

# Comparative assessment of extraction methods and quantitative HPLC estimation of Kaempferol in the leaves of *Prosopis Juliflora* Linn. Growing in Iraq.

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## ABSTRACT

Kaempferol is an active native flavonoid, has long been recognized for its curative effects. It has been observed to possess numerous pharmaceutical characteristics. High performance liquid chromatography (HPLC) was used as a quantitative evaluation method to determine the best extraction solvent for obtaining Kaempferol from *Prosopis juliflora* leaves. In this study, ethanol, ethyl acetate and methanol were used as solvents in the extraction of Kaempferol flavonoid from *Prosopis juliflora* by employing traditional methods of extraction (reflex and maceration). The plant leaves extract of *Prosopis juliflora* showed existence of Kaempferol compound. Among the three solvents used, it was obvious that ethyl acetate extract of leaves showed the most significant amount of Kaempferol (92.54 mg.L<sup>-1</sup>). In accordance to the HPLC results, reflex was the most efficient technique for extracting kaempferol from *Prosopis juliflora*, and ethyl ethanoate was the best solvent. This information may contribute to develop this flavonoid as a possible agent for the prevention and treatment of some diseases.

## 1. Introduction

The secondary metabolites found in herbs typically exhibit a broad spectrum of medicinal properties. As a result, the identification of flavonoids in plants facilitates the creation of drugs and their implementation in medical applications [1]. A lot of bioactive substances, including phenolic compounds, flavonoids, alkaloids, steroids, saponin tannins, terpenoids, glycosides, proteins, etc., are found in natural products and have a variety of medicinal benefits in the treatment of various diseases. Anthocyanides, flavones, flavanones, isoflavonens, and flavonoids were all categorized as polyphenolic components [2]. Flavonoids' practical hydroxyl groups contribute to their anti-inflammatory, hepatoprotective, anti-cancer,

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and antimicrobial properties as well as other aging-related diseases [3]. Studies were examined methods and conditions, especially for the separation of flavonoids from raw materials and products for technical, preparative, or manufacturing purposes [4]. for a long time due to the possibility and the significance of their anticipated use [5]. As a result, a number of flavonoid extraction methods were used to boost the amount produced of these important bioactive substances. Many methods, such as boiling, refluxing, soaking, maceration, percolation, Soxhlet, were suggested [6]. *Prosopis Juliflora* is a species belonging to the Leguminosae family and the Mimosoideae subfamily. It is a very widely distributed plant throughout India and around the globe with 44 species [7]. Numerous studies demonstrated the antibacterial, antioxidant, anthelmintic, antimalarial, larvicidal, antiemetic, insecticidal, and antitumor effects [8]. Ethno pharmacological studies revealed that *P. Juliflora* can be utilized as an astringent and in arthritic conditions in addition to being used as treatment for venomous snake and blisters caused by scorpions [9]. Alkaloids, flavonoids, and saponins are also abundant in this species. This plant's cultivating and toxicological properties of this plant highlighted its potential for use in the creation of herbicides [2]. the therapeutic value of kaempferol, a naturally active flavonoid, has long been recognized. Numerous pharmacological uses, such as antioxidant, anti-inflammatory, anticancer, neuroprotective, and cardioprotective qualities, were documented [10]. The aim of this research was to identify the best solvent for maximizing the extraction process in order to isolate kaempferol from *Prosopis Juliflora* L. leaves. This was accomplished by using high performance liquid chromatography (HPLC) to quantify kaempferol in various solvent extracts

## 2. Materials and Methods

Plant  
Family: Leguminosae  
Genus: *Prosopis*  
Species: *Prosopis juliflora*

### 2.1. Plant sampling

The leaves of *Prosopis Juliflora* L. plant were cultivated in an Garma Campas, University of Basrah, Basrah, Iraq. and identified by Assistant Professor. Dr. Ula AlMousawi, Pharmacognosy Department, Pharmacy College, University of Basrah. Raw plant sample material was gathered in August 2023, sanitized and shadow dried for seven days at room temperature before being grinded and stored in airtight boxes.

### 2.2. Chemicals

Standard Kaempferol was purchased from (Jinan Mtl Chemical Co., Ltd.). All other reagents used were of analytical grade and purchased from S.D. Fine Chemicals, India.

### 2.3. Extraction of Kaempferol

Two extraction techniques (cold and hot), a solvent (96% ethanol, 96% ethyl acetate, and 96% methanol), duration: 72 hours for cold extraction (maceration), 3 hours for hot extraction (reflux), temperature: room temperature for maceration, 60 °C for extraction reflux method, 10 g of grinded plant powder of *P. Juliflora* leaves were put in an empty glass vase with 100 milliliters of hexane, and after two hours of defatting and filtration using filter paper, the plant was taken out and allowed to dry at room temperature. Following the extraction processes of reflex and maceration using 200 ml of three distinct solvents (solid-liquid proportional 1:20 W.V<sup>-1</sup>), the resulting extracts went through a centrifuge for 20 minutes at 3000 rpm [11]. With some modifications. The purpose of the initial phytochemical screening tests was to identify any bioactive flavonoids present in the plant extract.

## 2.4. Chromatographic conditions

### 2.4.1. Identification of Kaempferol by Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) was carried out on pre-coated silica gel plates. In order to achieve the ideal conclusion of loaded samples, various solvent systems were designed. Diethyl ether: acetic acid: toluene (6:4:1 v.v-1) was found to be the most suitable mixture for TLC. After the plates had been created at room temperature, various bands were seen when exposed to UV light (254 and 366 nm).

### 2.4.2. High performance liquid Chromatographic for Kaempferol analysis

#### 2.4.2.1. Prepare the Kaempferol standard solution.

One mg.mL<sup>-1</sup> stock solution of Kaempferol was made using methanol of HPLC grade. After that, working solutions containing 100, 90, 70, 40, and 20 µg/mL were made and heated at room temperature. The standard solution underwent a 0.2 µm membrane filter filtering process before being subjected to HPLC analysis in order to determine its peak height at a static retention time. Next, a calibration plot was created to show the relationship between concentration (µg.mL<sup>-1</sup>) and peak area. The amount of kaempferol present in test samples was calculated using the linear equation derived from the standard plot.

#### 2.4.2.2. Sample solution preparation

Diverse solvent extracts of *P. Juliflora* leaves were made using various techniques, and one milligram per milliliter of the obtained mixture was filtered through a 0.2 µm membrane filter. HPLC analysis was then performed on 100 µL of this solution.

#### 2.4.2.3. Chromatographic conditions

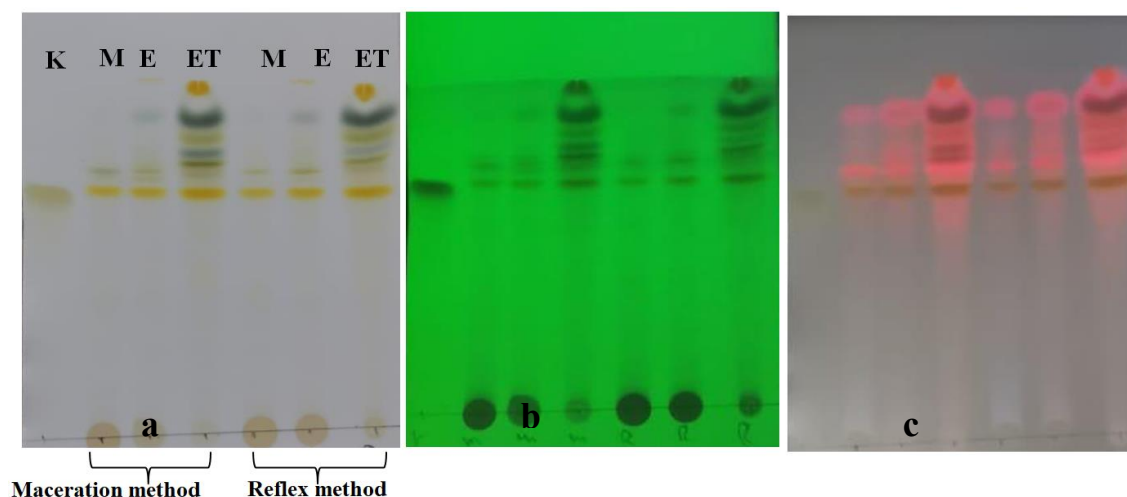
Reversed phase HPLC analysis was used to quantify single phenolic substances. A SYKAM HPLC chromatographic system with a UV detector was used, and the column used was -C18-OSD (25cm, 4.6mm) 30°C was the column temperature. Using eluent, A (methanol) and eluent B (1% formic acid in water (v.v-1)), the gradient elution method was carried out as follows: initial 0–5 min, 40% B; 6–15 min, 50% B; and flow-rate of 1.0 mL.min<sup>-1</sup>. Samples and standard were introduced at a volume of 100 µL each, effortlessly employing an autosampler. The spectra were obtained at 280 nanometers. By comparing the retention time of the crude extract to that of the standard solution, HPLC analysis was able to recognize kaempferol in combination with it. The calibration curve's final kaempferol concentration in the extracts was determined using an equation of linearity. FT-IR and TLC were used to identify the separated kaempferol.

## 3. Results

Phytochemical testing by using the ferric chloride and Shinoda tests, all of those available extracts- ethanol, ethyl acetate, and methanol-showed an existence of flavonoids [12].

### 3.1. Thin layer Chromatography analysis

Chromatography TLC for ethanol, ethyl acetate, and methanol extracts of the leaves of *P. Juliflora* are shown in Figure 1. ethyl acetate was determined to be the optimal extraction solvent showed the presence of different chemical compounds. Different mobile phases were used for separation such as *n*-hexane-ethyl acetate-methanol-water (0.8 : 0.9 : 1.2 : 1, V : V : V : V), ethyl acetate-methanol-water (8 : 2 : 1, V : V : V) [21] and toluene: diethyl ether: acetic acid (6 : 4 : 1, V : V : V). toluene: diethyl ether: acetic acid was the better separation mobile phase. The R<sub>f</sub> value in TLC with (toluene: diethyl ether: acetic acid system) was 0.6 for kaempferol marker, while (0.58- 0.62) for kaempferol spot identified in different extraction methods, respectively.



**Fig.1.** TLC chromatogram separation of kaempferol (**K**) in ethanol, ethyl acetate and methanol extracts of *P. Juliflora* leaves with toluene: diethyl ether: acetic acid (6:4:1 v.v-1) (**a**) real image; (**b**) UV image at 254 nm; and (**c**) UV image at 366 nm (**M**: methanol, **E**: ethanol, **ET**: ethyl acetate)

### 3.2. Extraction of kaempferol by various extraction techniques

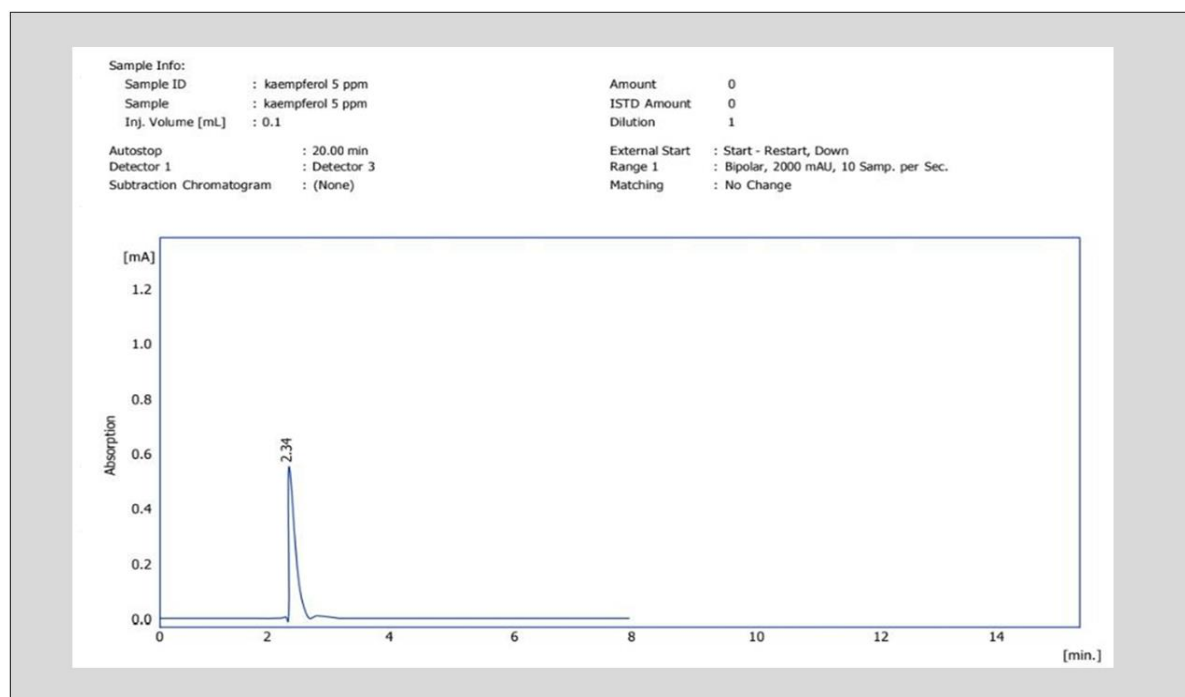
By applying methanol and eluent B (1% formic acid in water (v.v-1)), the HPLC technique was applied to the standard solution of kaempferol and the sample extracts. Reverse-phase HPLC chromatograms of crude extract and kaempferol standard solution are displayed in Figures 2–5. By comparing the retention times of the kaempferol flavonoid extracted from *P. Juliflora* L. to those of the reference standard, it was identified. In comparison to the retention time of standard kaempferol, which was 2.34 minutes, the retention time of the Kaempferol peak in various extracts was seen at (2.35-2.39) in the HPLC chromatogram.

To find the amount of flavonoids, a calibration curve for kaempferol was created using five dilutions of standard solution at concentrations ranging from 2 to 10 ppm. The linear regression equation that resulted was;

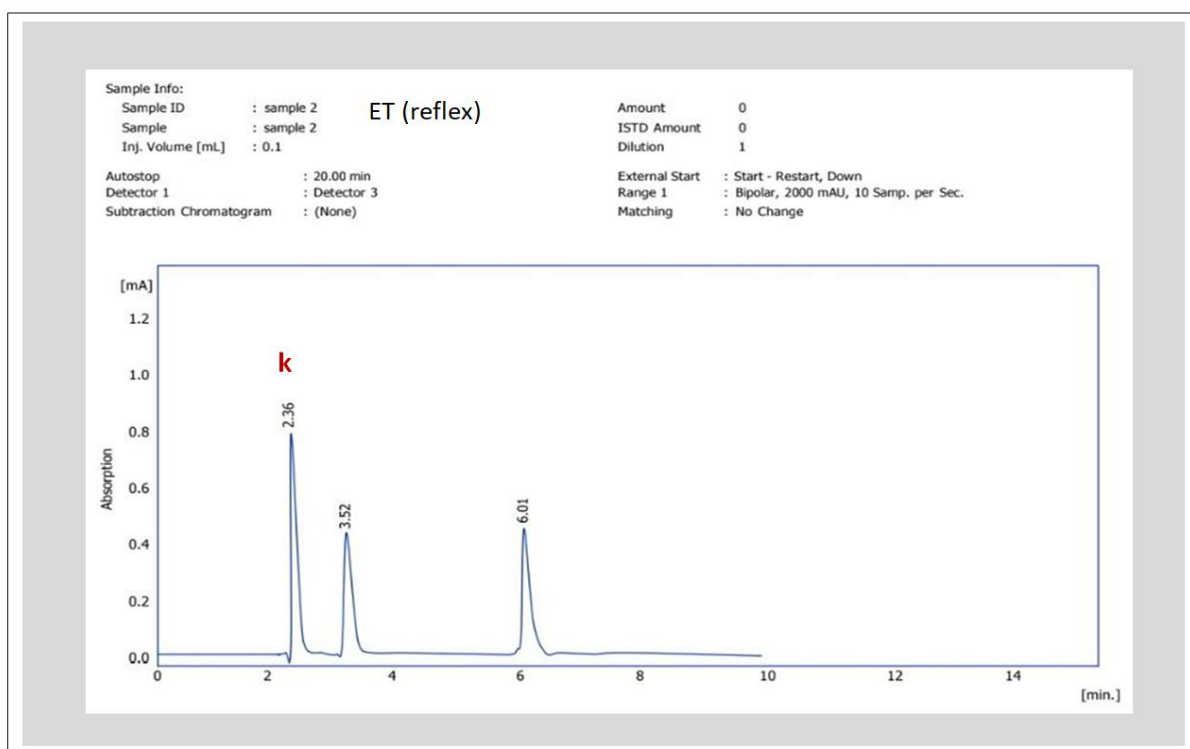
$$y=mx+c,$$

where x and y refer to the concentration and area under the curve, respectively Figure 6. regression equation and correlation coefficient were as follows:  $y=40.78182*x$ ;  $r^2=0.9998113$ .

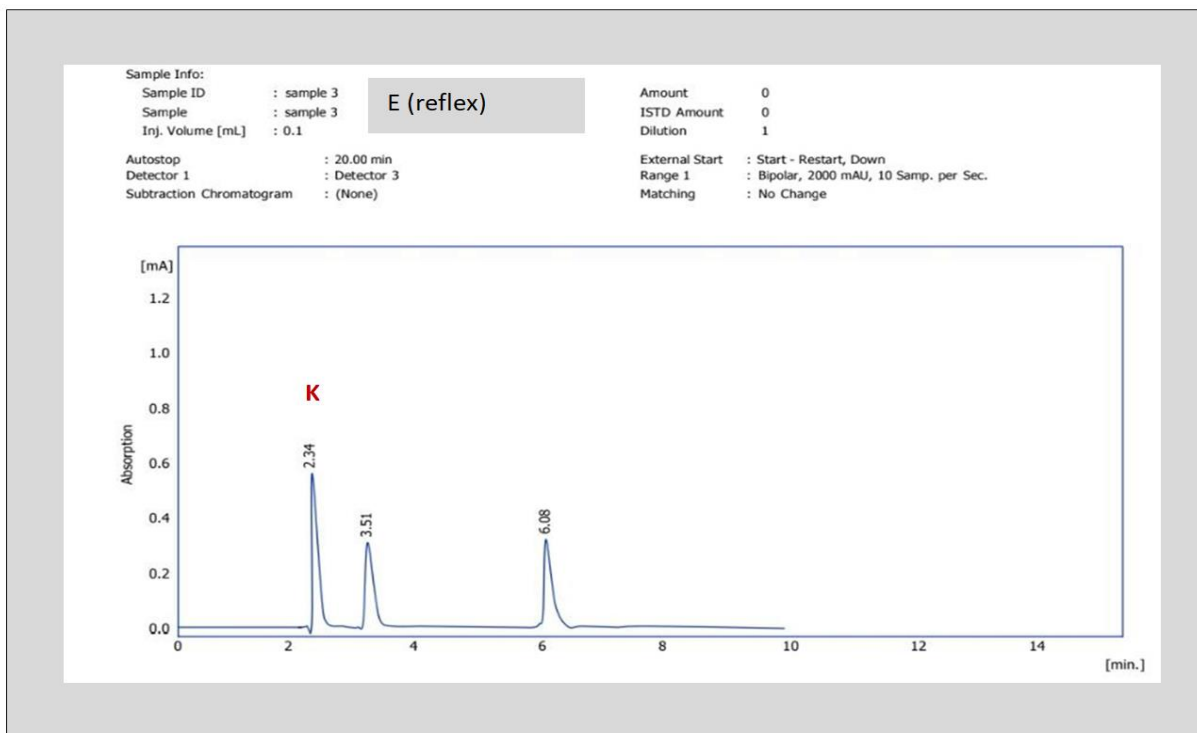
Table 1. displays the outcomes of the percentage extract produced in various solvents using different extraction techniques. As was to be anticipated, using various solvents and extraction methods produced varying extract yields. Reflex technique produced the maximum crude extraction yield of 1.66 grams with ethanol extract, while maceration produced the lowest yield of 0.5 grams with ethyl acetate extract. However, HPLC quantification of the various extracts showed that ethyl acetate was the most effective solvent for kaempferol extraction in the two distinct methods. The yield obtained by the isolated kaempferol from leaves of *P. Juliflora* was found to be 92.54 mg from 0.056 g ethyl acetate extract. Our study's results unmistakably showed that ethyl acetate is the best solvent for kaempferol extraction, and this conclusion is in line with the TLC's findings in Figure 1. The HPLC and TLC analysis and the  $R_f$  value of the isolated flavonoid was found to be 2.34 Figure 7a. exactly in similar with standard kaempferol.



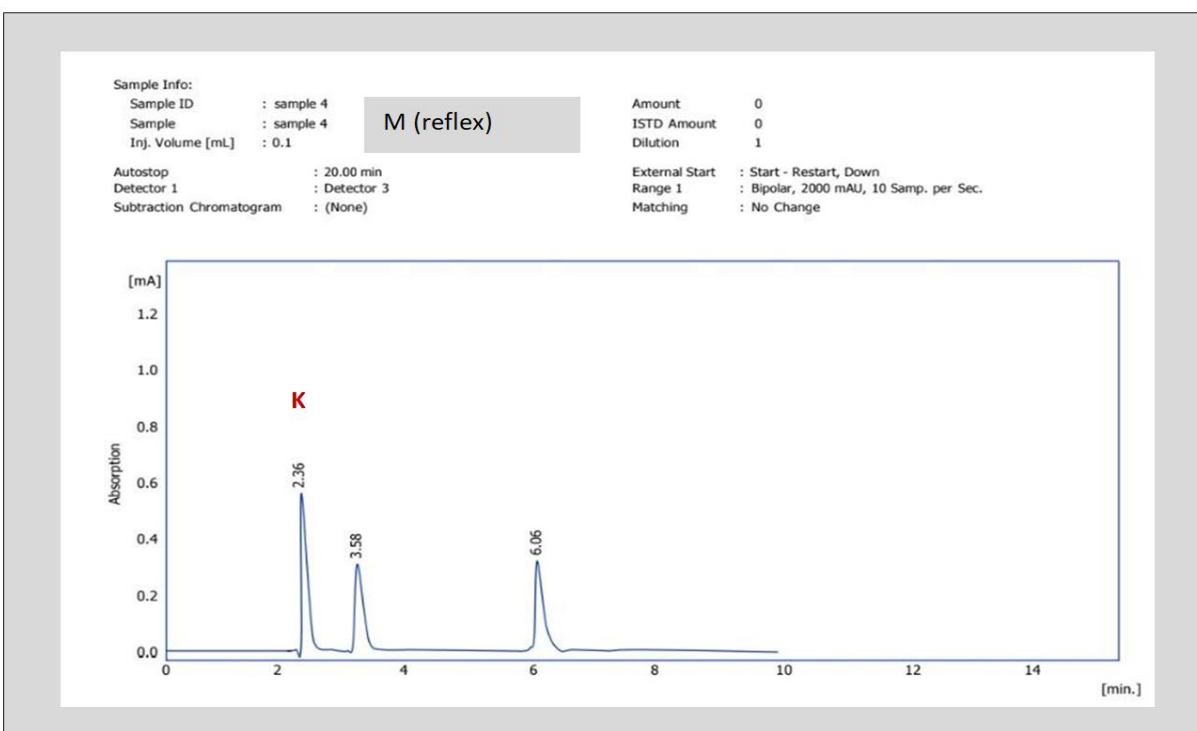
**Fig.2.** HPLC chromatogram of Kaempferol stander with retention time 2.34



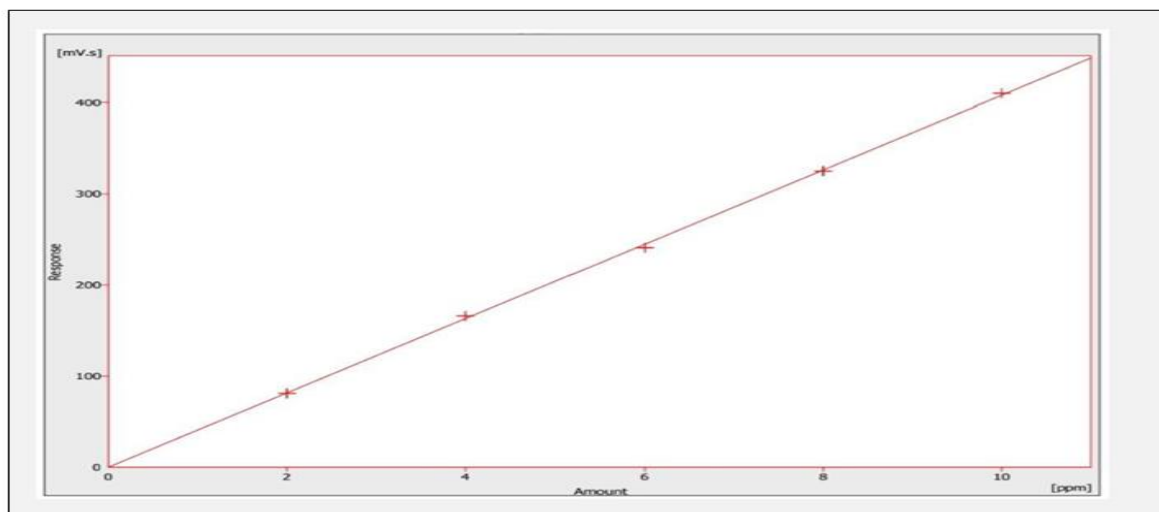
**Fig.3.** HPLC chromatogram of ethyl acetate of *P. Juliflora* leaf extract by reflux technique  
Kaempferol in extract with retention time 2.36



**Fig.4.** HPLC chromatogram of ethanol of *P. Juliflora* leaf extract by reflux technique. Kaempferol in extract with retention time 2.34



**Fig.5.** HPLC chromatogram of methanol of *P. Juliflora* leaf extract by reflux technique. Kaempferol in extract with retention time 2.36



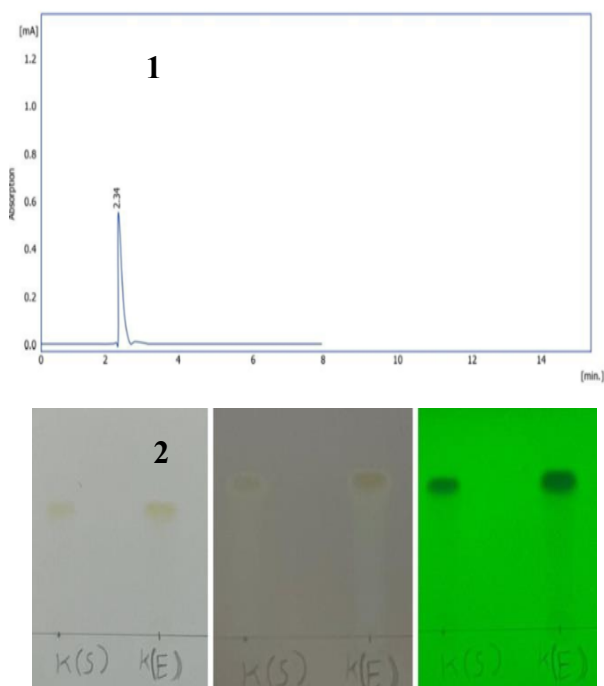
**Fig.6.** Calibration curve of the Kaempferol stander

$$\text{concentration of sample} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{conc. of standard} \times \text{dilution Factor}$$

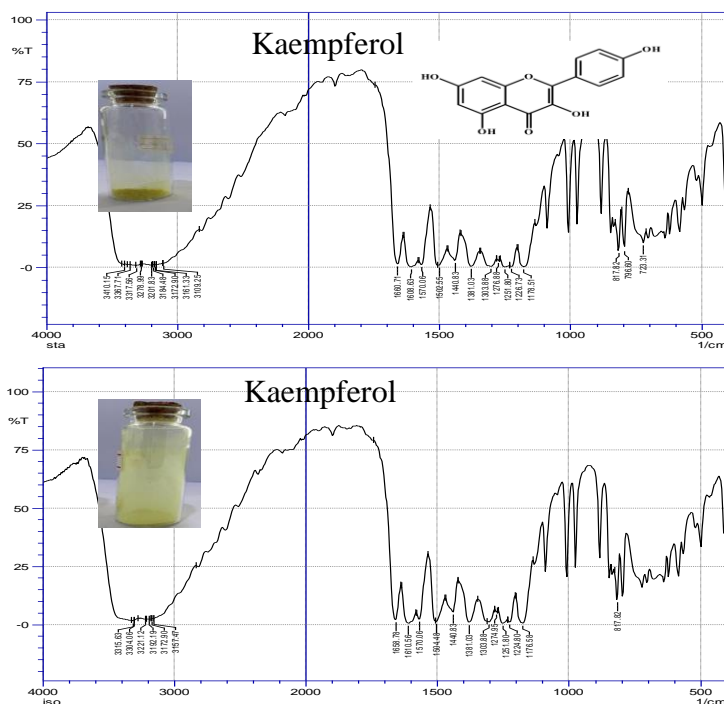
**Table 1.** kaempferol content in different extracts methods of *P. Juliflora* L.

Crude extracts	Extraction yield (g)	Kaempferol content (mg)
Maceration ethyl acetate	0.5	74.1
Maceration ethanol	1.59	39.98
Maceration methanol	1.65	40.81
Reflex ethyl acetate	0.67	92.54
Reflex ethanol	1.66	41.2
Reflex methanol	1.48	54.63

The FT-IR spectrum of isolated Kaempferol was recorded using (Shimadzu/84005) infrared spectrophotometer. In brief, previously dried samples (~2 mg) were weighed individually and transferred into agate mortar and pestle. The individually weighed samples were mixed uniformly with potassium bromide (KBr, FT-IR grade, ~200mg) to form a homogenous mixture. This mixture was compressed at a pressure of 10 Ton to obtain thin, transparent discs on a mini hand press (Model: MHP - 1, P/N - 200-66747-91, Shimadzu, Kyoto, Japan). The sample discs were scanned to obtain infrared spectra in the wavelength range of 4000 to 400  $\text{cm}^{-1}$  at a resolution of 4  $\text{cm}^{-1}$ . The obtained spectra were processed and analyzed using instrument software (IR solution FTIR control software, version 1.10) accompanying the instrument. as illustrated in Figure 8. A wide absorption peak was observed in the kaempferol spectrum at approximately 3304.06  $\text{cm}^{-1}$ , which was attributed to the stretching vibration of the hydroxyl groups. ketonic carbonyl group stretching vibrations was observed at 1658.78  $\text{cm}^{-1}$ . The other important peaks absorption at 1610.56, 1570.06 and 1440.38  $\text{cm}^{-1}$  were assigned to the aromatic C=C stretching vibrations. On the other hand, phenols' OH bending vibrations were detected at 1381.03  $\text{cm}^{-1}$ , indicating that the isolated substance is the flavonoid kaempferol. Regarding the molecular structure of kaempferol, such outcomes were in good accordance with the earlier literature [13].



**Fig.7.** (1) HPLC chromatogram of isolated Kaempferol with retention time (2.34) (2) TLC chromatogram of standard and isolated Kaempferol (KS: Kaempferol standard KE: Kaempferol isolate)



**Fig.8.** FT-IR spectrum of standard and isolated compound Kaempferol

#### 4. Discussion

Over the past several years, numerous innovative methods of extraction were created for the isolation of the biologically active elements of medicinal herbs. However, multiple investigations showed that the biological functions of the method of extraction used influenced the quality of the herbal extracts. Thus, this must be done for selecting an appropriate solvent and extraction method [14]. This research established the amount of Kaempferol in the leaves. The findings of flavonoids evaluation of leaves in various extracts of *Prosopis juliflora* revealed the presence of flavonoids. Before performing the HPLC examination, all extracts underwent chromatography TLC to determine the amount of kaempferol present. Thin-layer chromatography was proven to be a great instrument for the quick identification of plant material for chemotaxonomy [15]. Post-development derivatization of the TLC plate revealed the existence of flavonoids, which is consistent with Mehta, 2017 [16]. There are two methods for extraction of kaempferol. hot extraction method using reflex and cold extraction method using maceration. Hot solvent extraction methods were more effective approach for extracting flavonoids from plants than non-thermal methods because the increase in temperature provokes the solvent density to decrease, diminishing the solubility of interest compound. thus, temperature was the main parameter that influences selectivity and it was necessary to optimize it in order to increase yield. [17]. The Kaempferol compound was examined utilizing the identical HPLC condition. The spectral peak was measured by scanning the leaf extract samples at 280 nm wavelength. The  $R_f$  value of the peak obtained was compared with  $R_f$  value of standard Kaempferol ( $R_f = 0.234$  Fig.2). The Kaempferol content in ethyl acetate leaf extract was estimated as 92.54 mg /L. The content of Kaempferol in methanol leaf extract was 54.63 mg. mL<sup>-1</sup>, while with ethanol leaf extract 41.2 mg.L<sup>-1</sup> by reflex method. The ethyl acetate leaf extract exhibited an optimized quantity of Kaempferol compared to all other analyzed leaf extract of *P. juliflora*. The bands for isolated kaempferol from leaf extracts matched with Kaempferol standard compound (Fig.7), in both TLC and HPLC results. Furthermore, between the different methods used for kaempferol extraction and isolation, the reflex technique proved to be most successful. This agreed with [18] that found the ethyl acetate leaf extract exhibited an optimized quantity of Kaempferol compared to all other analyzed leaf extract of *Syzygium cumini*. The FT-IR spectrum of isolated Kaempferol compound was shown in (Fig.8) and their



corresponding characteristic peak positions were listed for Kaempferol standard demonstrated that the isolated compound was the flavonoid kaempferol. The present finding was in good accordance with the prior research on the molecular structure of Kaempferol [19]. For a very long time, spectroscopic methods, especially the FT-IR method, was widely used to analyze the nutritional content and composition of food as well as to predict the amount of phenolic compounds, mostly flavonoid composition, and total phenolic in fermented goods [20].

## 5. Conclusions

The extraction method and solvent influenced the separation and kaempferol yield of *Prosopis juliflora* leaf extract. The outcomes of the current study certainly will help the researchers in identifying the correct extraction method--ethyl acetate and reflux--and appropriate solvent for the purification of kaempferol from the *P. juliflora*. Kaempferol may also be used as an indicator in the evaluation and quality assurance of this drug leaves extract in the herbal industries due to the ease of use, effectiveness, sensibility, and rapidity of this HPLC method.

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## Confllict of Interest

All authors declare that there is no conflict of interest.

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## تقييم مقارن لطرق الاستخلاص وتقدير HPLC الكمي للكامبفيرول في أوراق *Prosopis juliflora* Linn. المتنامي في العراق.

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<sup>2</sup> قسم الكيمياء الصيدلانية، كلية الصيدلة، جامعة البصرة، البصرة، العراق.

ملخص	معلومات البحث
الكامبفيرول هو احد اهم الفلافونويدات الذي تم اكتشافه منذ فتره طويله لأغراضه العلاجية. وقد لوحظ أنه يمتلك العديد من الخصائص الصيدلانية. وقد تم استخدام كروماتوغرافيا سائلة عالية الأداء (HPLC) كطريقة تقييم كمي لتحديد أفضل مذيب استخلاص للحصول على الكامبفيرول من أوراق <i>Prosopis juliflora</i> . حيث انه في هذه الدراسة تم استخدام الإيثانول وخلات الإيثيل والميثانول كمذيبات في استخلاص فلافونويد الكامبفيرول من نبات <i>P. juliflora</i> باستخدام الطرق الشائعة للاستخلاص (الرفلكس، التنقيع). وقد أظهرت النتائج ان مستخلصات أوراق نبات <i>P. juliflora</i> تحوي مركب الكامبفيرول. من بين المذيبات الثلاثة المستخدمة، كان من الواضح أن مستخلص أوراق الإيثيل أسيتات أظهر أكبر كمية من الكامبفيرول (92.54 ملغم.ل-1). الاستنتاج: وفقا لنتائج HPLC، كان reflex هو الطريقة الأكثر كفاءة لاستخلاص الكامبفيرول من <i>P. juliflora</i> ، وكانت أسيتات الإيثيل هي أفضل مذيب. ونتيجة لذلك، يتم الحث على استخدام هذه الطريقة في الإنتاج والدراسات اللاحقة.	الاستلام 22 تشرين الأول 2024 المراجعة 12 كانون الأول 2024 القبول 17 كانون الأول 2024 النشر 31 كانون الأول 2024
	<b>الكلمات المفتاحية</b> كامبفيرول ، <i>Prosopis juliflora</i> ، تقنيات الاستخلاص HPLC.
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