosphate	30.00	12.00	0.75 ± 0.38	45.58 ± 1.98	41.23 ± 0.65
	36.00	13.20	0.74 ± 0.16	54.97 ± 1.94	37.30 ± 3.37
	41.90	14.50	2.82 ± 0.01	84.93 ± 0.05	24.10 ± 3.01
Citrate	30.00	11.00	2.03 ± 0.06	56.82 ± 5.38	28.91 ± 1.83
	34.30	11.70	2.44 ± 0.22	78.68 ± 7.90	33.97 ± 4.01
	38.50	12.30	2.47 ± 0.21	79.53 ± 6.24	24.69 ± 4.14
Sulfate	30.00	12.00	2.22 ± 0.48	72.36 ± 3.04	28.07 ± 1.57
	36.00	13.20	3.03 ± 0.68	83.03 ± 3.19	33.28 ± 1.81
	41.90	14.60	5.83 ± 0.48	90.49 ± 1.86	76.97 ± 2.94

Furthermore, the increase in the compositions of EL and salt in ATPS resulted in an overall increase in the *K* and *Y*. As the salt composition rises, the number of unbound water molecules in the bottom phase decreases (Chow et al., 2016). This decline resulted in greater hydrophobicity difference between both phases, thereby promoting favourable hydrophobic interaction between phenolic compounds and EL. Consequently, the recovery of phenolic compounds to the EL-rich top phase was increased. Among the EL-based ATPSs investigated, ATPS consisting of 41.9% (w/w) EL and 14.6% (w/w) sulfate salt demonstrated the greatest *K* of 5.83 ± 0.48, *Y* of 90.49% ± 1.86%, and DPPH scavenging activity of 76.97 ± 2.94. Hence, ATPS comprised of 41.9% (w/w) EL and 14.6% (w/w) sulfate was selected to evaluate the impact of sample loading in subsequent experiments.

3.2 Impact of sample loading

The impact of sample loading on the phenolic compounds' extraction efficiency (Figure 1a) was investigated to determine the maximum loading capacity of EL-sulfate ATPS. The highest *K* of 20.99 ± 0.42 was recorded at a maximum loading capacity of 1.4% (w/w) of *O. stamineus* leaves sample loading, along with the highest *Y* and DPPH scavenging activity of 96.33% ± 0.07% and 89.31% ± 0.23%, respectively.

As the sample loading increased from 0.2% (w/w) to 1.4% (w/w), the *K* and *Y* showed an upward trend. This rise may be attributed to the addition of higher amounts of *O. stamineus* leaves sample loading, resulting in a greater quantity of phenolic compounds being recovered at the top phase. The extraction efficiency decreased as sample loading increased from 1.4% (w/w) to 2.0% (w/w) due to the constant composition of EL (41.9% (w/w)) and sulfate salt (14.6% (w/w)) in ATPS, which restricted the phenolic compounds separation into the EL-rich top phase. Đorđević & Antov (2017) observed a comparable trend and found a significant drop in phenolic compounds' yield from wheat chaff in ethanol-salt ATPS when there was an increase in sample loading from 1.2% (w/w) to 2% (w/w).

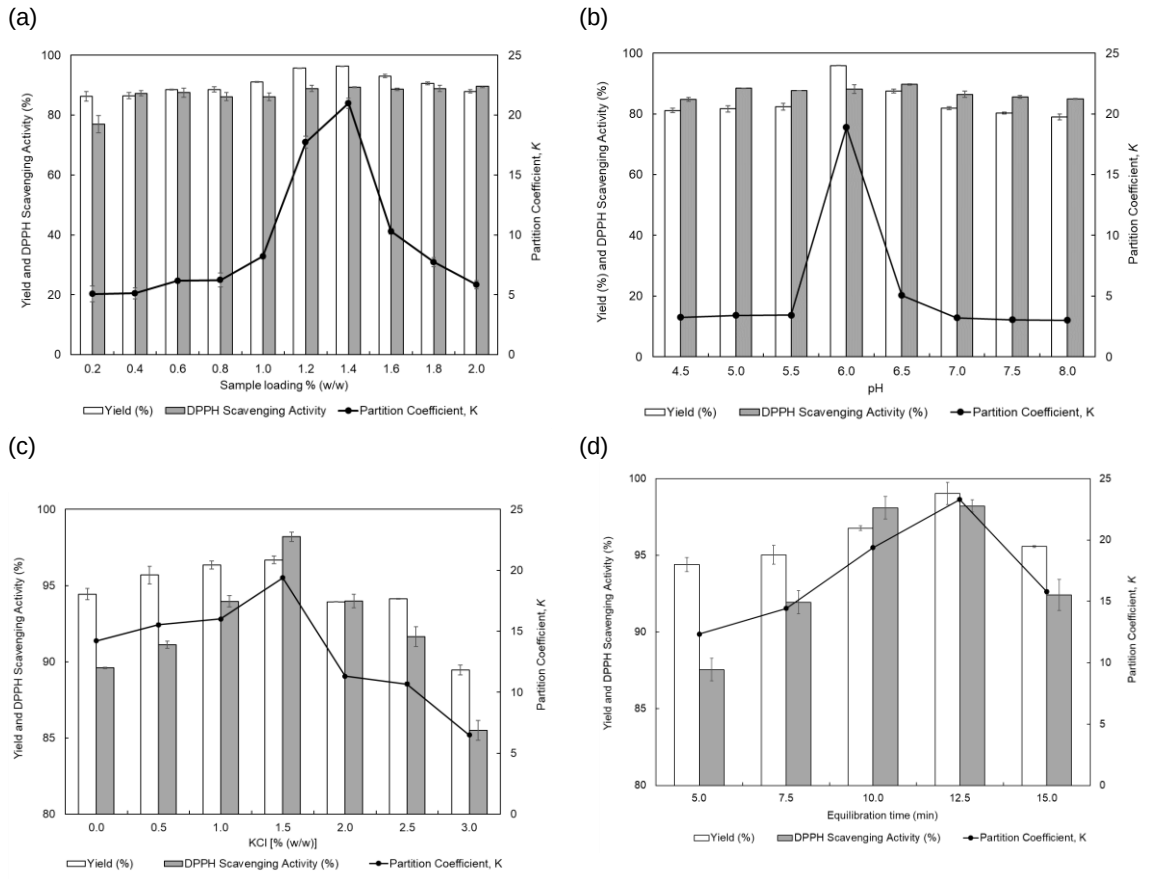

Figure 1: The effect of (a) O. stamineus leaves sample loading, (b) pH, (c) KCl concentration, and (d) equilibration time on the K, Y, and DPPH scavenging activity of phenolic compounds in the EL-sulfate ATPS

3.3 Impact of pH

The distribution of phase components in the ATPS and targeted biomolecules' net surface charge are affected by pH (Leong et al., 2015). The main phenolic compounds found in *O. stamineus* include caffeic acid derivatives (pKa of 4.62), flavanol glycosides, and lipophilic flavones (pKa values ranging from 6.66 to 8.47) (Numviyimana et al., 2019). Although the flavanol glycosides' sugar components make them more soluble in water at pH 6, the salting-out effect reduces their solubility in the aqueous phase and increases their distribution into the EL-rich top phase (Herrero-Martínez et al., 2005). Between pH 4.5 and 6.0, the lipophilic flavones remain relatively uncharged. Thus, the amphiphilic EL promotes a considerable amount of lipophilic flavones partitioning to the EL-rich phase. Given their lower pKa value, the carboxyl group of the caffeic acid and its derivatives were more deprotonated at pH 6, which increases their solubility in the EL-rich (Velho et al., 2022). Hence, this combined effect improved the phenolic compounds' extraction yield from 81.10% ± 0.73% at pH 4.5 to 95.94% ± 0.02% at pH 6 (Figure 1b). Along with the decline in pH from pH 6 to pH 8, there was a drop in the *K*, *Y* and DPPH scavenging activity, following the trend reported by Ng et al. (2017). This decline in extraction efficiency is ascribed to the increased rate of hydrolysis and oxidation of these compounds at alkaline pH, which negatively impacts their stability and partitioning behaviour in ATPS (Numviyimana et al., 2019).

3.4 Impact of Compositions of Neutral Salt

Figure 1c shows that the *K*, *Y*, and DPPH scavenging activity increased to 19.40 ± 0.09, 96.68% ± 0.00%, and 98.20% ± 0.15% with the addition of potassium chloride (KCl) neutral salts up to 1.5% (w/w) KCl. This result shows that adding KCl to the EL-based ATPS could enhance the extraction efficiency. The addition of neutral salt increased the hydrophobicity difference between the two aqueous phases, facilitating favourable interaction between the EL and relatively uncharged phenolic compounds (Chow et al., 2016). Nevertheless, a drastic decrease in *K*, *Y*, and DPPH scavenging activity to 6.49 ± 0.10, 89.46% ± 0.02%, 85.63% ± 0.17%, respectively, was recorded upon adding neutral salt up to 3.0% (w/w) KCl. The decline in extraction efficiency was caused by

the intensified electrostatic repulsion between the water structure breaking anion of the neutral salt, Cl^- and deprotonated caffeic acid derivatives, which affected the distribution of the latter to the EL-rich top phase. To examine the impact of equilibration time in subsequent experiments, ATPS containing 1.5% (w/w) KCl was chosen.

3.5 Impact of equilibration time

Theoretically, extended extraction duration increases phenolic compounds' yield due to prolonged solute-solvent interaction (Zhu et al., 2022). When the equilibration duration was extended from 5.0 min to 12.5 min, the *K* and *Y* increased from 12.33 ± 0.12 to 23.28 ± 0.12 and from 94.40% ± 0.45% to 99.03% ± 1.37% (Figure 1d), respectively. However, when the equilibrium duration was extended from 12.5 min to 15.0 min, the excluded volume effect led to a decrease in both *K* and *Y* values. The limited free volume in the EL-rich top phase expelled phenolic compounds to the salt-rich bottom phase, resulting in decreased extraction efficiency. Ng et al. (2017) also found that the *K* and *Y* values decreased as the equilibration time increased from 12.5 to 15 min when extracting phenolic compounds from black tea using ATPS composed of thermos-separating polymer and salt. Therefore, the ATPS approached complete phase separation and equilibrium at 12.5 min, as indicated by the maximum *K* (23.28 ± 0.12), *Y* (99.03% ± 1.37%), and DPPH scavenging activity (98.22% ± 0.40%) achieved.

3.6 Comparison of Ethyl Lactate-based ATPS with other Extraction Methods and Green Solvent

The comparison between EL-salt ATPS and a few other extraction methods, which include supercritical fluid extraction (SFE) (Radzali et al., 2020), maceration (Ashraf et al., 2020) and ultrasonic-assisted extraction (UAE) (Ho et al., 2014) for the phenolic compounds' yield from *O. stamineus* is shown in Table 2. The EL-salt ATPS and UAE showed comparable phenolic compounds' yield, suggesting efficient extraction under milder conditions and shorter timescales. Maceration produced the highest yield but required prolonged extraction time. SFE produced the lowest yield due to the phenolic compounds' poor solubility in the supercritical CO_2-ethanol mixture (Radzali et al., 2020). When compared to another green extraction solvent applied in ATPS, EL exhibited a higher yield compared to ChCl-1,4-BDO (Edrisi & Bakhshi, 2024) attributed to the EL's inherent amphiphilic nature. Hence, EL-salt ATPS demonstrated its potential as a green extraction solvent for extracting phenolic compounds.

Table 2: Comparison of EL-salt ATPS with various extraction techniques and green solvent for the extraction of phenolic compounds

Extraction Methods	Extraction Conditions	Yield of Phenolic Compounds	References
Maceration	Methanol and extraction time of 48 h.	173.3 mg GAE/g	(Ashraf et al., 2020)
Super Critical Fluid Extraction (SFE)	50% (v/v) ethanol and 4 mL/ min of liquid CO_2 at 60°C and 225 bar	3.42 mg GAE/g	(Radzali et al., 2020)
Ultrasonic-assisted extraction (UAE)	15.2% (w/w) ethanol, amplitude of 58.5%, and duty cycle of 0.7 W/s with extraction time of 8.3 min	12.84 mg GAE/g	(Ho et al., 2014)
Aqueous two-phase system (ATPS)	0.67% (w/w) chorine chloride-1,4-butanediol (ChCl-1,4-BDO) and 0.32% (w/w) tripotassium phosphate at a solid-liquid ratio of 18.97 mL/g and 40°C with extraction time of 30 min	88.54%	(Edrisi & Bakhshi, 2024)
	41.9% (w/w) EL-14.6% (w/w) sulfate at pH 6, 1.4% (w/w) of sample loading, 1.5% (w/w) of KCl, and 12.5 min of equilibration time	14.68 mg GAE/g and 99.03% ± 1.37%	This study

4. Conclusion

This study evaluated the efficacy of EL-salt ATPS in extracting phenolic compounds from *O. stamineus*, by assessing the impact of different ATPS parameters, such as the type of salts, phase composition, sample loading, pH, neutral salt concentration, and equilibration time, on the partition coefficient (*K*) and yield (*Y*). Among the salts investigated, increased sulfate salt composition could better improve the phenolic compounds' extraction efficiency due to its superior salting-out ability. Highest *K* of 23.28 ± 0.12, *Y* of 99.03% ± 1.37, and DPPH scavenging activity of 98.22% ± 0.40 were achieved in ATPS comprised of 41.9% (w/w) EL, 14.6% (w/w) sulfate salt at pH 6.0, 1.5% (w/w) KCl, and 1.4% (w/w) sample loading after an equilibration time of 12.5 min. Therefore, EL-sulfate ATPS could serve as an alternative extraction strategy for recovering phenolic compounds from other plant sources.

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