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Comparative Evaluation of Extraction Techniques and HPLC-Based Quantification of Luteolin from *Mentha piperita* Leaves

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ABSTRACT:

Background: Efficient extraction of bioactive flavonoids is critical for their downstream research and industrial applications.

Objective: This work systematically investigates the influence of extraction procedures and solvent polarity on the separation of Luteolin from *Mentha piperita* leaves.

Methods: Dried and powdered leaves were treated to maceration, reflux, and Soxhlet extraction using solvents of increasing polarity—methanol, ethanol, chloroform, and ethyl acetate. Extract yield and Luteolin content were quantitatively evaluated by High-performance liquid chromatography (HPLC) utilising a Lichrospher C18 column and a methanol–acetic acid mobile phase.

Results: The results indicated a substantial reliance of Luteolin recovery on both the extraction process and solvent employed. Among all combinations, reflux extraction with methanol provided the highest Luteolin concentration (5.57% w/w) and purity (98.97%). In comparison, the maceration and Soxhlet techniques generated relatively lower concentrations and purity levels. In general, methanol was the most effective solvent and reflux extraction was the optimum procedure.

Conclusion: The results might be used as a robust and optimised technique for the Luteolin extraction from *M. piperita*, facilitating its effective utilisation in phytochemical studies and industrial applications.



Mentha piperita, HPLC, Extraction techniques, Luteolin quantification

1. INTRODUCTION

Luteolin (3',4',5,7-tetrahydroxyflavone) is a naturally occurring flavone that is extensively distributed in medicinal plants, vegetables and dietary herbs. It has attracted substantial scientific interest due to its varied pharmacological actions such as antioxidant, anti-inflammatory, anticancer, antibacterial and neuroprotective properties [1]. Recent studies also have further demonstrated that Luteolin exerts its therapeutic benefits by modulation of several molecular targets and signalling pathways including NF- κ B, MAPK, PI3K/Akt and Nrf2, regulating oxidative stress, apoptosis and inflammatory cascades [1][2]. These pleiotropic bioactivities emphasise Luteolin as a high-value phytochemical for pharmacological, nutraceutical and functional food use. However, its translational and industrial utilisation vitally relies on efficient extraction techniques ensuring high yield, purity, repeatability and scalability. *Mentha piperita* L. (peppermint) is a perennial aromatic plant of the *Lamiaceae* family which is widely utilised in traditional and modern medicine for its antibacterial, antioxidant, anti-inflammatory and digestive qualities [3]. Besides essential oil components, peppermint leaves have a high content of phenolic chemicals, especially flavonoids, such as Luteolin and its glycosides [4]. Although the phytochemical richness and therapeutic significance of Luteolin from *M. piperita* have been acknowledged, comprehensive investigations targeted at optimising Luteolin extraction from *M. piperita* leaves stay limited. Most investigations have focused widely on total phenolics or essential oils with comparably little emphasis on selective and quantitative recovery of specific flavonoids. The extraction effectiveness of plant-derived flavonoids depends on various connected parameters, such as solvent polarity, extraction technique, temperature, duration, and the properties of the plant matrix. Conventional extraction procedures such as maceration, reflux and Soxhlet extraction are significantly different in terms of solvent penetration capacity, heat transfer efficiency, mass diffusion rates and probable destruction of thermolabile chemicals [5]. Recent improvements in phytochemical processing have highlighted the significance of solvent selection on the basis of polarity index and solubility characteristics to maximise the recovery of target chemicals and minimise the co-extraction of contaminants [6]. However, there is no consensus on the most appropriate solvent–method combination for the maximum yield and purity of Luteolin from *Mentha piperita*. However, comparative studies that comprehensively evaluate the connection between the extraction technique and solvent polarity for this particular molecule are lacking. Considering the growing need for standardisation of plant derived bio actives and evidence based phytopharmaceutical development, an optimised extraction technique for Luteolin from *M. piperita* is warranted both scientifically and industrially. A systematic comparative evaluation can provide critical insights into maximising extraction efficiency, improving analytical reproducibility, and supporting large-scale production feasibility.

Thus, we hypothesise in the present study that the solvent polarity and extraction methodology substantially influence the extraction efficiency of Luteolin from *Mentha piperita* leaves and that a well optimised solvent-method combination can remarkably increase the Luteolin yield and purity. For a systematic evaluation of this, different conventional extraction procedures, such as maceration, reflux and soxhlet, were comparatively evaluated using organic solvents of different polarity and then the concentration and purity of Luteolin were quantitatively determined by validated HPLC analysis. This inquiry merges the analytical standardisation with the methodological comparison to fill the current gap in the solvent/technique thorough evaluations for selective Luteolin separation.

The results of this work are anticipated to provide scientifically established and reproducible extraction process, hence contribute into improved phytochemical standardisation, quality control and scalable utilisation of *M. piperita* as a trusted source of high purity Luteolin for pharmaceutical and nutraceutical use

2. MATERIALS AND METHODS

2.1 Plant Sample Collection and Preparation

Mentha piperita L. Fresh, healthy leaves of The samples were gathered from Botanical Garden of Gujarat University, Ahmedabad, Gujarat, India during the proper vegetative stage. The plant specimen was taxonomically certified by a qualified botanist of the Department of Botany, Faculty of Science, Gujarat University. A voucher specimen was collected and preserved at the departmental herbarium of Gujarat University for future reference (Ref.No./GU/BOT/ BBM /1/09-2023). After collection, the leaves were carefully inspected and damaged or infected material was removed and the leaves were thoroughly cleaned to remove adherent soil, dust and extraneous materials. The samples were carefully cleaned with distilled water to eliminate surface contamination and then blotted dry with sterile absorbent paper. The cleaned leaves were dried in shade at room temperature (25–30 °C) with proper ventilation for about 7–10 days until uniform weight was obtained to

minimise microbial growth and destruction of thermolabile phytoconstituents such as flavonoids. The dried leaves were pulverised using a laboratory grade mechanical grinder to create fine powder. The powdered material was sieved through a standard mesh (40–60 mesh) to ensure uniform particle size, hence improving extraction efficiency and reproducibility. The produced powder was stored in airtight, light-resistant containers under dry conditions at room temperature until further extraction and analysis.

2.2 Chemicals and Reagents

Luteolin ($\geq 98\%$ purity) reference standard was obtained from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade organic solvents (methanol, ethanol, chloroform and ethyl acetate) were procured from (Merck Specialities Pvt. Ltd., Mumbai, India) and utilised without further purification. Glacial acetic acid and HPLC grade methanol were used throughout the chromatographic analysis for precision, accuracy and reproducibility of the results. All reagents and solvents employed in this study were of analytical or chromatographic grade and were used without further purification. Solvent handling, storage and disposal were performed according to established laboratory safety procedures. All experimental methods were performed utilising calibrated laboratory glassware and equipment to preserve uniformity and reproducibility of analytical measurements.

2.3. Comparative Evaluation of Extraction Method

Luteolin was isolated from powdered leaves of *Mentha piperita* by three traditional extraction methods, including maceration, reflux and Soxhlet extraction utilising solvents of different polarity, such as methanol, ethanol, chloroform and ethyl acetate. For each extraction, 10 g of dried leaf powder was mixed with 100 mL of solvent, maintaining a solvent-to-drug ratio of 10:1 (v/w) to guarantee sufficient solvent penetration and mass transfer.

In maceration procedure, the plant material was soaked in the corresponding solvent at room temperature and retained for 72 h occasional shaking to facilitate diffusion of phytoconstituents into the solvent medium. For reflux extraction, the powdered leaves were boiled with solvent in a refluxing equipment at 50–60 °C for 2–3 h with continuous boiling and condensation to enhance extraction efficiency. Exhaustive extraction was facilitated by Soxhlet extraction at similar temperature conditions (50–60 °C) for 2–3 h with continuous hot percolation.

All the solvent extracts were filtered through Whatman No. 1 filter paper after extraction and concentrated under reduced pressure using a rotary evaporator at 45 °C to get the crude extracts. The percentage yield of each extract was determined as the ratio of the dried extract weight to the original weight of plant material

2.4. High-Performance Liquid Chromatography (HPLC) Analysis:

High-performance liquid chromatography (HPLC) was employed for the quantitative determination of luteolin in the prepared extracts. Stock solution of luteolin standard (1 mg/mL) was prepared in HPLC grade methanol and then diluted to prepare the working standard solutions in the concentration 100, 125, 175, 200 and 500 $\mu\text{g/mL}$ for the calibration curve. All standard solutions were stored at $-20\text{ }^{\circ}\text{C}$ until analysis to maintain their stability and to avoid degradation. Dried extracts obtained from different extraction procedures and solvent systems were accurately weighed and reconstituted in HPLC-grade methanol to a final concentration of 1 mg/mL for the preparation of samples. The solutions were thoroughly vortexed to ensure complete dissolution and filtered through a 0.22 μm membrane syringe filter to eliminate particulate matter and protect the chromatographic system. The chromatographic analysis was performed on a Shimadzu quaternary HPLC system equipped with a UV–Vis detector and a Lichrospher C18 reverse-phase column (250 mm 4.6 mm, 5 μm particle size). Isocratic separations were performed with the mobile phase of methanol and 1% aqueous acetic acid (99:1, v/v) at a flow-rate of 1.0 mL/min. The temperature of the column was maintained at 30 °C to ensure the consistency and reproducibility of chromatographic performance. The compound was quantified in a sensitive and selective way by detection at 289 nm, the maximum absorption wavelength (λ_{max}) of luteolin. Quantification was done by comparing sample peak regions to the external standard calibration curve.

2.5 Quantitative Analysis

Quantitative evaluation of the Luteolin content and extraction efficiency of the produced samples was carried out by means of standardised analytical techniques. The efficiency of each solvent–method combination was assessed by calculating the % extract yield according to the following equation:

Extract Yield (%) = (Weight of dried extract / Weight of dried plant powder) × 100

This parameter was useful for the comparison of solvent extraction capacity and total recovery of phytoconstituents. HPLC analysis with an external standard calibration curve was used to quantify luteolin in each extract. Luteolin concentration was determined from peak area and expressed as % weight/weight (w/w) relative to the dried plant material. The purity of luteolin in each extract was determined by calculating the proportion of the quantified luteolin to the total weight of the corresponding crude extract. This assessment was the basis for comparison of selectivity and extraction efficiency of the different solvent systems and extraction methods applied. All experiments were expressed as mean ± standard deviation in triplicate to guaranty analytical reliability, reproducibility and accuracy.

3. RESULTS AND DISCUSSION

3.1 Extract Yield Analysis

The extraction yield was highly dependent on both the polarity of the solvent and the extraction technique (Table 1). Chloroform under reflux conditions gave the highest crude extract recovery (15.6%) followed by Soxhlet extraction using ethyl acetate (14.7%) and ethanol (14.5%). Conversely, maceration gave lower extraction yield between 4.6% (ethyl acetate) and 9.8% (methanol) at room temperature, showing a relatively poor extraction efficiency of the method. It is interesting to note that the highest crude recovery (15.6%) was obtained from the reflux extraction with chloroform, while the total extract yield was relatively low (4.9%) with the reflux extraction with methanol. This shows the extraction was selective rather than bulk. These results indicate that the use of reflux and Soxhlet techniques with high temperature and continuous solvent cycling enhanced overall mass transfer and phytochemical Solubilization. However, crude yield alone is not an indicator of Luteolin enrichment, as significant recovery may be due to co-extraction of non-target constituents [5]. Thus, compound-specific quantification is required for reliable evaluation of the extraction efficiency.

Table 1. Comparison of extract yield, luteolin content and purity from *Mentha piperita* leaves by different extraction techniques and solvents

Sr. No.	Extraction Method	Solvent	Extract Yield (%)	Luteolin Content (% w/w)	Purity (%)
1	Maceration	Ethanol	6.5	ND	ND
2	Maceration	Methanol	9.8	5.44	98.52
3	Maceration	Chloroform	5.7	0.48	41.89
4	Maceration	Ethyl Acetate	4.6	0.32	15.91
5	Soxhlet	Ethanol	14.5	0.007	0.26
6	Soxhlet	Methanol	9.1	3.21	98.04
7	Soxhlet	Chloroform	8.8	0.50	79.35
8	Soxhlet	Ethyl Acetate	14.7	0.09	10.42
9	Reflux	Ethanol	9.8	ND	ND
10	Reflux	Methanol	4.9	5.57	98.97
11	Reflux	Chloroform	15.6	0.13	48.62
12	Reflux	Ethyl Acetate	14.2	1.37	35.13

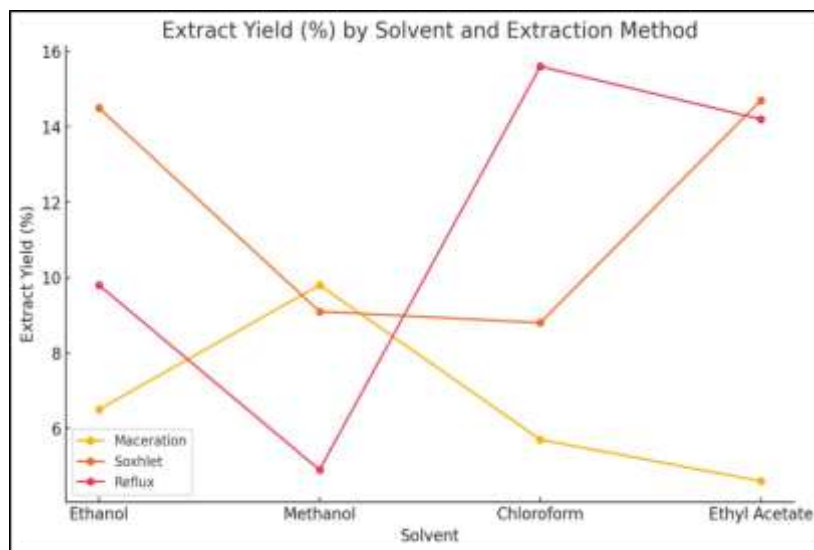


Figure 1. Effect of extraction process and type of solvent on the yield (%) of extract produced from *Mentha piperita* leaves.

3.2 Quantitative Estimation of Luteolin Content

Quantitative HPLC study (Table 1; Figure 2) indicated that Luteolin recovery was significantly affected by both solvent polarity and extraction process. Methanol was also shown to give the best extraction efficiency, with the reflux extraction giving the highest Luteolin content (5.57% w/w), closely followed by maceration (5.44% w/w) and Soxhlet extraction (3.21% w/w). As shown in Figure 2, the Luteolin content reduced significantly in less polar solvents, with chloroform and ethyl acetate having significantly lower concentration for all techniques. Luteolin was not detected or was present in very low amounts in ethanol extracts. The graphical trend clearly showed preferential enrichment of luteolin in methanolic extracts, especially when reflux extraction was used. It indicates that the high polarity of methanol and the controlled heating of the reflux increase the solvent's ability to breakdown plant matrices and to improve the solubilization and diffusion of luteolin in the extraction medium. The synergistic effect of solvent polarity and increased temperature resulted in improved mass transfer efficiency and thus higher recovery of luteolin. [7]. On the whole, reflux extraction with methanol was found to be the best method for maximising the recovery of Luteolin from *Mentha piperita* leaves.

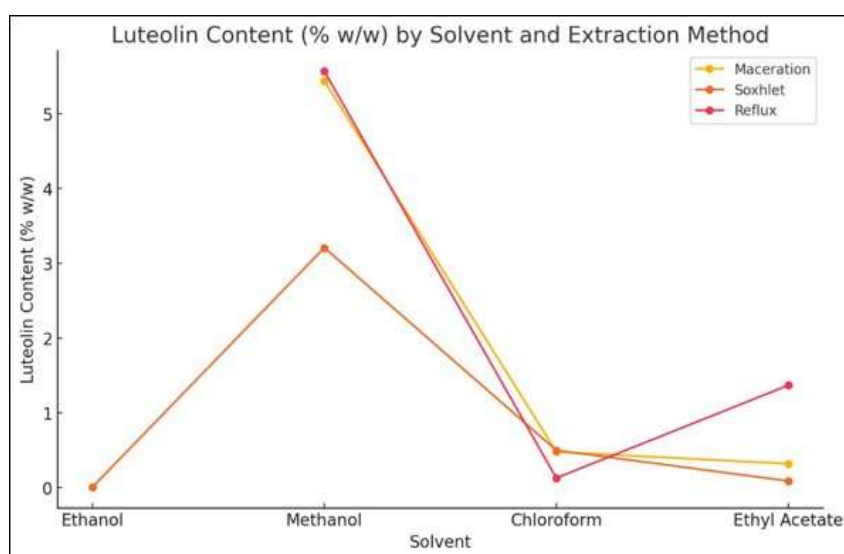


Figure 2. Effect of solvent and mode of extraction on %w/w Luteolin content in *Mentha piperita* leaf extracts. Highest recovery was obtained using methanol in reflux circumstances.

3.3 Purity Assessment of Luteolin in Crude Extracts

The purity profile of Luteolin was quite different based on the solvents and extraction procedures as shown in Table 1

and Figure 3. Of all the solvent systems that were examined, the methanol extracts consistently showed the highest levels of purity. Note that the highest purity (98.97%) was obtained by reflux extraction using methanol, followed by maceration (98.52%) and Soxhlet extraction (98.04%), which indicated that methanol was highly selective toward Luteolin enrichment. Whereas, chloroform and ethyl acetate extracts had relatively lower purity values for all techniques indicating the co-extraction of non-target phytoconstituents and less selectivity. No Luteolin was found in ethanol extracts of maceration and reflux, thus proving the unsuitability of ethanol for selective isolation of Luteolin. This is exemplified with methanol in Figure 3 – especially under reflux circumstances gives better purity – highlighting the need of both solvent polarity and controlled heat extraction in selective chemical recovery.

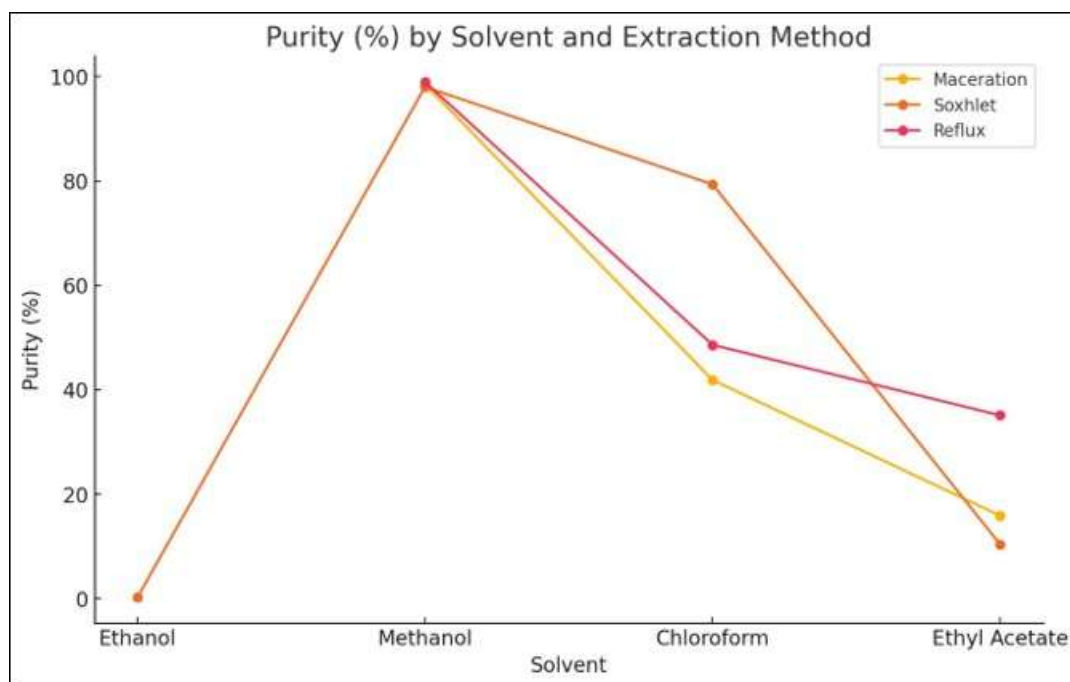


Figure 3. Effect of solvent and extraction procedure on Luteolin purity (%). Highest selectivity with methanol, especially under reflux conditions.

3.4 Comparative Evaluation of Extraction Techniques

The three extraction methods were compared in detail and the results showed significant differences in the efficiency and selectivity of luteolin extraction. Soxhlet and reflux methods generally yielded more crude extract, especially with chloroform (15.6%) and ethyl acetate (14.7%). However, the quantification by compound showed that reflux extraction with methanol was more selective to enrich Luteolin.

This combination resulted in the highest Luteolin concentration (5.57% w/w) and maximum purity (98.97%) implying better selectivity despite significantly lower total extract yield. This superior performance of the reflux method is due to controlled thermal energy, enhanced solvent penetration and efficient mass transfer kinetics that allows the solubilisation of thermally stable flavonoids like Luteolin without substantial co-extraction of interfering phytoconstituents [5][6]. In contrast, Soxhlet extraction, while efficient in achieving maximum bulk extraction, appeared to be less selective for Luteolin in some solvent systems, probably because the extended heating and exhaustive extraction favoured the co-extraction of non-target molecules. Maceration, despite being operationally easy and cost-effective, displayed relatively lower efficiency in extraction, which is due to the low diffusion rates and absence of heat enhancement [5]. Importantly, the present data underscore that increased crude yield does not necessarily translate into higher bioactive enrichment, thus highlighting the necessity of solvent–method optimisation led by compound-specific analytical validation. Similar observations of solvent polarity and thermal-assisted

extraction improving flavonoid recovery have been reported in studies of medicinal plants [1][3]. These results together confirm that the selection of solvent and extraction methodology is essential for achieving high-yield and high-purity isolation of Luteolin from *Mentha piperita* leaves.

4. DISCUSSION

The present study provides systematic and comparative evaluation of the conventional extraction methods for selective isolation of Luteolin from *Mentha piperita* leaves and hence fills an important methodological gap in phytochemical standardisation. The results clearly show that extraction efficiency is not only influenced by crude yield, but also by combined effects of solvent polarity and extraction dynamics. The highest Luteolin content (5.57% w/w) and purity (98.97%) were observed for reflux extraction with methanol as quantified by compound specific quantification when compared to Soxhlet and reflux methods, however, Soxhlet and reflux methods showed relatively higher bulk extract recovery. This distinction brings out an important analytical principle that maximal crude extraction may not necessarily lead to selective enrichment of target phytoconstituents. Such observations underscore the necessity to couple extraction optimisation with quantitative chromatographic validation to ensure reproducibility and compound specificity.

The higher performance of methanol is mechanistically understandable by its high polarity index and hydrogen-bonding capacity that allow for efficient solubilisation of polyhydroxylated flavonoids such as Luteolin. Previous studies have always shown methanol to be an efficient solvent for the extraction of flavonoids as it may interfere with plant cell matrices and enhance the diffusion of phenolic compounds [5][6]. In addition, Luteolin's stability at moderate thermal conditions supports controlled heating for efficient reflux extraction [8]. The reflux method guarantees longer contact between solvent and matrix, improved mass transfer and decreased solvent loss due to condensation, leading to better recovery selectivity and avoiding long exposure time or excessive degradation associated with exhaustive procedures such as Soxhlet extraction. Such mechanistic findings are in line with general principles of phytochemical extraction applied to medicinal plants [6][7].

While the therapeutic potential of Luteolin and its pharmacological relevance in modulating inflammatory and oxidative stress pathways have been demonstrated in several studies [1][9], fewer studies have focused on the optimisation of its selective extraction from specific botanical sources. Most research on *Mentha* species has focused on essential oils and total phenolic content over compound-specific standardisation [3]. The present study extends on existing literature by providing a systematic comparison of solvent–method interactions through the direct comparison of maceration, Soxhlet and reflux methods across polarities of solvent. This comparison approach provides a reproducible strategy to maximise the production and purity of Luteolin and thereby contribute to the methodological refinement in phytochemical research. The findings from this investigation have broader implications beyond laboratory scale experiments. With the increasing demand for plant derived bio actives worldwide, in pharmaceutical, nutraceutical and functional food industries, there is a need for standardised extraction techniques to assure batch-to-batch uniformity, regulatory compliance, and scalability [6]. The method was determined to be the best extraction method and the practical benefits of a methanol-based reflux extraction were highlighted in terms of reduced extraction times, improved selectivity and potential cost effectiveness for large-scale applications. The results obtained emphasize the significance of the choice of extraction solvent and controlling the extraction parameters to achieve maximum purity and yield of phytochemical chemicals. Moreover, the present study emphasizes the importance of systematic optimization of extraction and provides a reliable basis for future process development, scale-up strategies and industrial production of luteolin from *Mentha piperita*.

5. CONCLUSION

This study indicates that the use of methanol with the reflux extraction procedure is the most effective way to separate Luteolin from peppermint leaves. Other methods may provide more crude extract but this yields the highest real Luteolin (5.57%) and an excellent 98.97% purity. So with regulated heat, constant circulation, and the chemical characteristics of methanol, it becomes quite efficient at grabbing only the component you want. This gives a very dependable, easily scalable technology that the pharmaceutical and wellness businesses can adopt with confidence.

6. AUTHOR CONTRIBUTIONS

The project was planned and designed by R. Jatvada, who also executed the experiments, conducted data analysis and produced the report. T. Shah helped in drafting the manuscript. C. Roat oversaw the research work, contributed to the improvement of

experimental design, validated the analytical approach, critically evaluated the text for intellectual content, and approved the final version for publication.

7. CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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