



Chromatographic Detection and Quantification of Fisetin in Tomato Extract: A Comparative Solvent Study

Ahmed Abbas Almaliki ^{1*}, Rafeef Amer Abdul-Jabar ², Ahmed Najim Abood ³

¹ Iraqi Ministry of Health, Basrah Health Directory, Basrah, Iraq

² Dept. of Clinical and Laboratory Sciences, College of Pharmacy, University of Basrah, Basrah, Iraq

³ Dept. of Pharmaceutics, College of Pharmacy, University of Basrah, Basrah, Iraq.

*Corresponding Author

Ahmed Abbas Almaliki

Abstract: -

Background: Flavonoids are natural bioactive constituents in many plants. Fisetin (3,3',4',7-tetrahydroxyflavone), is a known key flavonoid found naturally in tomatoes, it has gathered interest worldwide for its potential prevention of chronic diseases.

Aim: Using simple and low-cost methods for the extraction, identification, and measurement of the concentration of naturally occurring fisetin in tomatoes.

Method: Two solvent mixtures were used for the extraction of fisetin, methanol/water (80:20) v/v and ethanol/water (50:50) v/v, followed by the measurement the total contents of phenols and flavonoids, in addition to the analysis of fisetin via thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC).

Results: The contents in the methanol extract (ME) and ethanol extract (EE) were 5.15g and 2.65g, respectively. Although there was no significant difference ($p > 0.05$) in total phenolic content between the two extracts, total flavonoid content was found to be significantly increased in EE ($p \leq 0.05$). The presence of fisetin in the sample was confirmed by TLC, while HPLC showed higher values in EE (4.40 mg/g) than in ME (3.18 mg/g).

Conclusion: Ethanol is a more effective solvent for fisetin extraction, providing a simple and low-cost approach for recovering a good amount of fisetin from tomato.

Keywords: Extraction, Fisetin, Flavonoids, HPLC, TLC, Tomato.

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Introduction

Naturally bioactive secondary metabolites, such as flavonoids, are substantially responsible for the health benefits in plants, which have long been essential sources of medicine. These naturally occurring polyphenolic chemicals are extensively dispersed and support human health in addition to plant defense and coloration [1,2]. A crop of global importance, tomatoes (*Solanum lycopersicum*) are especially abundant in phytochemicals such as flavonoids, carotenoids, and vitamins. Crucially, it has been found that around 98% of the fruit's total polyphenolic content is present in the tomato peels, which makes them a useful part of the tomato for study on functional foods and even bioactive materials [3]. A lot of peel (which is a plentiful, affordable, and sustainable raw material for the recovery of bioactive compounds) is produced in the processing of tomatoes to make juices, sauces, and pastes. This is particularly significant when discussing the important value of food waste [4]. Recent research in the literature suggests that the antioxidant, anti-inflammatory, antibacterial, and possibly anticancer properties of flavonoids derived from tomato peels may give them the ability to treat some chronic diseases and increase nutritional value [5-7].

Fisetin (3,3',4',7-tetrahydroxyflavone), a bioactive flavonoid family member, is found naturally in various fruits and vegetables, including tomatoes. Like other flavonoids, it has strong antioxidant, anti-inflammatory, neuroprotective, and anticancer characteristics. Furthermore, fisetin is defined by a C6–C3–C6 chain and numerous (OH) atoms, and has gained growing interest in its potential as a treatment [8]. In addition, fisetin has recently gained global interest for the potential ability to prevent chronic diseases such as cancer, some neurological diseases, and a possible positive effect on metabolic syndromes. However, due to its quite low natural abundance and the complexity of phytochemical combinations, the process of its extraction and quantification from plants may need strong analytical procedures [3,8].

According to previous studies, many identification and extraction methods for the isolation of flavonoids from plant matrices have been employed, these may include solvent extraction, ultrasonic-assisted extraction, and supercritical fluid extraction. These methods are often followed by several purification steps, such as column chromatography and solid-phase extraction [9-11]. Although a wide range of polyphenolic compounds can be recovered by these approaches, likely, they require multiple additional steps for purification, the addition of large volumes of organic solvents, and processing times are often long, which limit their efficiency and reproducibility [12,13]. Among the developed methods for active plant compounds isolation and identification, high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) have emerged as simpler, very reliable, and precise techniques for the separation and quantification of flavonoids, including fisetin [14,15].

The chromatographic method (HPLC) is important for the extraction, separation, and quantification of fisetin, which is frequently subjected to multi-step purification techniques [15]. Similar to HPLC, TLC is a useful, less resource-intensive technique that is mainly used to compare and track fractions obtained during initial purification steps such as primary silica-gel column chromatography. By evaluating their composition, TLC helps to guide the overall extraction and purification procedure of many important plant compounds specially the flavonoids [15]. Even though tomatoes are known to contain flavonoids [16], there is still little specific studies on how to extract, separate, and measure fisetin from tomatoes. In order to provide both qualitative and quantitative insights, the current study aims to extract fisetin from tomato and detect it using TLC and HPLC techniques.

Materials and Methods

Tomato Powder Preparation

Tomatoes were washed, sliced, de-seeded, and dried naturally in the dark at room temperature. Then, the dry pieces were ground to form a fine powder. This powder was subjected to defatting by the maceration process with hexane overnight. After that, the previously macerated powder was collected and air-dried for 48 hrs. The resulting powder (defatted) was the one used in this study for further extractions [17].

Crude Extraction Preparation

Two separate samples, weighing 10 g each, of the defatted powder were used for extraction with two different solvent systems by the maceration process. First, methanol extract (ME): the first sample was macerated with a methanol: water (80:20, v/v) mixture. Second, ethanol extract (EE): the second sample was macerated with an ethanol: water (50:50, v/v) mixture, and the maceration extraction process was carried out over three days. Following extraction, the mixtures were filtered, and the resulting materials were allowed to air dry at room temperature in the dark by using petri dishes. This step yielded the crude extracts of ME and EE [15].

Liquid-Liquid Extraction

For further extraction and purification of the less polar flavonoids, each crude sample was dissolved in a small amount of water first. Then, each aqueous solution was mixed with 1:1 ratio (v/v) of ethyl acetate using a separator funnel. The mixture was then strongly and repeatedly shaken and allowed to separate into two layers. The ethyl acetate organic layer, containing less-polar compounds, such as fisetin, was collected and dried at room temperature to gain the final samples for analysis [15].

Qualitative Analysis of Phytochemical Constituents

An initial qualitative analysis of phytochemicals was conducted on the ethyl acetate fractions of the ME and EE to establish the presence of key secondary metabolites (polyphenols and flavonoids). The tests were carried out as in (Table 1).

Table 1. Qualitative screening methods for flavonoids and polyphenolic compounds [18]

Secondary Metabolite	Test Performed	Procedure and Indication
Flavonoids	Shinoda test (magnesium-hydrochloric acid reduction)	A small amount of the extract was dissolved in ethanol, and a few pieces of magnesium ribbon were added, followed by the addition of concentrated hydrochloric acid.
Flavonoids	Lead acetate test	The extract solution was treated with lead acetate solution of 10%.
Polyphenols/Tannins	Ferric chloride test	A solution of the extract was treated with a few drops of 5% ferric chloride solution.

The Quantitative Analysis of Phenolic Compounds in Tomato Extract

The Folin-Ciocalteu (FC) colorimetric assay was used in this study for the determination of the total phenolic content (TPC) of the ME and EE. First, working solutions of both ME and EE were prepared at a concentration of 100 mg/mL. 1 mL of the sample was mixed with 1 mL of FC's reagent. After 3 min of reaction time, saturated sodium carbonate was added (1mL), and the solution volume was adjusted to 10 mL with the addition of distilled water. Then, the solution mixture was incubated for 90 minutes in the dark. Furthermore, standard solutions of gallic acid (GA) were prepared in different gradual concentrations ranging from 0.01-2.00 mg/mL. For calibration curve creation. The absorbance of all prepared solutions was measured against the blank using a UV-Vis spectrophotometer at 765 nm. Results were expressed as GA equivalents (GAE) per 1 g of dry extract [19].

The Quantitative Analysis of Flavonoid Content in Tomato Extract

The total flavonoid content (TFC) of the ME and EE samples was determined using a modified sodium nitrite-aluminum chloride method [19]. Solutions of both ME and EE were prepared at a concentration of 2 mg/mL. First, 0.5 mL of the sample extract is mixed with 0.3 mL of 5% sodium nitrite solution. After 5 min, 0.3 mL of 10% aluminum chloride was added to the mixture. Following that, by 6 min, 2 mL of 1 M sodium hydroxide was also added to the solution. Then, the volume was made up to 5 mL with distilled water. Quercetin was used as a standard for calibration by preparing various concentrations (0.05-2) mg/mL. The absorbance of all prepared solutions was measured against the blank with a UV-Vis spectrophotometer at 510 nm. The TFC was expressed as mg of quercetin equivalent (QE) per 1 g of extract.

Thin-Layer Chromatography (TLC)

TLC was conducted in this study to qualitatively confirm the existence of fisetin in the ME and EE by comparing its retention factor (R_f) to the R_f of a known pure fisetin standard.

1. Preparation of Stationary Phase

The sample separation was conducted on a plate consisting of Silica Gel 60 F₂₅₄ with dimensions of 20x20 cm. To ensure an optimum separation and activity of the plate, it was pre-activated by first drying, then heated in an oven at 120 °C for 20 minutes, then allowed to cool down prior to use. Finally, an accurate baseline for samples and a solvent front line was drawn on the activated plate [20].

2. Preparation of Mobile Phase

The mobile phase system was adjusted specifically for the detection of fisetin, consisting of a four-component mixture of solvents, ethyl acetate: methanol: water: toluene in a volumetric ratio (10:1.5:1.3:0.2). This solvent system was moved into the TLC developing chamber for saturation with its vapors before the plate was immersed in it [21].

3. Sample Application

Four separate samples (2 of ME and 2 of EE) were carefully drawn into the baseline by using a capillary tube. Furthermore, the fisetin standard (2 mg/mL) was also applied to the samples for comparison. After application, the plate was left for a few minutes to ensure the evaporation of the spotting solvent completely before beginning the separation process [20].

4. Sample Detection

Following development, the plates were removed from the TLC chamber, air-dried completely, and the separated spots of sample and standard were visualized under UV light at 254 nm. The R_f for the resolved spots was calculated as described by Pandey et al. [22], using the formula [21]:

$$R_f = \frac{\text{the distance traveled by the compound spot}}{\text{distance traveled by the solvent}}$$

Quantitative Determination by High-Performance Liquid Chromatography (HPLC)

The concentration of fisetin in the prepared tomato extracts was accurately quantified using a SYKAM Liquid Chromatograph (Germany) by following the manufacturer's guidelines.

1. Instrumentation and Column

In this analysis, The Chromatograph was equipped with a sample injector, quaternary gradient pump, a column oven, and a UV-Vis detector. The process of compound separation from the prepared solutions was completed on a C-18 ODS reverse-phase column (Nucleodur 250 x 4.6 mm i.d., 5 µm, Macherey Nagel) [23].

2. Standard and Sample Preparation

Before analysis, the standard solution of fisetin was prepared at a concentration of 1 mg/L. The analytical run (RP-HPLC) included the injection of the fisetin standard for the detection of its retention time (RT). Then, extracted samples were injected for quantification. The injection volume used for all samples was 20 µl [23].

3. Chromatographic Conditions

The isocratic elution method (the mobile phase should remain fixed throughout the entire separation process) was used for the separation of fisetin under the following optimal conditions: mobile phase: consisted of a solution mixture of acetonitrile (A) and 0.2 M orthophosphoric acid (B) in a fixed 30:70 (v/v) ratio, flow rate: constant at 1 mL/min, detection wavelength: fisetin was detected and quantified at 362 nm, and column oven temperature 30 °C [23].

4. Quantification

Fisetin's initial identification in the crude extract was confirmed by the comparison of its RT to that of a pure, known fisetin standard. After the identification, the concentration of fisetin was quantitatively measured by a calibration curve (plotting the known pure fisetin concentration range (5–1000 µg/mL) against the resulting peaks detected after HPLC analysis). The graph was then used directly to find the unknown fisetin concentration present in both EE and ME [23].

Statistical Analysis

Data in this research are presented as the mean. Student T-tests were conducted by utilizing the SPSS program to assess the results from the quantitative analysis of TPC and TFC. Differences were considered statistically significant at a p-value that is ≤ 0.05 .

Results

Crude Extraction Yields

The yield extracted from 10g tomato powder using the two hydro-organic solvent systems of the ME and EE was expressed as dry mass weight. Weights and their corresponding percentages are expressed in (Table 2). The ME has resulted in a higher yield (5.15 g) when compared to the EE (2.56 g).

Table 2. Summary of extracts, their dry mass weights, and percentages

Extract Name	Dry Mass weight	Percentage
Methanol Extract (ME)	5.15 g	51.5%
Ethanol Extract (EE)	2.65 g	26.5%

Qualitative Analysis of Phytochemicals

The preliminary qualitative screening was performed on the methanol and ethanol extracts in order to confirm the general class that fisetin belongs to. The results are presented in (Table 3).

Table 3. Qualitative Phytochemical Analysis of Tomato Extracts

Test Name	Observations
Shinoda Test	+++ (pink color)
Lead Acetate Test	++ (Yellow precipitate)
Ferric Chloride Test	+++ (blockish-green color)

(+++)= abundant, (++) = trace presence

Quantitative Determination of Total Phenolic Compounds

In the ME, the TPC was estimated by the calibration equation derived from the GA standard and it was estimated to 1.5467 mg GAE/g, while the TPC in the EE was 1.9333 mg GAE/g. Statistical analysis showed no significant difference ($p > 0.05$) between the extracts.

Quantitative Analysis of Total Flavonoid Content

In contrast, the TFC showed a clear difference between extracts. The ME recorded 13.9233 mg QE/g, whereas the EE resulted in 47.6867 mg QE/g, indicating a significant difference ($p < 0.05$), with the ethanol extract showing the higher flavonoid concentration. The total phenolic and flavonoid contents of the ME and EE are summarized below in (Table 4).

Table 4. Quantitative summary of total phenolic and flavonoid contents in methanol and ethanol extracts

Sample	Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mg QE/g)
Methanol Extract (ME)	1.5467	13.9233
Ethanol Extract (EE)	1.9333	47.6867

Qualitative Analysis by Thin-Layer Chromatography.

TLC analysis confirmed the presence of the fisetin bands in both extracts with nearly the same retention factors for the extracts and standard fisetin. The results are summarized in (Table 5).

Table 5. Thin-layer Chromatography Retention Factor (Rf) Comparison of Fisetin Standard and Tomato Extracts MF and EF

Sample	Retention Factor (Rf) Value
Fisetin Standard	0.87
Methanol Extract (MF)	0.889
Ethanol Extract (EF)	0.889

Both the MF and the EF analysis resulted in a prominent component with a 0.889 Rf value that was highly comparable to the Rf value of the fisetin standard used in this study (0.87), therefore, there was a confirmation of the presence of fisetin in these tomato extracts. As demonstrated in (Fig. 1).

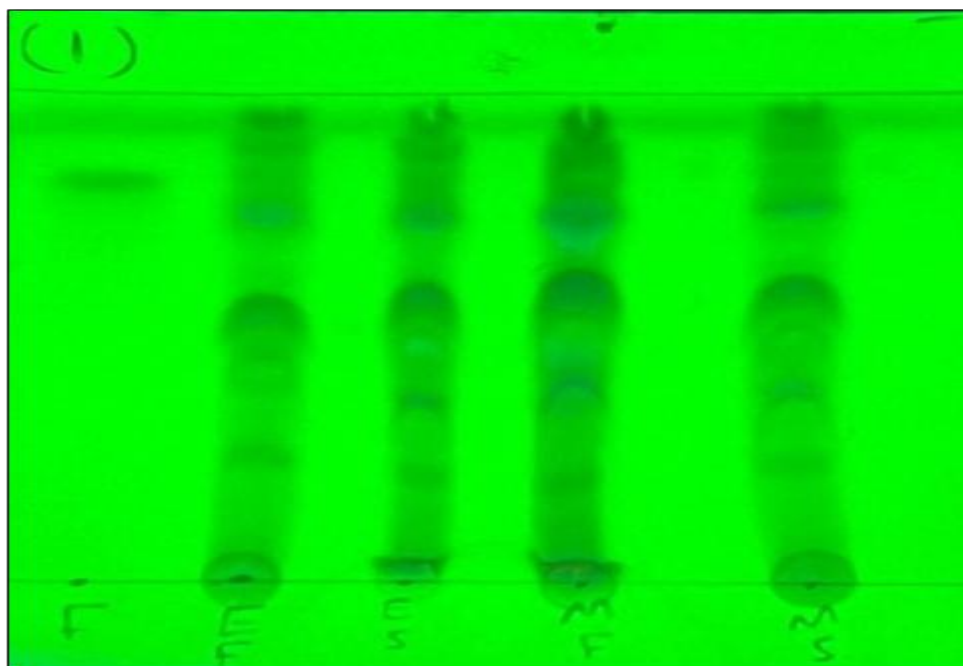


Fig. 1. Thin Layer Chromatography Profile of Fisetin Standard and other Tomato Extracts. F, fisetin standard; EF, ethanol extract; ES, ethanol extract (2nd sample); MF, methanol extract; MS, methanol extract (2nd sample)

Quantitative Analysis of High-Performance Liquid Chromatography

The quantitative HPLC test was used in this study to determine the fisetin content in the ethyl acetate fractions of the tomato extracts.

Fisetin was identified by its characteristic RT and further quantified by its peak area using the specified detection wavelength of 362 nm. The pure fisetin standard Rt was registered at (5.08

minutes), as illustrated in (Fig. 2). The watery methanolic fisetin (MF) showed a peak nearly matching RT to that of standard fisetin at (5.05 minutes), as shown in (Fig. 3). This confirmed the presence of the fisetin compound. Similarly, the watery ethanolic fisetin (EF) displayed a peak exactly matching the Rt of the fisetin standard (5.08 minutes), therefore, validating the presence of fisetin in both extracts, as shown in (Fig. 4).

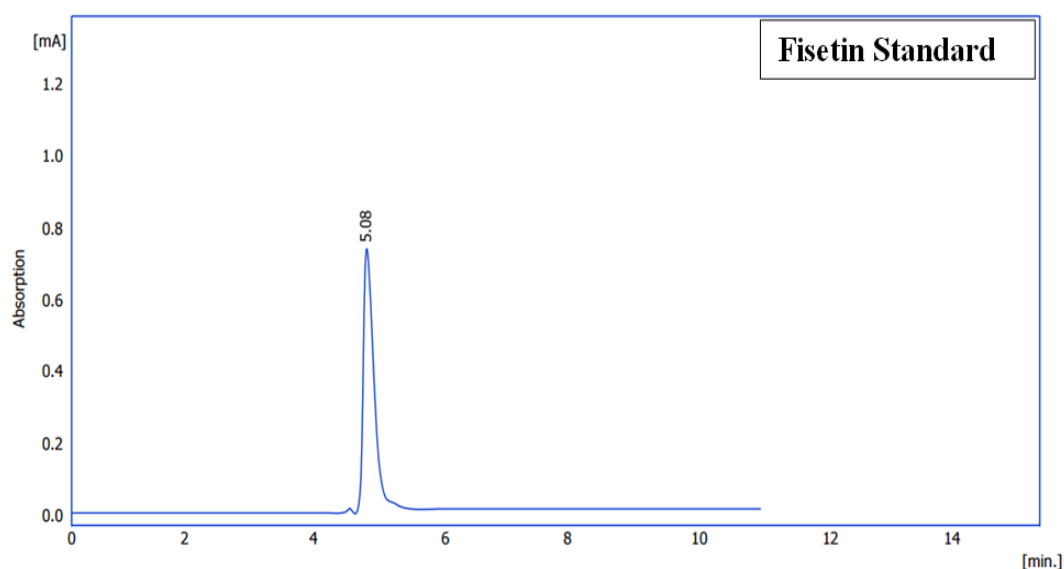


Fig. 2. HPLC Spectrum of fisetin standard. The detection was monitored at 362 nm

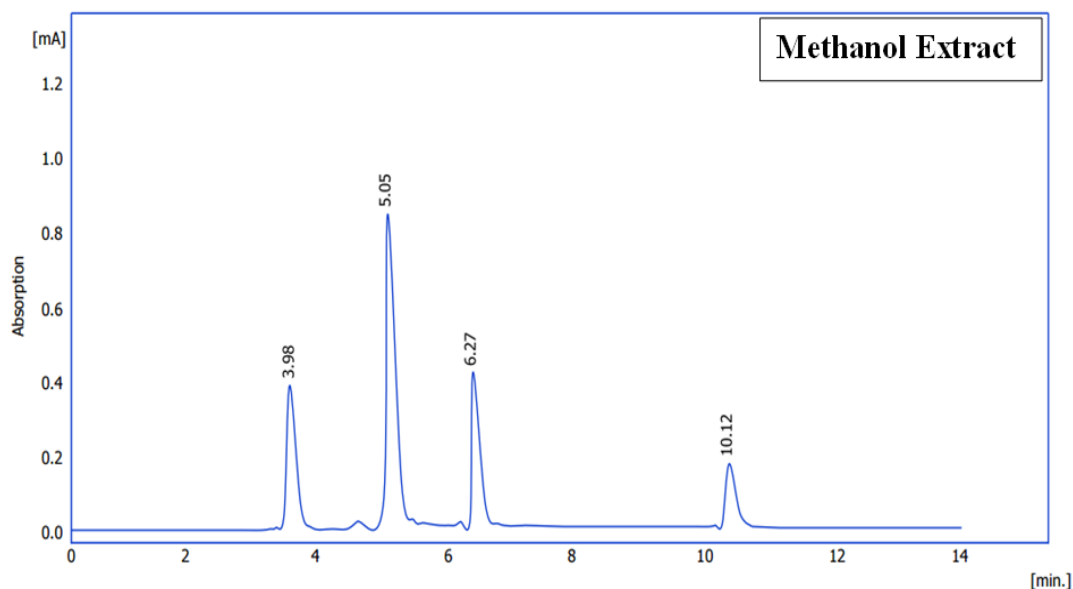


Fig. 3. HPLC spectrum of methanol extract, the detection was monitored at 362 nm

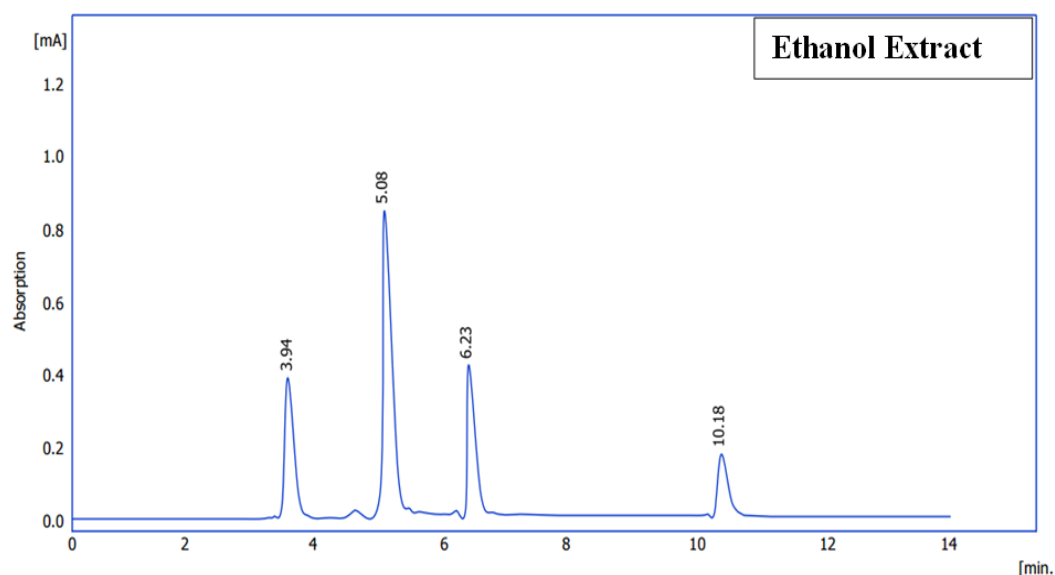


Fig. 4. HPLC Spectrum of Ethanol Extract. The detection was monitored at 362 nm

Fisetin Concentration

The fisetin quantification using HPLC was based on a calibration curve prepared from different concentrations of the pure standard fisetin solutions. The concentrations range (5 to 1000 $\mu\text{g/mL}$) were used to attain their respective peak area (mAU), as illustrated in (Table 6). The calibration curve was constructed based on this data, as shown in (Fig. 5), which assisted in the calculation of fisetin concentrations in the unknown extracted samples. A strong linear correlation was demonstrated between concentration and peak area in the constructed calibration curve, which confirms the accuracy of the used quantification method in this study. Based on this analysis, the EF contained a higher fisetin concentration of 4.40 mg/g of dry extract, whereas the MF showed a slightly lower concentration of 3.18 mg/g.

Table 6. Concentrations and peak areas used in the calibration curve of fisetin

Fisetin Concentration ($\mu\text{g/mL}$)	Peak Area (mAU)
1000	25364.4
500	13343.193
100	2170.674
50	905.333
10	220.613
5	74.317

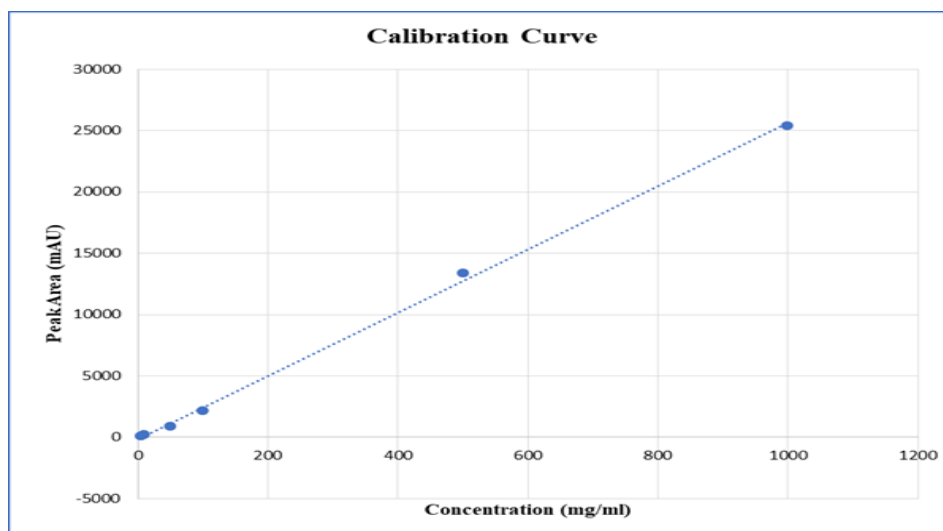


Fig. 5. Standard calibration curve of fisetin

Discussion

Throughout history, natural plants have been an important source of many bioactive compounds, which were used worldwide as medication. The identification and extraction of such plant constituents have been instrumental in the discovery of novel pharmaceutical resources [24]. Fisetin is a naturally occurring flavonoid that is normally found in various vegetables and fruits, due to its outstanding biological activities, it has attracted significant research interest globally due to possessing range of health benefits [15]. The process of extracting phenolic compounds from plants is usually challenging because many factors can affect the amount of the final yield and the sample integrity. The amount of the extract is dependent on many factors, such as the solvent of extraction, time duration, heat application, the utilized methods, level of maturity of the plant used, etc. [25]. To avoid any obstacle during this research, and to ensure maximum, unharmed composition, fresh tomatoes were utilized, and high heat was avoided during processing.

The selection of a solvent system with the optimal extraction efficiency is critical in this study to maximize the yield of the chemically diverse group of polyphenols [26]. Organic solvents, including methanol and ethanol, and their aqueous mixtures, are used in many studies for the extraction of plants. Their high polarity allows them to solubilize many compounds, including flavonoids [27]. Furthermore, water molecules has the ability to enhance polyphenols diffusion through plant tissues, and the swelling impact of water on plant tissues increases the surface area required for interaction, which leads to efficient extraction in the used aqueous solutions [2]. Accordingly, this study used an aqueous-organic mixture of solutions for extraction. Two hydro-organic systems were used methanol in water (80:20, v/v) and ethanol in water (50:50, v/v) to compare their extracting efficacy. Although both alcohols are considered polar, nevertheless, methanol is slightly more polar (0.762) than ethanol (0.654) [9,27]. This slight difference in polarity might lead to differences in extraction efficacy for a specific compound [26].

In this study, fisetin was successfully extracted from tomato powder using room-temperature maceration with methanol and

ethanol solvent systems. Since maceration is a mild technique of extraction that operates without applying heat, it minimizes the damage of thermal degradation for sensitive flavonoids such as fisetin [28]. Utilizing ethyl acetate (organic solvent) for LLE was highly effective in fisetin extraction because the latter is an aglycone flavonoid, and highly alkylated aglycones show a preference for organic solvents [16,29]. Furthermore, a previous work by Kim Huh, supports our method, which demonstrated that ethyl acetate has the highest ability to recover less-polar flavonoids among various solvent fractions tested [15]. The tomato TPC analysis demonstrated no statistically significant difference between the ME and the EE in this research. The lack of a statistically significant difference ($p > 0.05$) in TPC between the used aqueous extracts can be mainly explained by the polarity resemblance of the aqueous systems. Methanol and ethanol are polar organic solvents that are used for the extraction of polyphenols, which are themselves polar because of their hydroxylated aromatic chemistry [30,31]. While the polarity of pure methanol is originally slightly higher than that of pure ethanol, the addition of water as cosolvent decreases this marginal difference, enhances their polarity, and facilitates the extraction of molecules that are soluble in both components [27,32]. Therefore, the overall extraction efficiency for the general pool of phenolics becomes statistically comparable.

A previous study by Boonkasem et al., in which a comparative analysis of TPC from different tomato varieties showed that hydro-alcoholic forms of methanol and ethanol produced similar extraction efficiencies [33]. These findings support the present results. Furthermore, the findings of another previous study by Sultana et al. [30], are consistent with our current results, showing that the use of methanol and ethanol in their aqueous forms did not result in significant values in TPC for several plant materials. In contrast, TFC showed a significant increase in the EE ($p \leq 0.05$) when compared to ME. The current finding is in agreement with results published in a study by Mohammed et al. [26]. This outcome can be clarified by the selective solubility of flavonoid subclasses. Flavonoids in nature can widely vary in their polarity depending on the presence or absence of glycoside groups [34]. The moderate polarity of ethanol makes it more effective in the solubilization of the less-polar aglycones and glycosides content in

flavonoid, while methanol tends to solubilize more polar phenolic acids and lower-molecular-weight compounds. Therefore, the current study concludes that ethanol shows better flavonoid selectivity for extraction, leading to a higher TFC. The TLC test successfully confirmed the existence of fisetin in both extracts. The fisetin Rf values in both the methanol (0.889) and ethanol (0.889) extracts were almost identical to the Rf of the fisetin standard (0.87). The Rf value of a compound under specific conditions serves as a valuable aid to identity [35]. This approach is consistent with other research that used TLC to confirm fisetin's presence in various plants [15]. To the best of our knowledge, this is the first report on the detection of fisetin using TLC in tomato extracts. In the present study, fisetin was successfully identified in both methanol and ethanol extracts through comparison of RT with that of the pre-prepared standard fisetin. The standard exhibited an RT of 5.08 minutes, while the MF and EF showed peaks at 5.05 and 5.08 minutes, respectively. The near-identical RT values of extracts confirmed the presence of fisetin in both of them.

The quantification of fisetin was based on a calibration curve, which was derived from standard pure fisetin solutions. It demonstrated a strong linear correlation between concentration and peak area, which confirmed the accuracy of the analytical method. The EF resulted in a higher fisetin concentration (4.40 mg/g) when compared to the MF (3.18 mg/g). This difference can be explained by the principle of "like dissolves like", when the target compound's polarity is close to the solvent, it tends to extract it more efficiently [2]. Fisetin is moderately polar with poor aqueous solubility (<1 mg/mL); therefore, it is more soluble in aqueous ethanol, which has an optimal balance regarding polarity and formation of hydrophobic interactions [36,37]. Tissue swelling caused by ethanol could facilitate better solvent penetration, therefore enhancing the crossing of fisetin from plant cells into the solvent [2,31]. Several studies, such as those by Sultana et al. [30] and Do et al. [32], also demonstrated that ethanol-based systems generally yield higher flavonoid concentrations compared to methanol, supporting the findings of this study.

Conclusion

In this study, simple and efficient extraction methods were successfully applied to obtain fisetin from tomato powder, showing that a good amount of yield of this important flavonoid can be obtained from a small and inexpensive plant source, tomato. Fisetin presence in both ME and EE were confirmed qualitatively through TLC and quantitatively by HPLC, showing higher amounts in ethanol.

Conflicts of Interest

The authors declare that they have no conflicts of interest related to this study.

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Authors' contributions

Conceptualization: Rafeef A. Abdul-Jabar, methodology: Rafeef A. Abdul-Jabar, software: Ahmed N. Abood, validation: Rafeef A. Abdul-Jabar & Ahmed N. Abood, formal analysis: Rafeef A. Abdul-Jabar, investigation: Ahmed Abbas Almaliki, resources: Ahmed Abbas Almaliki, data curation: Ahmed Abbas Almaliki, project administration: Rafeef A. Abdul-Jabar & Ahmed N. Abood,

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