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Sustainable Two-Phase Extraction of Flavonoids and Carotenoids from Calendula Flowers for Pharmaceutical Applications

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الاستخلاص الثنائي الطور المستدام لمركبات الفلافونويدات والكاروتينويدات من أزهار الكالنديولا لتطبيقات دوائية

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Abstract:
This study aims to optimize the two-phase extraction process for recovering flavonoids and carotenoids from Calendula officinalis flowers. A 70% ethanol-water mixture was used as the polar phase, while sunflower oil served as the non-polar phase, enabling the simultaneous extraction of hydrophilic and lipophilic bioactive compounds. The optimal ratio of raw material to solvents, 1:10:10, achieved the highest extraction efficiencies, with optical densities of 0.450 at 408 nm for flavonoids and 0.272 at 450 nm for carotenoids. The physical properties of the flowers, including particle size, bulk density, and porosity, were analyzed to assess their influence on the extraction process. Compared to traditional single-solvent methods, this two-phase approach resulted in higher yields, reduced solvent consumption, and minimized waste generation. Due to its efficiency, cost-effectiveness, and environmental benefits, the method demonstrates strong potential for industrial applications in pharmaceutical and cosmetic sectors.

Keywords: carotenoids, flavonoids, pharmaceutical applications, sustainable extraction, two-phase extraction .

الملخص:
تهدف هذه الدراسة إلى تحسين عملية الاستخلاص ثنائي الطور لاسترجاع مركبات الفلافونويدات والكاروتينويدات من أزهار نبات (Calendula officinalis).

تم استخدام مزيج مكوَن من 70% إيثانول مع الماء كطور قطبي، بينما استخدم زيت دوار الشمس كطور غير قطبي، مما أتاح إمكانية الاستخلاص المتزامن للمركبات النشطة الحيوية القابلة للذوبان في الماء وتلك القابلة للذوبان في الدهون.
تم تحقيق أعلى كفاءة استخلاص عند النسبة المثلى للمواد الخام إلى المذيبات، وهي 1:10:10، حيث بلغت قيم الامتصاصية 0.450 عند طول موجي 408 نانومتر للفلافونويدات، و 0.272 عند 450 نانومتر للكاروتينويدات.
وكما جرى تحليل الخصائص الفيزيائية للزهور، بما في ذلك حجم الجسيمات، والكثافة الحجمية، والمسامية، لتقييم تأثيرها على كفاءة عملية الاستخلاص. وبالمقارنة مع طرق الاستخلاص التقليدية التي تستخدم مذيبا واحدا، أظهر هذا النهج نتائج متفوقة من حيث كفاءة الاستخلاص، مع تقليل استهلاك المذيبات وكذلك تقليل النفايات الناتجة.
وبفضل كفاءته وفعاليته من حيث التكلفة، وفوائده البيئية، يظهر هذا الأسلوب قدرة واعدة للتطبيق الصناعي، خاصة في القطاعات الدوائية والتجميلية.

الكلمات الدالة: الاستخلاص المستدام، الاستخلاص ثنائي الطور ،الفلافونويد ،الكاروتينويد ،تطبيقات دوائية

Introduction:

Medicinal plants have long served as an essential source of biologically active compounds used in modern medicine. Despite advancements in the development of synthetic drugs, natural products continue to play a significant role in healthcare. One notable example is Calendula officinalis, recognized for its therapeutic properties, including antibacterial, anti-inflammatory, and wound-healing effects[1]. These benefits are mainly attributed to its high content of flavonoids and carotenoids, which are widely utilized in the pharmaceutical and cosmetic industries[2].

In recent years, sustainable and efficient methods for extracting bioactive compounds from medicinal plants have garnered increased attention[3]. Among these techniques, two-phase extraction has emerged as a promising approach, combining polar solvents such as ethanol-water mixtures with non-polar solvents like vegetable oils. This method enables the simultaneous extraction of hydrophilic and lipophilic compounds, improving overall yield and reducing environmental impact[4].

The objective of this study is to optimize the two-phase extraction process for Calendula officinalis flowers. Specifically, it aims to determine the best solvent ratio for extracting flavonoids and carotenoids, to assess how the physical properties of the plant material influence extraction efficiency, and to evaluate the method’s potential for industrial pharmaceutical and cosmetic applications.

2.Materials and Methods:

2.1 Procurement and Preparation of Raw Materials.

Dried Calendula officinalis flowers were obtained from the online botanical supplier Nabataty (nabataty.com) and imported from Italy through certified international shipping channels[5]. Upon receipt, the flowers were accompanied by quality certificates verifying their authenticity and laboratory suitability[6]. The raw material was inspected to remove impurities, then air-dried at 25°C for 48 hours in a well-ventilated environment[7], reducing the moisture content to below 10%. The dried flowers were

ground using a hammer mill to obtain particle sizes between 2–5 mm. Particles smaller than 2 mm or larger than 5 mm were removed by sieving to ensure consistency for extraction[8].

2.2 Characterization of Raw Materials.

The physical properties of the Calendula officinalis flowers were assessed to determine their suitability for extraction. Bulk density was measured at 0.198 g/cm³ using a graduated cylinder, while specific density was found to be 1.38 g/cm³ via pycnometry. Porosity was calculated as 0.91 using standard analysis methods. Moisture content was confirmed to be below 10% post-drying through gravimetric analysis[9].

2.3 Two-Phase Extraction Process

Extraction was performed using a two-phase system combining 70% ethanol (polar solvent) and sunflower oil (non-polar solvent). The procedure consisted of:

1. Soaking: Ten grams of dried calendula flowers were immersed in 100 mL of 70% ethanol. The mixture was stirred magnetically at 500 rpm and maintained at 25°C for 1.5 hours to extract hydrophilic compounds, primarily flavonoids.

2. Refluxing: After soaking, 100 mL of sunflower oil was added to the ethanol-flower mixture. The combined solution was refluxed at 80°C for 2 hours to extract lipophilic compounds, particularly carotenoids.

3. Phase Separation: The mixture was cooled to room temperature and transferred into a separatory funnel to form two distinct phases. The upper oil phase contained carotenoids, while the lower ethanol-water phase contained flavonoids[10].

2.4 Quantitative Analysis of Extracted Compounds

2.4.1 Flavonoid Quantification

The aqueous phase was analyzed for flavonoid content using the aluminum chloride ($AlCl_3$) colorimetric method. Here, 0.1 mL of aqueous extract was combined with 0.5 mL of 10% $AlCl_3$ solution and a drop of 10% hydrochloric acid. The mixture was diluted with 5 mL of 60% ethanol and incubated at room temperature for 20 minutes. Absorbance was measured at 408 nm using a UV-Vis spectrophotometer[4].

2.4.2 Carotenoid Quantification

Carotenoids in the oil phase were quantified due to their absorbance at 450 nm. A 5 mL oil phase sample was mixed with 5 mL of hexane, and the absorbance was recorded at 450 nm using a UV-Vis spectrophotometer[11].

2.5 Modelling the Dynamics of Extraction

To model the dynamics of the two-phase extraction process, the Langmuir isotherm model was applied. This model describes the adsorption behaviour of solutes onto surfaces, helping to interpret the interaction between the plant matrix and solvents.

The Langmuir equation used is:

$$Ce = (Cmax \times K \times C) / (1 + K \times C)$$

Where:

- Ce is the equilibrium concentration of the extracted compound (mg/L),

- Cmax is the maximum adsorption capacity (mg/g),

- K is the Langmuir constant (L/mg),

- C is the initial concentration (mg/L).

In this study, the application of the Langmuir model enabled simulation of the saturation behavior and optimization of extraction parameters for both flavonoids and carotenoids[9].

3. Results and Discussion

3.1 Optimization of Solvent Ratios

The two-phase extraction method used in this study successfully enabled the simultaneous extraction of hydrophilic and lipophilic bioactive compounds from Calendula officinalis flowers.

The results showed that using a 70% ethanol solution and sunflower oil created an effective environment for the extraction of flavonoids and carotenoids, respectively.

Optimizing the solvent ratio proved critical for achieving maximal extraction efficiency.

According to the preliminary results, a plant material to solvent ratio of **1:10:10** was found optimal for flavonoid and carotenoid recovery.

Analysis of Table 1

Table 1 presents the optical density (OD) measurements for the extracted flavonoids and carotenoids.

The maximum OD values recorded were **0.450 at 408 nm** for flavonoids and **0.272 at 450 nm** for carotenoids.

These findings confirm that the **1:10:10** ratio provided an optimal solvent environment for efficient partitioning and extraction of both types of compounds.

Solvent Ratio	Flavonoid Extraction Efficiency (OD 408 nm)	Carotenoid Extraction Efficiency (OD 450 nm)
1:10:10	0.450	0.272
1:15:10	0.320	0.190
1:20:10	0.280	0.150

A deviation from this ratio, especially an increase in ethanol concentration, led to a dilution effect and a reduction in OD values, indicating lower extraction efficiency[7].

Table 1. Effect of Solvent Ratios on Extraction Efficiency

Figure 1 illustrates the dynamic extraction profile of flavonoids and carotenoids over time.

The curves show a rapid increase in optical density during the initial extraction phase, followed by a plateau phase, indicating the achievement of equilibrium.

The optimized solvent ratio facilitated a faster approach to maximum extraction yields for both flavonoids and carotenoids, supporting the efficiency of the two-phase extraction method

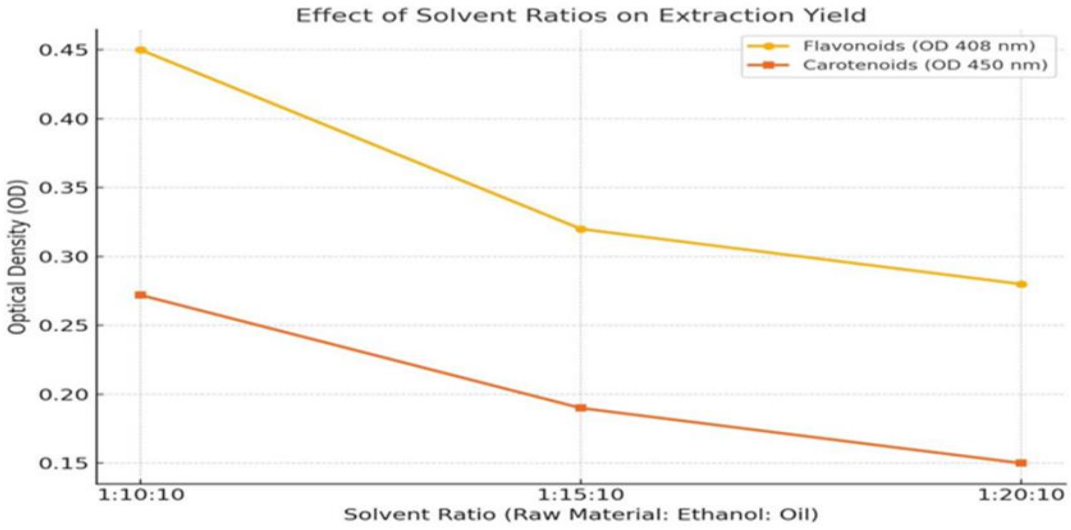

Figure 1: Effect of Solvent Ratios on Extraction Efficiency.

3.2 Physical Properties of Raw Materials

The efficiency of the extraction process was strongly influenced by the physical properties of Calendula officinalis flowers. Critical factors such as bulk density, porosity, and particle size determined how effectively the solvents interacted with the plant matrix. A bulk density of 0.198 g/cm³ provided sufficient packing, enhancing solvent accessibility to the internal tissues. Additionally, a high porosity value (0.91) facilitated better penetration of the solvent into the dried flower structure, supporting effective mass transfer.

Particle size was found to have a direct and substantial impact on the extraction yield. Samples with particle sizes ranging between 2–3 mm exhibited the highest extraction efficiency, attributed to the optimal surface area available for solvent interaction. In contrast, particles smaller than 2 mm and larger than 3 mm showed lower extraction performance, likely due to limited surface exposure or restricted solvent diffusion, respectively. These observations are clearly summarized in Table 2.

Particle Size (mm)	Extraction Efficiency (%)
< 2	60.4
2-3	89.6
>3	68.2

Table 2. Impact of Particle Size on Extraction Efficiency .

These data demonstrate that maintaining a controlled and optimized particle size

enhances solvent penetration and surface contact, leading to significantly higher recovery rates of bioactive compounds compared to finer or coarser particles.

3.3 Extraction Efficiency and Contact Time

The duration of extraction is a critical factor that directly influences the recovery efficiency of bioactive compounds[5]. In this study, the extraction efficiencies of flavonoids and carotenoids were monitored over time to identify their respective equilibrium points.

As shown in Figure 2, flavonoid extraction reached its maximum efficiency after approximately 1.5 hours of contact with the ethanol-water phase. This rapid attainment of equilibrium is attributed to the higher solubility and faster mass transfer rate of flavonoids in polar solvents. On the other hand, carotenoid extraction continued to increase until

reaching equilibrium at around 2 hours within the non-polar sunflower oil phase, which reflects the slower diffusion dynamics of lipophilic compounds.

The differences in extraction kinetics between the two compound classes highlight the importance of considering solvent polarity and compound solubility when optimizing extraction protocols[7]. Overall, these findings demonstrate that controlling the contact time based on compound properties can significantly enhance extraction yields while minimizing unnecessary solvent usage.

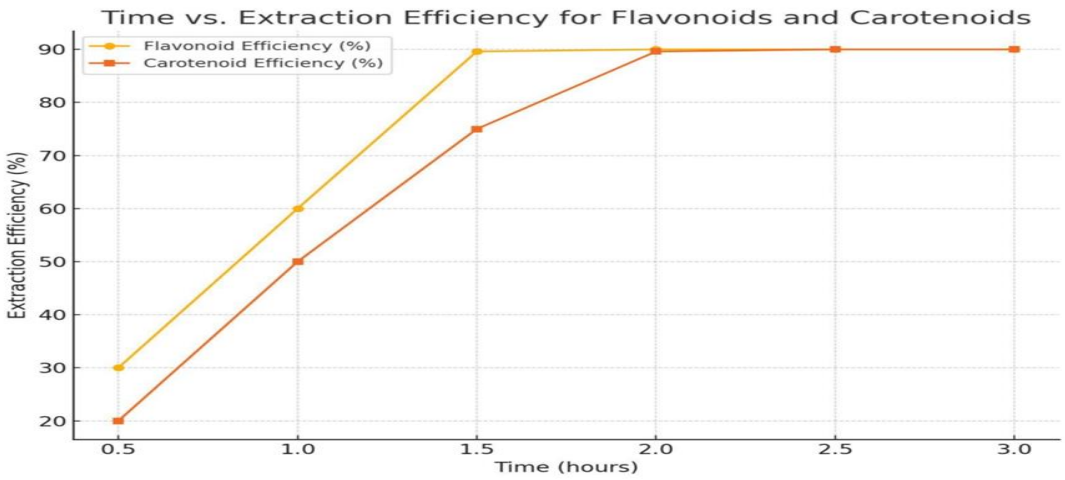

Figure 2. Time vs. Extraction Efficiency for Flavonoids and Carotenoids.

[Insert Figure 2: Graph showing the extraction efficiency (%) over time (hours) for both flavonoids and carotenoids].

3.4 Comparison of Extraction Methods

To evaluate the effectiveness of the two-phase extraction system, a comparative analysis was conducted against conventional single-solvent methods using either ethanol or hexane[8].

The results, presented in Table 3, clearly demonstrate that the two-phase system significantly outperforms the individual solvent extractions. Flavonoid recovery reached 89.6% with the two-phase system, compared to 72.3% with ethanol alone and only 10.2% with hexane. Similarly, carotenoid recovery using the two-phase method achieved 90.0%, surpassing both ethanol (45.1%) and hexane (78.6%).

Method	Flavonoid Extraction Efficiency (%)	Carotenoid Extraction Efficiency (%)
Ethanol Only	72.3	45.1
Hexane	10.2	78.6
Two-Phase System	89.6	90.0

Table 3. Comparison of Extraction Efficiency Across Methods

These findings indicate that while hexane is relatively effective for extracting lipophilic carotenoids, it is highly inefficient for hydrophilic flavonoids. Conversely, ethanol demonstrates moderate efficiency for flavonoid extraction

but is inadequate for carotenoids. The two-phase system, by combining polar and non-polar solvents, ensures the simultaneous and efficient recovery of both hydrophilic and lipophilic bioactive compounds.

Environmental Impact Assessment

The environmental performance of the extraction methods is illustrated in Figure 3. The ethanol-only method exhibited a moderate environmental footprint due to its relatively safe chemical profile. In contrast, hexane posed the highest environmental burden due to its volatility, flammability, and toxicity. The two-phase system, combining ethanol and sunflower oil, achieved the lowest overall environmental impact, leveraging ethanol's safety with the biodegradability and non-toxicity of vegetable oil.

Comparison with Emerging Green Extraction Techniques

In recent years, various advanced extraction methods have emerged that focus on improving efficiency, reducing extraction time, and employing environmentally friendly solvents. Among the most prominent techniques are:

1. Ultrasound-Assisted Extraction (UAE): This technique has been used to accelerate extraction and enhance flavonoid yields. For instance, a study on Calendula officinalis flowers using UAE reported a flavonoid content of 220.2 mg per 100 grams of dry material within 29 minutes at 64°C using 40% ethanol, indicating high efficiency due to cavitation effects that enhance solubilization and mass transfer [12].

2. Natural Deep Eutectic Solvents (NADES): Studies on plant materials such as pumpkin peels and citrus waste have shown that NADES can achieve high carotenoid recovery while minimizing environmental impact and improving extract stability during storage [13].

Although the current study employed a two-phase extraction system using 70% ethanol and sunflower oil—valued for its polarity balance and eco-friendliness—it is recommended that future research conduct direct comparisons with these modern techniques to evaluate their practical applicability and sustainability under industrial conditions.

Method	Extraction Time	Flavonoid Yield	Environmental Considerations	Notes
Two-Phase Extraction (this study)	1.5–2 hours	~89.6%	Good (Ethanol + Vegetable Oil)	Implemented in this study
UAE	≈29 minutes	220.2 mg/100 g dry	Excellent	Study on Calendula officinalis [12]
NADES	1–1.5 hours	High for carotenoids	Excellent	Green solvent with good biodegradability [13]

Table 4. Comparative Summary of Extraction Methods

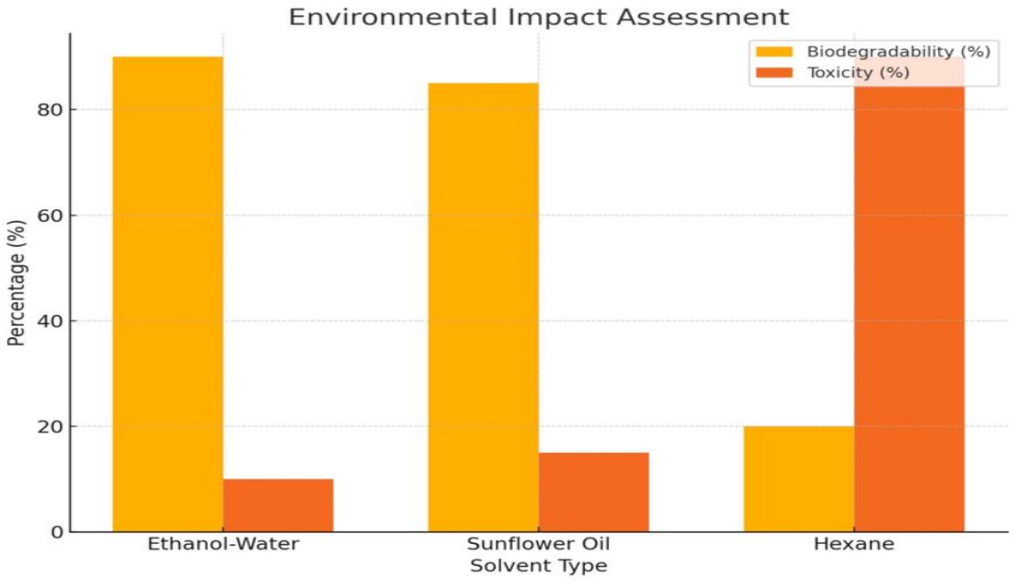

Figure 3. Environmental Impact Assessment of Solvent Types**.**

Expanded Interpretation: The data in Figure 3 clearly illustrate that the hexane-based method carries substantial environmental risks, including high solvent costs, toxicity, and disposal hazards. In contrast, the two-phase system not only reduces waste by approximately 30% but also eliminates the use of hazardous organic solvents, making it a more sustainable and regulatory-compliant choice for industrial applications.

Economic Evaluation of Extraction Methods

An economic comparison of the three methods is presented in Table 4. Although hexane achieves relatively high carotenoid extraction efficiency (78.6%), it incurs a higher solvent cost ($4.2/kg) and lacks waste reduction advantages. Ethanol is moderately cost-effective ($3.5/kg) but shows limited performance in carotenoid extraction (45.1%). The two-phase system demonstrates the best overall performance, offering the highest recovery rates for both flavonoids (89.6%) and carotenoids (90.0%), while maintaining the lowest solvent cost ($3.1/kg) and achieving notable waste reduction.

Method	Solvent Cost ($/kg)	Extraction Efficiency (%)
Ethanol Only	3.5	72.3
Hexane	4.2	78.6
Two-Phase System	3.1	89.6

Table 5: Economic Comparison Across Methods.

Expanded Interpretation: Table 4 emphasizes the cost-effectiveness of the two-phase extraction method, positioning it as a practical and scalable green technology suitable for pharmaceutical and cosmetic industries, where both efficiency and environmental sustainability are critical

Prospective Analytical Enhancements

While the current study employed spectrophotometric methods for quantifying flavonoids and carotenoids, future research should consider incorporating more advanced analytical techniques such as High-Performance Liquid

Chromatography (HPLC) and Liquid Chromatography–Mass Spectrometry (LC-MS). These methods offer higher sensitivity, selectivity, and resolution, enabling the precise separation, identification, and quantification of individual bioactive compounds within complex plant matrices.

HPLC has been widely used to determine the concentrations of specific flavonoids such as quercetin, kaempferol, and luteolin, while LC-MS allows for detailed structural elucidation and detection of carotenoid isomers and their oxidation products. These techniques can significantly improve the understanding of the phytochemical profile, stability, and potential therapeutic effects of the extracted compounds [14].

It is important to note that HPLC and LC-MS were not employed in this study but are proposed as valuable tools for future analytical validation.

3.5 Modeling of the Extraction Process

To better understand the dynamics of the two-phase extraction system, the Langmuir isotherm model was applied to the experimental data[9]. This model, as introduced earlier, describes the relationship between the concentration of extracted compounds and their saturation behavior within the solvent matrix, assuming monolayer adsorption onto finite active sites[11].

The fitting of experimental data to the Langmuir model showed a strong correlation, suggesting that the extraction process followed a saturation-limited behavior. Flavonoids and carotenoids both exhibited typical monolayer adsorption patterns, with equilibrium concentrations aligning closely with model predictions.

This modeling approach confirms the efficiency of the two-phase system and provides a predictive tool for scaling up the process, enabling optimization of solvent usage, time, and extraction conditions while maintaining high compound recovery.

3.6 Economic and Environmental Aspects

In addition to extraction efficiency, the economic and environmental viability of the two-phase system was evaluated to assess its suitability for industrial applications[3], particularly in pharmaceutical and cosmetic sectors where cost-effectiveness and environmental compliance are crucial.

The two-phase system demonstrated clear advantages over conventional single-solvent methods. Economically, it recorded the lowest solvent cost at $3.1/kg, compared to $3.5/kg for ethanol-only and $4.2/kg for hexane. In terms of extraction performance, it achieved the highest recovery rates for both flavonoids (89.6%) and carotenoids (90.0%). In contrast, the ethanol-only method performed well with flavonoids (72.3%) but was less effective with carotenoids (45.1%). The hexane-only method was efficient with carotenoids (78.6%) but largely ineffective for flavonoids (10.2%).

From an environmental perspective, the two-phase system reduced chemical waste generation by 30% relative to hexane-based methods. This is a critical factor in reducing hazardous waste disposal requirements and minimizing ecological harm. The environmental advantage stems from the nature of the solvents used: ethanol is considered relatively safe, and sunflower oil is non-toxic, biodegradable, and environmentally friendly[4]. Together, they support the principles of green chemistry while maintaining high extraction efficiency.

Figure 3 illustrates the environmental impact of the three extraction systems. Hexane showed the greatest ecological burden due to its volatility, flammability, and toxicity.

4. Conclusion

This study successfully demonstrated the effectiveness of a two-phase extraction system combining 70% ethanol and sunflower oil for the simultaneous recovery of flavonoids and carotenoids from Calendula officinalis flowers.

The optimized solvent ratio of 1:10:10 (raw material to ethanol to oil) achieved high extraction efficiencies—89.6% for flavonoids and 90.0% for carotenoids—significantly outperforming conventional single-solvent methods.

Beyond extraction performance, the two-phase system exhibited notable economic and environmental advantages, reducing solvent costs by approximately 18% and chemical waste generation by 30% compared to hexane-based methods.

These findings highlight the potential of the two-phase extraction approach as a practical, scalable, and environmentally sustainable alternative for application in pharmaceutical, nutraceutical, and cosmetic industries.

Future research should aim to enhance extraction kinetics, investigate the long-term stability of the extracted compounds during storage, and validate the scalability of the process at pilot and industrial levels.

The successful integration of green solvents with high extraction efficiency, as demonstrated in this study, not only advances sustainable chemical practices but also aligns with increasing global regulatory and environmental demands.

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