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Development of a high-performance liquid chromatography (HPLC) method for coumarin quantification in medicinal plants extracted *via* Soxhlet

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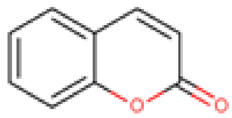
In this study, it was aimed to develop a new high-performance liquid chromatography (HPLC) method for the efficient extraction and quantification of coumarin compound from *Helichrysum arenarium*, *Origanum onites* and *Achillea millefolium* plants by Soxhlet extraction method. Deionized water, methanol, ethanol, acetone, n-hexane and toluene were used as solvents in Soxhlet extraction and the most suitable solvent was determined by comparing the extraction efficiencies of the solvents. Experimental results revealed that there were significant differences in coumarin extraction efficiency depending on solvent type and chemical structure of the plant. Deionized water provided the highest extraction efficiency in general, while the highest coumarin concentration (0.0339 mg/mL) was obtained for *Helichrysum arenarium* when methanol was used. *Achillea millefolium* was found to have the lowest overall coumarin content. The HPLC method developed within the scope of the study provided high sensitivity, accuracy and reproducibility for coumarin quantification. The findings indicate that the choice of solvent for the analysis of coumarin-containing herbal extracts should not be based solely on polarity, but should be evaluated together with the matrix properties of the plant.

Keywords: Coumarin extraction, Soxhlet extraction, high-performance liquid chromatography (HPLC), solvent selection

INTRODUCTION

Plants produce not only primary metabolites, which are vital for their functions, but also secondary metabolites that help them adapt to their environment and defend themselves [1]. Flavonoids, a valuable group of secondary metabolites, can be found in many plant sources, especially fruits, herbs, vegetables, stems, grains, nuts, flowers and seeds. Their antioxidant capacity, ability to change enzymatic activity and impact on cellular processes have made them central to research in nutrition, pharmacology and food science [2, 3]. Both *in vitro* and *in vivo* (animal and cell model-based) studies supporting the positive effects of flavonoids on health suggest that these compounds may be promising strategic components in preventive medicine [2, 4]. Coumarin is a phenolic compound commonly found in plants [5, 6]. It is an aromatic organic compound with formula $C_9H_6O_2$. 2D structure of coumarin is given in Scheme 1. Its molecule is a benzene molecule with an unsaturated lactone ring replacing two hydrogen atoms. It belongs to the benzopyrone class and is a lactone. This class of compounds has a wide spectrum of pharmacological effects such as anti-inflammatory, analgesic, antimicrobial and lipid peroxidation-inhibitory [6, 7]. However, it has also been reported that long-term or excessive use of coumarins may have adverse effects on health [8, 9]. Therefore, accurate quantification of these compounds is of great importance for potential therapeutic applications and safety assessments.



Scheme 1. 2D structure of coumarin

Helichrysum arenarium commonly known as sandy everlasting, is a coumarin-rich plant widely used in traditional medicine for treating conditions such as edema, digestive disorders, and joint pain [10]. *Origanum onites* is a highly valued herb with about 100 species distributed worldwide. Thymus species are native plants of Europe and Asia. They are also abundant in the Mediterranean region, southeastern Italy, and northwestern Africa. The genus Thymus comprises a number of highly useful medicinal plants with a range of therapeutic activities [11]. *Achillea millefolium* is a medicinal plant belonging to the Asteraceae family, widely found in the flora of Turkey and used for various purposes in traditional medicine. *Achillea millefolium* is notable for its rich content of volatile oils, phenolic compounds and flavonoids. The potential health effects of flavonoids obtained from this plant are also scientifically supported [12].

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Efficient synthesis of these compounds from plant materials is critical to the effective use of plant resources and to increasing the bioavailability of flavonoids. In this context, although different extraction methods have been developed, Soxhlet extraction (SE) still stands out as a reference method despite its classical structure. This technique, which is based on continuous solvent recycling, effectively breaks down plant cell walls and enables flavonoids to be extracted with high yields. Recent studies have examined SE in terms of flavonoid yield of different plant materials and used the method as a basic standard to compare with innovative techniques such as ultrasonic (UAE), microwave (MAE) or supercritical fluid extraction (SFE) [13].

High-performance liquid chromatography (HPLC) is a preferred technique for analyzing complex mixtures such as natural products. Thanks to its high sensitivity, specificity and ability to separate components, it is an ideal method for determining the quantity of compounds such as coumarin. However, validation of HPLC methods is necessary to ensure that the results obtained are reliable and accurate. Method validation is the set of experimental studies that confirm that an analytical method is suitable for its intended use [14, 15]. In this context, studies on the HPLC determination and validation of coumarin from *Helichrysum arenarium*, *Origanum onites* and *Achillea millefolium* will contribute significantly to the scientific understanding of the chemical composition and potential benefits of the plant.

EXPERIMENTAL

Materials

Helichrysum arenarium, *Origanum onites*, and *Achillea millefolium* plants, which grow around Uşak province in the Aegean region of Turkey, were supplied in dried form by Amcahafızoğlu, a local herbalist in Uşak. Deionized water (CAS 7732-18-5), ethanol (CAS 64-17-5), methanol (CAS 67-56-1), acetone (CAS 67-64-1), n-hexane (CAS 110-54-3), toluene (CAS 108-88-3) were supplied by Beyanlab. Potassium dihydrogen phosphate, acetonitrile, methanol, standard coumarin (CAS 91-64-5) were supplied by Boston Chemical.

Extraction

Helichrysum arenarium, *Origanum onites* and *Achillea millefolium* leaves (5 g) were separated and weighted. They were then wrapped in filter paper, placed in a closed package inside the Soxhlet cartridge. The cartridge was placed inside the Soxhlet extractor. Solvents (deionized water, ethanol, methanol, acetone, n-hexane and toluene) were measured at 250 ml each and loaded into the solvent chamber under the Soxhlet. A reflux condenser was connected to the extractor. Cold water was passed through the condenser using a pump. After operating the heated mantle under the flask, the solvents were siphoned 15 times at an extraction temperature of 70 °C, and the solvent was allowed to reflux.

The extraction efficiency was calculated according to Eq. 1:

$$\text{Extraction efficiency (\%)} = \frac{(W-Z)}{W} \times 100 \qquad (1)$$

where: W is the initial dry plant sample (g); Z is the remaining solid after extraction (g).

Development and validation of the HPLC method for the quantification of coumarin

Shimadzu LC-2030C Plus system was used for the HPLC. The analytical column was a C18 Inertsil ODS3 5 × 4.6 × 250 mm. Acetonitrile, methanol, potassium dihydrogenphosphate and coumarin were used as chemical substances in the analysis. The method was created as a gradient phase. The mobile phase was a 60% buffer solution (0.5 g of potassium dihydrogenphosphate per 1000 ml of deionized water), a 30% acetonitrile and 10% methanol mixture, and the methanol was used as the solvent.

RESULTS AND DISCUSSION

Extraction results

After 15 siphoning cycles, the highest extraction yield for *Helichrysum arenarium* was obtained using deionized water as the solvent (22.9%). This can be explained by the high polarity and numerous hydroxyl groups present in the phenolic and flavonoid compounds found in the plant. Polar protic solvents such as deionized water, methanol, and ethanol can act as both hydrogen bond donors and acceptors due to the hydroxyl (–OH) groups in their content; this increases solubility by forming strong interactions with phenolic compounds. Deionized water has been quite effective in dissolving phenolic and flavonoid compounds in plants. Methanol and ethanol provided moderate yields of 20.72% and 17.76%, respectively. Although these solvents are soluble in phenolic substances, their yields were lower than those obtained using deionized water. Extractions using acetone (polar aprotic solvent), n-hexane, and toluene (apolar solvents) yielded very low yields (7.34%, 2.20%, and 1.70%, respectively). This indicates that *Helichrysum arenarium* contains more hydrophilic compounds than apolar compounds.

The highest extraction yield for *Origanum onites* was still obtained using deionized water (32.00%).

This result indicates that the polyphenols in *Origanum onites* are highly soluble in deionized water and that the plant matrix contains components that are easily soluble in deionized water. Methanol and ethanol provided relatively low yields of 20.60% and 14.72%, respectively. Although these solvents are effective in extracting medium-polar components, the main target components in *Origanum onites* are likely more soluble in deionized water. Extraction using solvents such as acetone (polar aprotic solvent), n-hexane, and toluene (apolar solvents) resulted in extremely low yields (3.18%, 0.94%, and 2.74%, respectively). This indicates that the target components in *Origanum onites* are not apolar.

The highest extraction yield for *Achillea millefolium* was obtained using deionized water (23.02%). This indicates that the plant contains highly polar compounds that can be extracted with deionized water. Methanol and ethanol provided relatively high yields (21.34% and 19.66%, respectively). Acetone, toluene, and n-hexane provided yields of 9.46%, 2.38%, and 4.38%, respectively. Although acetone showed a higher yield of 9.46% in this plant compared to the apolar solvents, this value is still lower than that of deionized water or alcohols. The effect of different solvents on extraction yield is shown in Fig 1.

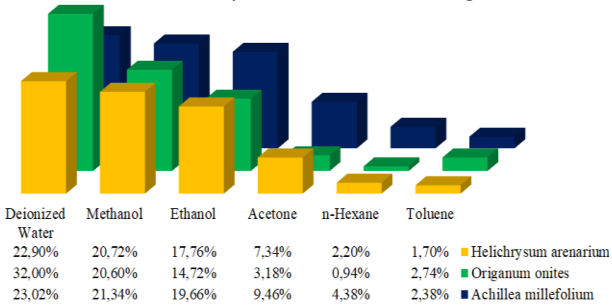

Fig. 1. Percent total phenolic compound extraction efficiency of plants by solvent type

Quantification of coumarin

A coumarin standard solution (0.2 mg/ml) was used to optimise the HPLC quantification method. Different percentages of mobile phase were trialled, then the ratio of buffer solution: acetonitrile: methanol = 60:30:10 was selected because it had good separation efficiency and met system conditions. The results of the system suitability test (SUT) and the analysis conditions are shown in Table 1.

The linearity range of the method was found to be 0.02 g/L - 0.15 g/L. The R^2 value of the method linearity data was found to be 0.992. The analytical procedure was performed according to ICH standards and the chromatogram of coumarin is given in Figure 2.

Table 1. HPLC system analysis conditions

Device	HPLC Shimadzu LC2030C PLUS
Detector	UV-Vis Detector
Column	GL Sciences Inertsil ODS-3 C18 (5 µm, 200 × 4.6 mm)
Wavelength	272 nm
Mobile phase	*Buffer solution: acetonitrile: methanol (60:30:10, h/h)
Column temperature	25 °C
Injection volume	10 µL
Analysis time	20 min
Flow rate	0.9 ml min^{-1}

*Buffer solution: 0.5 g potassium dihydrogen phosphate/1000 ml deionized water

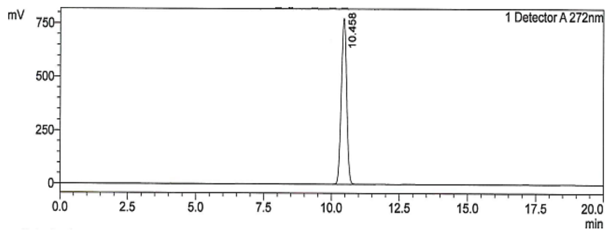

Fig. 2. Chromatogram of coumarin quantification analysis

The coumarin amounts determined using the method developed in HPLC are shown in Figure 3. When the results are examined, it is seen that the coumarin amount is low in all three plants. The highest coumarin amount, 0.033887 mg/mL, was obtained from the *Helichrysum arenarium* plant extracted with methanol solvent. Coumarin is a moderately polar compound. Moderately polar solvents, such as ethanol, dissolve these compounds more effectively than deionized water does. While the overall extraction efficiency with deionized water is high, the solubility of certain compounds, such as coumarin, may vary depending on the solvent used.

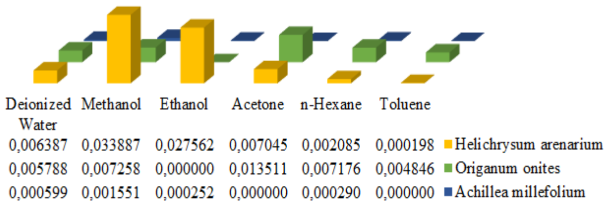

Fig. 3. HPLC results - concentration in coumarin equivalents (mg/mL)

CONCLUSION

In this study, the quantification analysis of coumarin in *Helichrysum arenarium*, *Origanum onites* and *Achillea millefolium* plants, which are used in many medicinal treatments, was carried out by HPLC and the developed method was validated. The linearity range of the method was found to be 0.02 g/L-0.15 g/L. The R^2 value of the method linearity data was found to be 0.992. As a result, the developed method and the obtained quantitative data provide an analytical approach that will contribute to the quality control processes of similar herbal products.

Furthermore, data obtained by traditional Soxhlet extraction in the presence of different solvents clearly demonstrate the effect of solvent's polarity on extraction efficiency. Pure polar solvents, in particular, were the most effective extraction medium for all three plants studied. In this context, environmentally friendly, non-toxic, economical solvents such as deionized water are notable for their environmental sustainability and high extraction efficiency. Combining traditional yet effective methods, such as Soxhlet extraction, with these solvents is an approach that aligns with green chemistry principles.

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