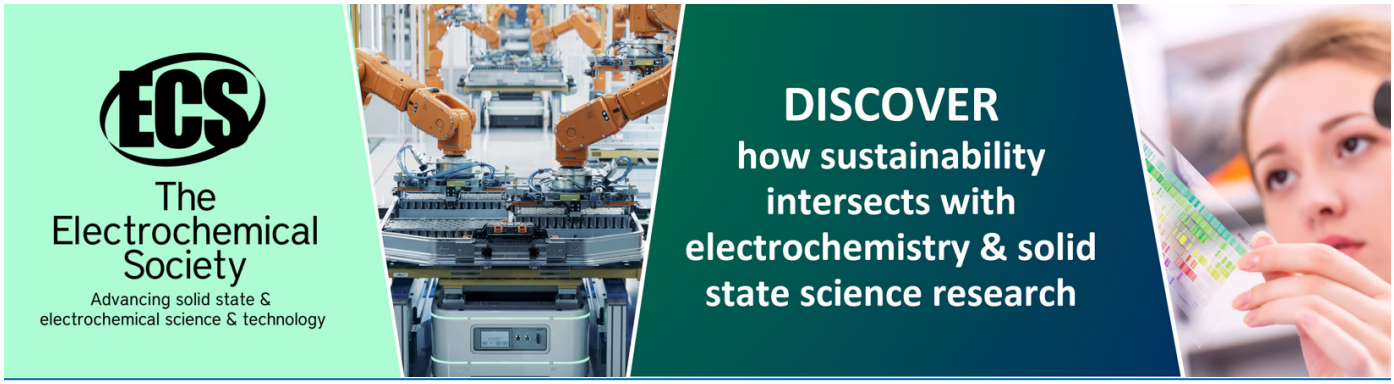
Extraction, Isolation and Identification of Luteolin Flavonoid from *Vitex pseudonegundo leaves*



Extraction, Isolation and Identification of Luteolin Flavonoid from *Vitex pseudonegundo leaves*

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Abstract. Objective: Using HPLC as a quantitative estimate technique, the optimal extraction solvent for luteolin from *Vitex pseudo-negundo* was identified. Methods: leaves *V. pseudonegundo* were gathered, washed, and dried. employeing methanol and ethanol as the extraction solvents (both with varied strengths), as well as the extraction techniques of maceration, reflux, and Soxhlet. The amount of luteolin in each sample extract was measured using a preparative HPLC technique Results: Methanol was the best solvent, and the best extraction method was the reflex extraction method, based on HPLC results Conclusion: According to the results of HPLC, reflex was the superior method for extracting luteolin from *V. pseudonegundo*, and the optimum solvent was methanol. thus, advising using this approach in industry and future study.

Keywords. Luteolin, *Vitex pseudonegundo*, Extraction techniques, HPLC.

1. Introduction

The Vitex plant is a plant that lives more than two years that consider genus of to Lamiaceae family [1]. Vitex pseudonegundo roughly 3 meters tall and is a shrub with woody bases [2]. Its native habitat frequently consists of moist areas beside streams, and this plant is especially common in numerous wetland regions of Asia [3]. Over 250 species of Vitex (Lamiaceae) may be found, the majority of which are frequently used medicinally [4]. According to recent research that have described their phytochemistry, the plant species in this genus generate iridoids, terpenoids, flavonoids, and steroids. [5]. Due to its hormone-like effects, this plant is particularly used to treat hyperprolactinemia and premenstrual difficulties [6]. It also has significant therapeutic benefits. Traditional medicine it is used as diuresis enhancer, fungal killer, Anxiolytics ,sexual desire improvement, analgesic, emmenagogues, suppresses muscle spasms, sleep inducer, reduction in the production of prolactin [1].

Around 4000 naturally occurring polyphenolic chemicals collectively referred to as flavonoids are exclusively of plant origin [7]. Flavonoids are divided into numerous subgroups based on the differences in functional groups and the skeleton's 15-carbon structure's relative positions (aglycons), comprising chalcones, anthocyanidin, flavone, flavanone, flavonol, isoflavonoid, and flavone. Several of these flavonoids have demonstrated anticancer action in cell and animal models of cancer [8].

Important natural antioxidants called flavonoids, such luteolin, have strong anticancer effects in vitro and in animals [9]. Luteolin, artlessly existing flavonoid (3,4,5,7-tetrahydroxy flavone, Figure.1) widely distributed among many plant species [10]. Vitex pseudonegundo in particular exhibits its presence [11] .

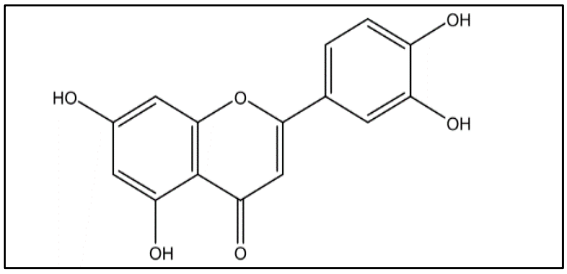

Figure 1. Chemical structure for luteolin.

Recent research has shown that luteolin can impede the carcinogenesis process by interfering with the course of the cell cycle, preventing proliferation, inducing apoptosis, and limiting the migration and invasion of malignant cells. [12]. With the use of simple extraction methods, luteolin may be recovered from plants, but due to its difficulty in obtaining, only a tiny amount is accessible for biological investigations. [13]. Analysis of luteolin in various extracts using chromatography technique (HPLC), the study sought to identify the best suitable vehicle to improve the extraction process for luteolin isolation from *Vitex pseudonegundo* leaves.

2. Materials and Methods

2.1. Plant

Vitex pseudonegundo were procured in July during the blooming stage (Sulaymaniyah, Iraq) . At the Pharmaceutical University in Basrah, Iraq, Assistant Professor Dr. Ula Almousawi identified and verified the plant species.

2.2. Chemicals

The standard luteolin was bought (Med ChemExpress). The study employed only analytical-grade chemicals and reagents, all of which were obtained from reputable businesses and institutions.

2.3. Extraction of Luteolin

To get rid of any adherent foreign material, the leaves of *Vitex pseudonegundo* were thoroughly cleaned. The leaves were partially ground using a household grinder after being air dried in the shade The leaves were then subjected to a 24-hour hexane treatment before being utilized to extract luteolin (defating).

Three distinct extraction techniques—maceration, reflux, and soxhlation—were used, as well as two different solvents with varied degrees of polarity and strength, including ethanol and methanol [14]. 60 grams of leaf powder were utilized in total (after defating with hexane). Drug to solvent ratio: 17.7:1 (mL/g) [15]. The quantity of extract in dry powder, or extract yield, was used as a benchmark to assess how well the various extraction methods performed. To determine the presence of flavonoids in all the produced extracts, Shinoda, fecl3 chemical assays were performed.

2.3.1. Extraction Through Maceration

Method of extraction illustrated in table 1. extraction carried out at ambient temperature.

Table 1. Extraction by maceration.

Weight (gm)	Solvent type	Solvent Strength	Volume of solvent	Duration hr
10	Methanol	100%	177ml	24
10	Methanol	80%	177ml	24
10	Ethanol	90%	177 ml	24

After centrifuging the menstruum for 15 minutes at 3000 rpm with No. 1 Whatman paper, the filtrate was then evaporated to produce the residue.

2.3.2. Reflex Extraction

Method of extraction illustrated in table 2. Using a reflux system, hot extraction was carried out for two hours at 50 °C.

Table 2. Extraction by reflex

Weight in gm	Solvent type	Strength	Volume in ml
10	Methanol	100%	177 ml
10	Methanol	80%	177 ml
10	Ethanol	90%	177 ml

The extracts were then treated in a similar way to how maceration was done previously.

2.3.3. Soxhlet Extraction

Method of extraction illustrated in table 3. Using a Soxhlet system, continuous hot solvent extraction was carried out for three hours at 50 °C. The extracts were further processed in the same way as previously reported.

Table 3. Extraction by Soxhlet.

Weight in gm	Solvent	Strength	Volume in ml
10	Methanol	100%	177 ml
10	Methanol	80%	177 ml
10	Ethanol	90%	177 ml

2.4. Thin Layer Chromatography (TLC)

The TLC was perform using conventional techniques The substances were solubilized in their corresponding solvents in very small amounts (2 mg/ml). Standard luteolin (1 mg) was solubilized in methanol. The screening procedure included a variety of mobile phases with varied concentrations, and the combination of n-butanol, acetic acid, and water was chosen because it allowed for unambiguous separation of flavonoids. Upon drying, all plates were viewed in the UV TLC viewer using ultraviolet at 254 nm and 366 nm. Using luteolin as a flavonoid standard, the Rf value of the several point that were seen was determined.

Table 4. Mobile phases were tested.

Mobile phase	Percent
n-butanol: acetic acid**:** water	6 **:** 1.5 **:**7.5
Tolune **:** ethylacetate **:** methanol **:**acetic acid	4 **:** 3 **:** 2 **:** 0.5

2.5. High Performance Liquid Chromatography

Luteolin was identified and separated using the HPLC technique. For Luteolin identification, we used analytical HPLC, in which we first injected standard luteolin to identify its peaks in detail, followed by a diluted extract. We then compared the two spectra to confirm the presence of Luteolin in the extract. We employed preparative HPLC for separation, in which we first inject luteolin standard to assess luteolin retention before injecting the whole extract to achieve separation. German HPLC system from Knauer systems elements. According to the reference [16], separation and detection were carried out.. The separation was performed using a C18 column (Knuaer, Germany) with a 250 x 4.6 mm internal diameter and 80 pore sizes. The thermostat had been set at 28°C for the column., the injection volume was maintained at 20 l, and the mobile phase contained 1% aq. acetic acid solution (Solvent A) and acetonitrile (Solvent B). The flow rate was set to 1 ml/min. By adjusting ratio of solvent B to solvent A, a gradient elution was carried out. The gradient elution was altered linearly from 10% to 40% B for 28 minutes, from 40 to 60% B for 39 minutes, and from 60 to 90% B for 50 minutes. Before injecting another sample, the mobile phase composition was returned to its starting state (solvent B: solvent A: 10: 90) after 55 minutes of operation (table 5). Three different wavelengths of HPLC chromatograms

were detected using a photo diode array UV detector (272, 280 and 310 nm). Each compound was identified by comparing the standards' retention times and absorbance spectra, and the concentration was established by serially varying the amounts of external standards to create a calibration curve between the concentration and the corresponding peak area.

Table 5. The gradient program for the estimation of luteolin flavonoid.

Time (min)	Mobile A (%)	Mobile B (%)	Flow rate ml/min
0	90	10	1ml/min
28	60	40	1ml/min
39	40	60	1ml/min
60	10	90	1ml/min

3. Results

3.1. Phytochemical Testing

By Shinoda, fecl3 tests, all produced extracts, i.e., methanol absolute and ethanol, demonstrated the presence of flavonoids [17].

3.2. Extraction of Luteolin by Various Extraction Techniques

The estimation of HPLC yield using various extraction methods and solvents has been given in (table 6). As predicted, a range of extract yields were produced using various extraction methods and solvents. Reflux approach produced the highest yield for methanol extract (21.58 ug/ml), whereas maceration technique produced the lowest yield for 90% ethanol. A maximum quantity of extracted luteolin yields with methanol by reflux method, as indicated by an HPTLC chromatogram, supported the findings of TLC. The quantity of isolated luteolin yield from 3.5 mg was 3 mg (table 7).

Table 6. Luteolin content in different extracts of V. pseudo negundo.

Method	**Solvent**	**ug/ml in sample**
Reflex	Methanol absolute	21.58
	Methanol 80%	17.67
	90% ethanol	15. 77
Soxhlet	Methanol absolute	17.45
	Methanol 80%	15.67
	90% ethanol	13.38
Maceration	Methanol absolute	9.47
	Methanol 80%	8.67
	90% ethanol	4.69

Table 7. The quantity of isolated luteolin.

Method	**Solvent**	**3.5 gm of extract /30 ml of solvent**
Reflex	Methanol absolute	3mg

3.3. Identification by TLC, UV, IR and HPLC of luteolin

Sample luteolin gave Rf value compared to standard luteolin in solvent system showed in (table 8) and (figure 2&3). The IR spectrum of isolated luteolin showed a strong band of OHstr at 3398 cm-1, CH aliphatic at 3089 cm-1, C=C aromatic at 1613, 1571, 1506 cm-1, C=Ostr at 1665 cm-1, C-Ostr at 1262, 1304, 1205, 1159, and 1118 cm-1 similar to standard luteolin (figure 4 A&B). The standard solutions of luteolin and sample extracts were subjected to HPLC technique using 1% aq. acetic acid solution (Solvent A) and acetonitrile (Solvent B) as mobile phase, in flow rate (1 mL/min). Calibration curve of luteolin was made with five dilutions of standard solution at a concentration ranged from (0 to 100 µg/mL). The regression equation and correlation coefficient were as follows: y=55.268x-49.86; r^2 =0.9996 (figure 5). The relative quantity of luteolin in the prepared extracts was then calculated

from this equation and the results are shown in (table 6). Luteolin peak was identified in the extracts by comparing the retention time with that obtained from the standard solution (Figure 6), by spiking the standard solution into the sample solution and by a comparison of its UV spectra with that of the standard. UV spectra measured for the peaks showed maximum absorbance at approximately 340, 286, and 265 nm for luteolin and its standard(figure 7). The retention time of isolated Luteolin peak was observed at 18.7 minute, compared to 18.9 in case of Luteolin standard (figure 8 A&B). Our study showed the Rf value of isolated Luteolin obtained from *V. pseudonegundo* leaves has the same value with luteolin standard shown in (figure 8C).

Table 8. Rf values for isolated compound and standard.

Mobile phase	Percent	Rf of sample	Rf of standar
n-butanol: acetic acid: water	6 : 1.5 :7.5	0.83	0.83
Tolune : ethylacetate : methanol :acetic acid	4 : 3 : 2 : 0.5	0.84	0.84

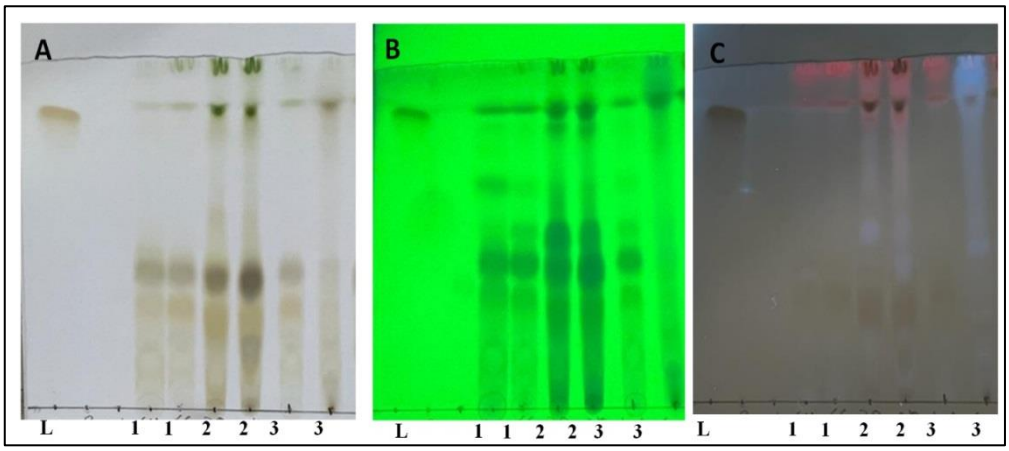

Figure 2. Detection of luteolin flavonoid TLC chromatograms obtained from methanol absolute plant extracts (leaves) of *V. pseudonegundo* and standard L: luteolin (sample codes as 1: Soxhlet, 2: reflux, 3: Maceration technique), B: Visualization was under UV light of wavelength 254 nm C: Visualization was under UV light of wavelength 366 nm. Mobile phase (n-butanol: acetic acid: water).

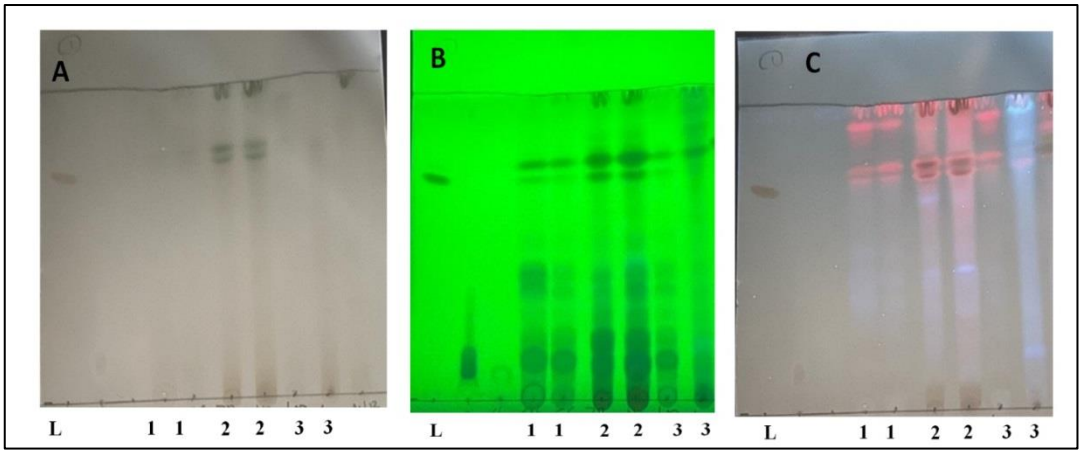

Figure 3. Detection of luteolin flavonoid TLC chromatograms obtained from methanol absolute plant extracts (leaves) of *V. pseudonegundo* and standard L: luteolin (sample codes as 1: Soxhlet, 2: reflux, 3: Maceration technique), B: Visualization was under UV light of wavelength 254 nm C: Visualization was under UV light of wavelength 366 nm. Mobile phase (Tolune : ethylacetate : methanol :acetic acid).

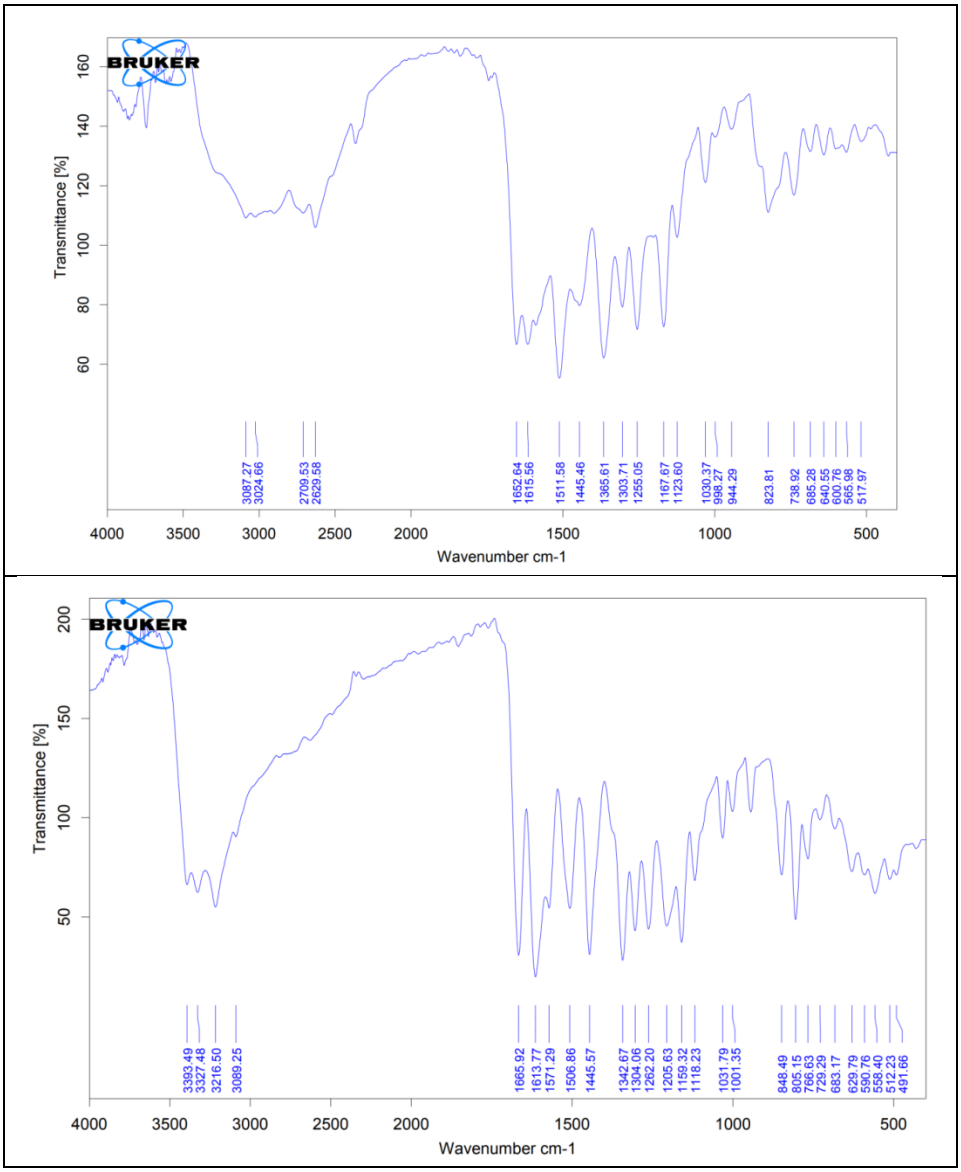

Figure 4. FTIR spectra of luteolin, A: represent isolated while B: represent standard.

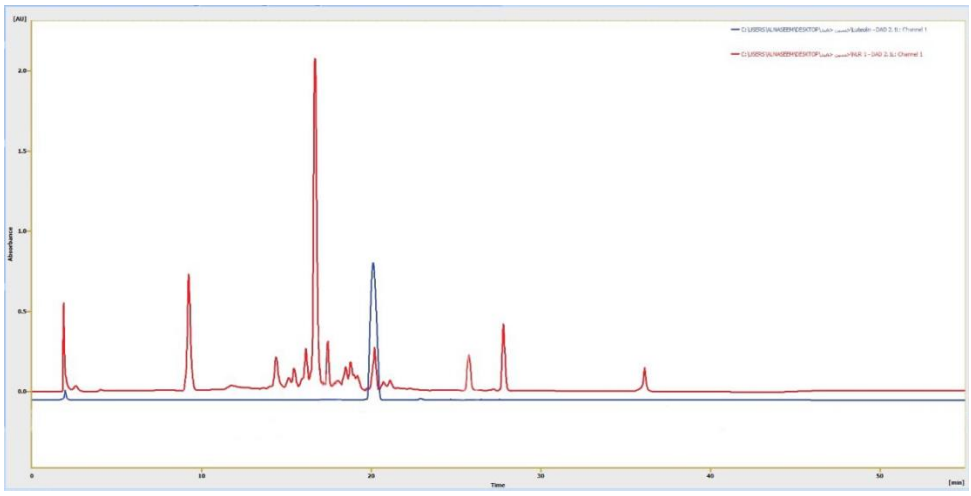

Figure 5. Chromatogram of luteolin standard (blue) and a chromatogram of showing separation of luteolin.

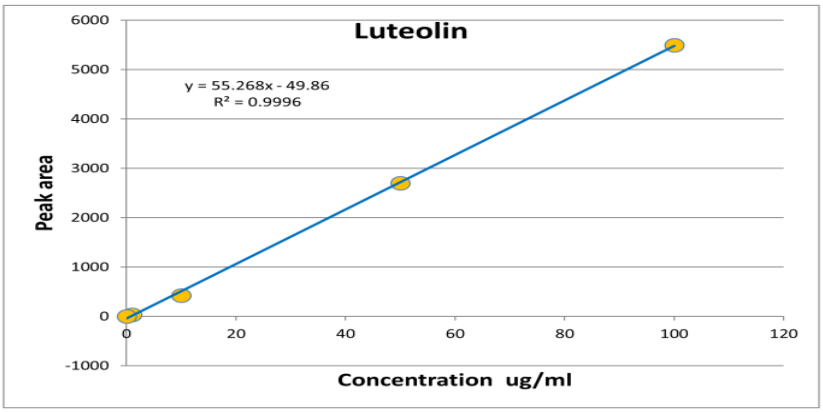

Figure 6. Calibration plot of luteolin.

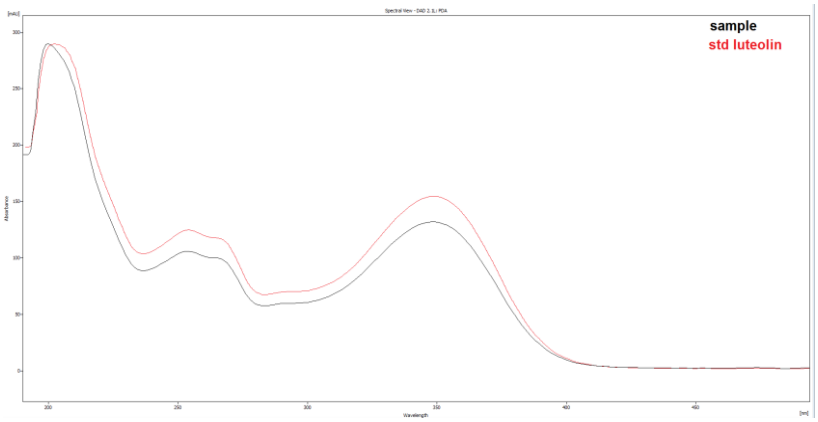

Figure 7. Luteolin spectrum in standard (red line) and sample (black line).

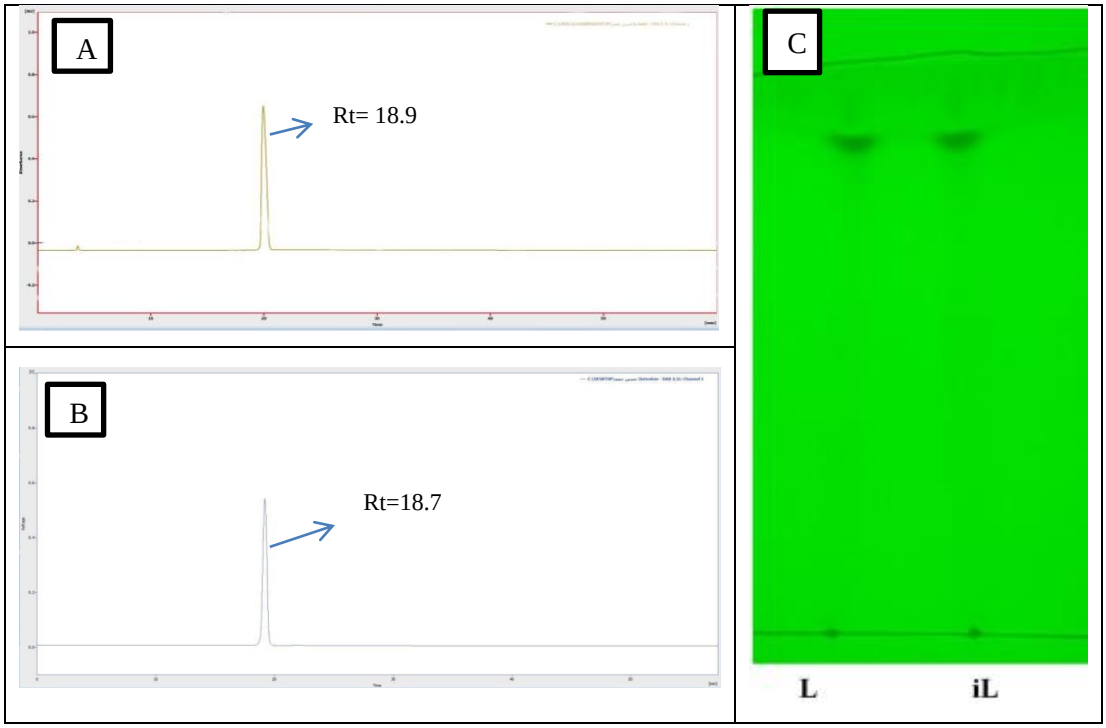

Figure 8. A&B. The retention time of isolated Luteolin peak (lower) was observed at 20 min, compared to 18.7 in case of Luteolin standard (upper).

4. Discussion

Achievement the separation of the bioactive molecules from medicinal plants, several innovative extraction procedures have been developed during the past few decades [18]. However several investigations have revealed that the extraction methods used have an impact on the biological activities of extracts [19]. Hence, depending on the physico-chemical properties of the sample matrix, it is crucial to choose an appropriate vehicle as well as an extraction process that is least impacted by the presence of interference-causing substances [20] . Luteolin is a significant component of certain plants and is well recognized to have many medicinal advantages [10]. It is necessary to know how much of it is present in the plant. The luteolin in this plant is found in the leaves. Hence, its measurement was done using the HPLC method. Due to its reputation as the most practical approach, Studies of flavonoids in plant materials using both qualitative and quantitative methods are typically conducted using the chromatographic method known as HPLC [21]. The greatest% yield was reached using the reflux approach, while extract yields varied depending on the solvents and/or extraction methods. The variations in the percent yield of leaf extracts may result from variations in the components that may be extracted using various solvents and extraction techniques. It may be concluded that hot extraction techniques are preferable to cold techniques like maceration for extract production from ***Vitex pseudonegundo*** leveas. [15]. Also, the findings of the HPLC analysis revealed that, regardless of the extraction method used, the luteolin concentration was greatest in the methanol extracts. This shows that the vehicle of choice for extracting luteolin from V. pseudo negundo is methanol.

Conclusions

The separation and letuolin yield from the extract of ***Vitex pseudonegundo*** leaves may therefore be inferred to be affected by the extraction method and solvent. The results of this research will undoubtedly aid the investigators in choosing the right extraction method—the methanol and reflux method—and suitable solvent for the isolation of luteolin from the V. pseudonegundo .Due to the simplicity, selectivity, sensitivity, and speed of this HPLC approach, luteolin may also be employed as a indicator in the comparability and quality assurance of this drug's leaves extract in the herbal industries.

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