

Comparison of the effects of cellulase, pectinase, and xylanase enzymes on the total flavonoid content of *Helichrysum arenarium* extract obtained by enzymatically pre-treated Soxhlet extraction

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Flavonoids are plant compounds that have various benefits for human health. Their benefits for human health have been supported by studies and their use has become widespread in the nutraceutical, pharmaceutical, medical and cosmetic sectors. In the present work, the flavonoid extraction from *Helichrysum arenarium* using Soxhlet extraction and single or a combination of several enzymes was achieved and the influence of enzymes types (cellulase, pectinase, and xylanase) on the flavonoid extraction efficiency was explored. The total flavonoid content was determined based on the aluminum chloride colorimetric method in terms of quercetin equivalent (mg QE/mL). Experimental data show that xylanase treatments increased the total flavonoid content to the range of 55.32–58.45 mg QE/g, yielding the highest values (58.45 mg QE/g in EA9) and providing the most significant increase compared to the control group. The enzyme-assisted extraction method has demonstrated higher extraction capacity compared to traditional Soxhlet extraction, offering high efficiency.

Keywords: *Helichrysum arenarium*, total flavonoid content, Soxhlet extraction, enzyme-assisted extraction, xylanase, cellulase, pectinase.

INTRODUCTION

Helichrysum arenarium is a medicinal plant species belonging to the Asteraceae family and is known for its high flavonoid and phenolic content. The plant's rich bioactive profile, consisting of flavanones and various polyphenolic compounds, supports its widespread use in traditional medicine [1–3]. *Helichrysum arenarium*, as a medicinal plant belonging to the Asteraceae family, is known for its diuretic, choleric, spasmolytic, hepatoprotective and anti-inflammatory properties, can also be used as a detoxification agent, and exhibits antiviral, antibacterial, antifungal and antioxidant activities, wound healing, anti-aging, antitumor and anti-atherosclerotic effects, supporting its traditional uses [4–6]. Various phytochemical and pharmacological studies emphasize that *Helichrysum arenarium* has a rich phenolic compound content, especially flavonoids [1, 6]. However, the cellulose, hemicellulose, and pectin matrix in the cell wall forms a physical barrier that limits the passage of flavonoids into the solvent during conventional solvent extraction; the literature emphasizes that the enzymatic hydrolysis of this structure facilitates the release of bioactive compounds [5, 6]. Enzyme-assisted extraction of flavonoids is a potential alternative to traditional solvent-based extraction methods. Enzymes, by their ability to break down or disrupt cell walls and membranes, enable more

efficient extraction of bioactives, thus offering a more environmentally friendly extraction method [7–9].

This study aims to improve the degradation of cell wall components and increase extraction efficiency by comparing the effectiveness of cellulase, pectinase, and xylanase enzymes individually and synergistically. These enzymes accelerate the transfer of intracellular compounds to the solvent by degrading plant tissues under mild conditions and improve extraction kinetics [10]. In this study, extraction was performed from *Helichrysum arenarium* plants using the Soxhlet extraction method after pretreatment with enzymes to increase the total flavonoid content (TFC) in terms of quercetin equivalent. The enzymes were added individually or in mixtures in various volumes, with the total activity fixed at 50 U/ml.

EXPERIMENTAL

Materials and method

The leaves of the *Helichrysum arenarium* plant, sourced from a local herbalist called Amcahafizoğulları in Uşak, were separated from the branches and stored in an airtight bag at room temperature. Commercial enzymes cellulase (SQzyme CSL, 10,000 U/mL), pectinase (SQzyme PCL, 60,000 U/mL), and xylanase (SQzyme XSL, 50,000 U/mL) were obtained as samples from

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Suntaq International Limited. Sodium hydroxide (CAS: 1310-73-2) and hydrochloric acid (CAS: 7647-01-0) were obtained from Boston Chemical, and citric acid (CAS: 5949-92-1) was obtained from Akbel Kimya, for the purpose of preparing the buffer solution. *Helichrysum arenarium* leaves were pre-treated with enzymatic treatment and then subjected to Soxhlet extraction, and the resulting extracts were analyzed using a UV spectrophotometer. Distilled water was preferred as the solvent in the Soxhlet extraction, and it was carried out at an extraction temperature of 70 °C for 4 h.

Preparation of buffer solution

A pH 5 buffer solution was prepared according to the procedure described in European Pharmacopoeia 5.0, containing 20.1 g/l citric acid and 8.0 g/l sodium hydroxide. The pH was adjusted with dilute hydrochloric acid [11].

Enzymatic pretreatment

Enzymatic pretreatment was performed by adding 99 mL of pH 5.0 citrate buffer and 1 mL of

diluted enzyme solution (to ensure 50 U/mL enzyme activity in each test flask) to 5 g of dry *Helichrysum arenarium* sample for each experiment; the ratio of “dry plant mass: volume of enzyme-added buffer solution” was prepared as 1:20, the total volume in each test flask was adjusted to 100 mL, and it was applied in a shaking water bath at 37 °C for 1 hour at a stirring speed of 100 rpm.

Cellulase (10,000 U/mL), pectinase (60,000 U/mL), and xylanase (50,000 U/mL) enzymes were used, and the target activity was brought to a level of 50 U/mL in diluted solutions; in each experiment, 99 mL of buffer and 1 mL of diluted enzyme solution were applied with the enzyme loads specified in Table 1, while no enzyme was added in the control group (EA10). After the enzymatic pretreatment was completed, the buffer solution was removed from the plant leaves *via* a rotary evaporator (Heidolph Hel-VAP), and only trace amounts of buffer solution extract were obtained and included in the Soxhlet extractor along with the *Helichrysum arenarium* leaves.

Table 1. Experimental design of enzymatic pretreatment application.

Sample No.	Experimental group / enzyme ratio	Cellulase, mL	Pectinase, mL	Xylanase, mL	Total enzyme volume, mL	Total enzyme load, U	Final enzyme activity, U/mL
EA1	Cellulase	0.1	0	0	0.1	1000	50
EA2	Cellulase	0.2	0	0	0.2	2000	50
EA3	Cellulase	0.3	0	0	0.3	3000	50
EA4	Pectinase	0	0.1	0	0.1	6000	50
EA5	Pectinase	0	0.2	0	0.2	12000	50
EA6	Pectinase	0	0.3	0	0.3	18000	50
EA7	Xylanase	0	0	0.1	0.1	5000	50
EA8	Xylanase	0	0	0.2	0.2	10000	50
EA9	Xylanase	0	0	0.3	0.3	15000	50
EA10	Control	0	0	0	0	0	0
EA11	Enzyme mixture (1:1:1)	0.1	0.1	0.1	0.3	12000	50
EA12	Enzyme mixture (2:1:1)	0.2	0.1	0.1	0.4	13000	50
EA13	Enzyme mixture (1:2:1)	0.1	0.2	0.1	0.4	18000	50
EA14	Enzyme mixture (1:1:2)	0.1	0.1	0.2	0.4	17000	50

Soxhlet extraction

Helichrysum arenarium leaves, pre-treated with enzymatic methods, were loaded into a Soxhlet extractor chamber. A heated mantle placed under the solvent chamber of the Soxhlet extractor was activated, and 250 mL of distilled water was evaporated at 110 °C. The water in the vapor phase, striking the reflux surface, condensed and collected in the extraction chamber. The plants were extracted at an extraction temperature of approximately 70 °C, and 15 extraction cycles were applied for each experiment. The extracts obtained after extraction were transferred to a rotary evaporator (Heidolph Hel-VAP). Excess water was removed by the rotary evaporator, the extracts were concentrated and prepared for UV analysis, and stored in amber glass bottles at 4 °C.

Determination of total flavonoid content

To determine the total flavonoid content in terms of quercetin equivalents by colorimetric analysis using a UV-Vis spectrophotometer (Perkin Elmer Lambda 35 UV-Vis), a 12.5 mg/mL stock solution was prepared by dissolving 625 mg of standard

quercetin in 50 mL of distilled water. This stock solution was then diluted to concentrations of 10; 7.5; 5; 2.5; 7.5; 5; 2; 1.5; 1.25; 1; 0.5 and 0.1 mg/mL, yielding quercetin solutions at various concentrations. Using these solutions, a calibration equation of $y = 0.3809x + 0.0697$ was obtained via UV-Vis analysis at 510 nm, with an R^2 value of 0.9919. To each 10 mL tube, 1 mL of extract (each extract obtained from the extraction processes was added separately), 4 mL of distilled water and 0.3 mL of 5% (w/v) sodium nitrite solution were added; the mixture was then thoroughly mixed and left to stand at room temperature for 5 minutes. Subsequently, 0.3 mL of a 10% (m/v) aluminium chloride solution was added to the tubes; after allowing the mixture to stand for a further 1 minute, 2 mL of 1 M sodium hydroxide and 2.4 mL of distilled water were added to adjust the total volume to 10 mL. The absorbance values of the prepared mixtures were measured at a wavelength of 510 nm using a UV-Vis spectrophotometer (Perkin Elmer Lambda 35 UV-Vis) [12], and the obtained values were applied to the calibration equation to calculate the results in terms of quercetin equivalents (mg QE/mL).

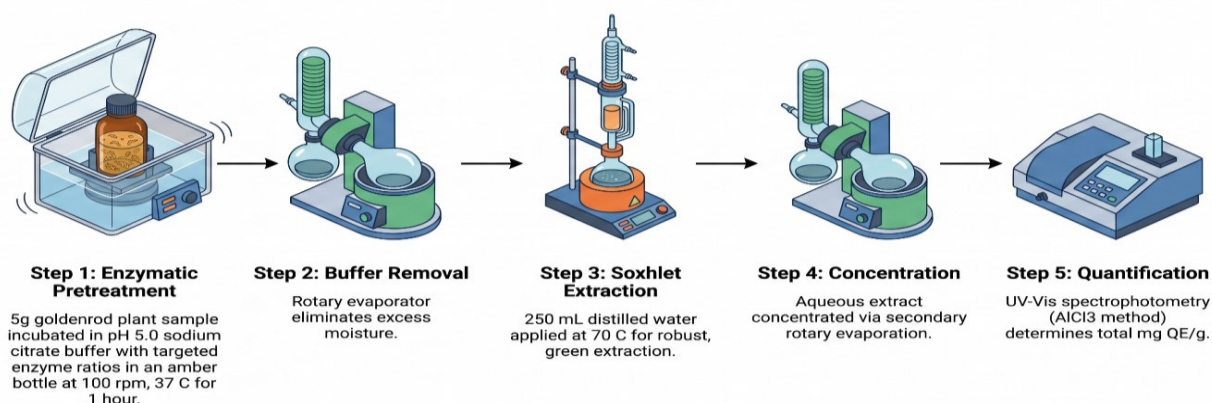


Figure 1. Experimental procedure for enzymatically pre-treated Soxhlet extraction and UV analysis

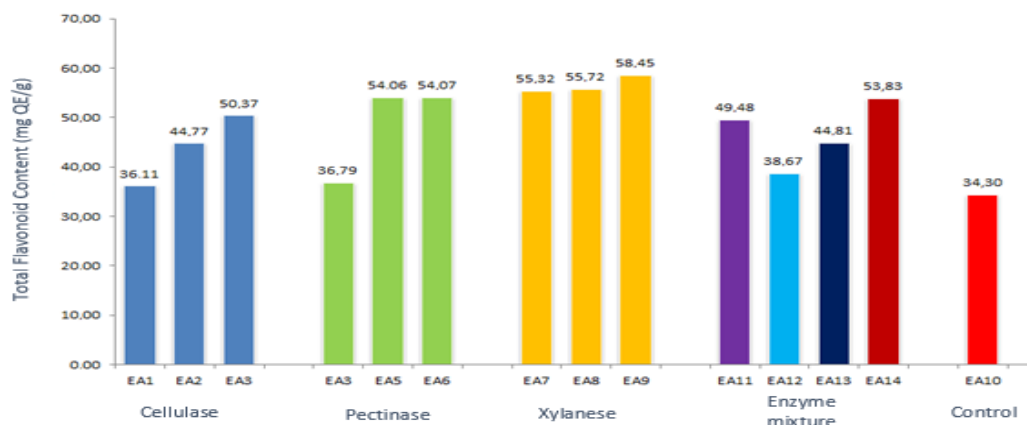


Figure 2. Effect of single enzymes and their mixtures on the extraction yields of total flavonoids.

RESULTS AND DISCUSSION

Extraction of total flavonoids from *Helichrysum arenarium* was conducted with three enzymes, cellulase, pectinase, and xylanase. A series of preliminary experiments were conducted to determine the most effective concentration and ratio for all three enzymes, both individually and in mixtures. The effect of enzyme type, enzyme quantity and ratio on flavonoid extraction yield is shown in Figure 2. It was observed that every single enzymatic condition lead to a clear and significant increase in flavonoid extraction. All enzymes significantly improved the yield, with increasing activity leading to higher flavonoid extraction. Among them, xylanase showed the strongest effect, reaching up to 58.45 mg QE/g, corresponding to 70.41% improvement. Interestingly, although multi-enzyme combinations also perform well, they do not surpass the performance of alone xylanase at optimal conditions. In the specific cellular architecture of *Helichrysum arenarium*, the hemicellulose network serves as the primary trapping matrix for phenolic compounds. Xylanase efficiently hydrolyzes the xylan structure, thereby completely isolating the structurally bound flavonoids that remain intact during Soxhlet extraction.

CONCLUSION

The main findings of this study are as follows: Every enzymatic variation tested resulted in a meaningful improvement over the baseline Soxhlet extraction. Although multi-enzyme systems perform well, xylanase provided the highest yield in this study. First, enzymatic pretreatment substantially enhances extraction performance, yielding 70.41% increase compared to direct Soxhlet extraction. Second, the results indicate a clear matrix-specific effect, where selective degradation of the hemicellulose fraction -particularly via xylanase- plays a dominant role in improving flavonoid accessibility.

Finally, this approach to improving the ability to obtain high flavonoid content using a Soxhlet extraction system with distilled water has the potential to be used as a simplified and efficient process alternative that does not require the use of toxic solvents.

The present study demonstrates xylanase to be a highly efficient enzyme to assist the extraction of flavonoid compounds in mild solvents like water. Overall, these findings demonstrate that targeted enzymatic pretreatment represents an effective strategy for intensifying flavonoid extraction from plant matrices.

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