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# Validated HPLC Method for Quantitative Analysis of Gallic Acid and Rutin in Leaves of Moringa Oleifera Grown in Egypt

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**Abstract**

*Moringa oleifera* is one of miracle tree and great interested medicinal plant which belongs to Moringaceae. It is widely used as a nutritive herb and possesses valuable pharmacological activities. In our study a rapid, sensitive and accurate HPLC method for qualitative and quantitative determination of gallic acid and rutin in two different solvent methanol and ethanol, extracts of *Moringa oleifera* leaves cultivated in Egypt was investigated. The separation was performed and validated by isocratic method utilizing C18 column with a mobile phase 10% (v/v) acetonitrile and 1.0% aqueous acetic acid mixture for gallic acid and 60% (v/v) methanol and 1.0% aqueous acetic acid mixture for rutin. Those bioactive components, gallic acid and rutin, were detected at 280 and 335 nm, respectively. The calibration graph was found to be linear in the concentration ranges 5.0-40.0 and 5.0-60.0 µg/mL for gallic acid and rutin, respectively. The higher concentrations of gallic acid were observed in the 50% methanolic and ethanolic extracts, while the higher concentrations of rutin were observed in the 100% methanolic and ethanolic extracts. Moreover, a successfully spectrophotometric quantification of the total phenolics and flavonoids content in the plant extract was achieved, where the equivalent amount to gallic acid in methanolic and ethanolic extracts and the equivalent amount to rutin in methanolic and ethanolic extracts were calculated.

**Key words:** *Moringa oleifera, Gallic acid, Rutin, HPLC*

## Introduction

Medicinal plants have been a very important source of drugs for majority of the world population due to their therapeutic potential which used against diverse diseases [1]. Moringaceae which known as "Drumstick tree" is a single genus family with fourteen known species of these, *Moringa oleifera* Lam is the most widely known and utilized species [2]. *Moringa oleifera* is a highly valued plant, distributed in many countries of the tropics and subtropics, mainly in India, tropical Africa, Malaysia and Mexico [3]. Different parts of this plant contain a profile of various important minerals and are a good source of proteins, vitamin A, C and E, β-carotene, and various poly phenolics [4]. The leaves are rich source of essential amino acids such as methionine, cystine, tryptophan, and lysine with a high content of proteins [5], so they are consumed as vegetables and are preferred to be eaten as salad greens, cooked, and used in soup and sauce. Also their extracts can be used against bacterial or fungal skin complaints [6]. Such plants are capable to display different extent of antioxidant activities owing to the presence of varied amount of free phenolic and flavonol contents [7]. The most active phenolic components in leaves of *Moringa oleifera* are gallic and chlorogenic acids which are used as anti-fungal, anti-viral and antioxidant. This may help in protecting our cells against oxidative damage [8]. Also leaves are rich of flavonoids such as rutin, kaempferol, quercetin which are well known compounds for their properties as anticancer, anti-allergic, anti-spasmodic, anti-inflammatory [9], anti-thrombitic, hepatoprotective, anti-diabetic, antiviral, cardiotonic agents [10] and also for the treatments of stomachaches, sprains and fever [11]. In the past few decades, many analytical methods have been used, like thin layer chromatography and gas chromatography, for the extraction quality assessment and

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quantification of the gallic acid and rutin from medicinal plants. The later are non-specific, time consuming and more expensive [12-14]. So high-performance liquid chromatography (HPLC) was used to characterize the bioactive compounds in the *Moringa oleifera* leaves [4, 15-18]. According to our knowledge, no publications were found on the estimation of rutin and gallic acid in *Moringa oleifera* leaves cultivated in Egypt. The aim of this study is to optimize HPLC conditions to estimate rutin as an example of flavonoids and gallic acid representing the phenolics components in *Moringa oleifera* leaves cultivated in Egypt. The analytical conditions for the reversed-phase HPLC with UV detection are studied. The method can be used to monitor the batch-to batch content in the extracts of *Moringa oleifera*.

## Materials and Methods

### Reagents

Double distilled water was used for preparing mobile phase solutions. HPLC grade acetonitrile and methanol were obtained from Fisher chemical (UK). Glacial acetic acid (analytical reagent), reference standards gallic acid, and rutin were obtained from Techno Pharmachem (SDFCL, India).

### Instruments

The HPLC system (Hewlett Packard, series 1050, USA), composed of vacuum degasser, quaternary pump, thermostatted column compartment, variable wavelength detector, manual injector and processing system to calculate statistical analysis was used (Chemistation software). Ultrasonic Cleaner, [J.P SELECTA, S.A. Spain] for degassing solution before injection, electronic balance [DENVER Instrument AA-250 Germany] and drying oven [ED115, Binder, Germany], glass vacuum mobile phase filtration system (Phenomenex, USA) consisting of AHO-1566 filter system, 47 mm, 300 mL funnel with 1 L vacuum flask. Analysis was performed on Phenomenex hypersil C18 column (DDS) (Analytical 250 × 4.6 mm, 5 µm).

### Preparation of standard solutions

Accurately weighed amounts (50.0 mg) of reference standard of gallic acid or rutin were dissolved in HPLC grade methanol and diluted to 50 mL to obtain stock solution (1.0 mg/mL).

Working standard solutions were obtained by diluting the standard stock solutions by HPLC grade methanol in 50 mL volumetric flask. The final concentrations of gallic acid or rutin were made to be 5.0, 10.0, 20.0, 30.0, 40.0, 50.0, 60.0, and 70.0 µg/mL by suitable dilutions then stored in refrigerator.

### Sample preparation

### Collection of plant material

Fresh, green *Moringa oleifera* leaves were collected on April 2018-2019 from a *Moringa* farm in Elsharkia, Egypt. The botanical identification of the plant was confirmed by Dr. Abo El Fotoh Mohammed Abdu Allah, the president of the Egyptian Moringa Scientific Association. Firstly, foreign matters were removed from the collected leaves, washed thoroughly with water to remove dust and then they were dried under the shade at room temperature for 5 days. The dried leaves were ground using [IKA, WERKE, (USA)] kitchen blender to obtain the course powder, then transferred to a labeled Ziploc bag and kept at 4 °C in refrigerator till further use.

### Sample preparation for spectrophotometry

Two samples of the dried powdered leaves of Moringa oleifera, 1.0 g, were prepared [19]. The first one was soaked in 100 mL 70% methanol for 72 hour and then the residue was dried and accurately weighed, 0.137 g. The second sample was soaked for 72 hour in 100 mL 70% ethanol and then the residue was dried and weighed, 0.0585 g.

### Spectrophotometric determination of total phenolic and flavonoid contents

The amounts of phenolic compounds in the extracts of the *Moringa oleifera* leaves were estimated by using Folin–Ciocalteau [20] method; it depends on the oxidation of phenolic groups with phosphomolybdic and phosphotungstic acids, where a green blue complex was formed. A stock solution of gallic acid was prepared in methanol; containing 1.0 mg/mL of standard gallic acid. Serial dilutions were prepared with concentrations of 500.0, 250.0, 125.0, 62.5, 31.2, 15.6 and 7.8 µg/mL. In a series of calibrated tubes, 0.4 mL of the extract was taken, mixed with 2 mL of 1.0 N Folin–Ciocalteau reagent and 1.6 mL of 20% sodium carbonate. After shaking the mixture, it was kept for one hour before measurement. Each of the seven standards and two samples were pipetted in the plate wells in six replicates and the absorbance of the resulting solutions was measured at 630 nm against reagent blank using microplate reader "Fluostar Omega".

For the determination of the total flavonoid compounds in the Moringa oliefera leaves, rutin is used as a standard. The colorimetric method applied for their determination is carried out by using aluminum chloride. The principle of the method is that

aluminum chloride forms acid stable complexes with keto or hydroxyl groups of flavones [4]. A stock rutin solution was prepared in methanol containing 1.0 mg/mL of standard rutin, then six serial dilutions were prepared in the concentrations of 1000.0, 250.0, 150.0, 100.0, and 50.0 µg/mL. Each of the six standards and two samples prepared above were pipetted in the plate wells in six replicates and the absorbance of the resulting solutions was measured at 510 nm against reagent blank using microplate reader "Fluostar Omega". The standard calibration curve was prepared by plotting absorbance versus concentration. The concentration of total phenol and flavonoid in the test sample was determined by extrapolation from the calibration graph where the total phenolic and flavonoid contents were calculated and expressed as gallic acid and rutin equivalent in mg/g of extracts, respectively.

**Preparation of extracts for HPLC**

All samples of the dried powdered leaves of *Moringa oleifera* were accurately weighed (1.0 g), and then soaked in 100 mL solvent with different percentages of methanol and ethanol for 72 hour in a refrigerator, where the extraction efficiency was dependent on the extraction time and temperature. The macerates were filtered, and the filtrates were dried at low temperature (40 °C) under vacuum. The residue was re-dissolved in 3 mL of HPLC grade methanol, and then kept in dark for further analysis.

Conventional solid-liquid extracts were performed using different percentages of solvent (methanol or ethanol) to water [100% solvent, 70**:**30% solvent**:**water, 50**:**50% solvent**:**water, 70**:**30% solvent**:**water, and 30**:**70% solvent**:**water ] were tried to select the suitable solvent:water ratio for the highest extraction efficiency. All of the standards and extracts were filtered through 0.45 µm PVDF syringe membrane filter before injection.

**Optimization of the chromatographic conditions**

The HPLC measurements was performed on Hewlett Packard, series 1050, (USA) system using a manual sample injection valve equipped with 25 µL loop. Isocratic elution was performed on Phenomenex hypersil C18 (DDS) column (Analytical 250 × 4.6 mm, 5 µm). The chromatographic conditions were optimized to separate peaks of each sample with good resolution. Different flow rates (0.5, 1.0 and 1.5 mL/min) were carried out using a UV-visible detector at three different absorbing detection wavelengths, 250, 280, and 300 nm for gallic acid, and 330, 335, and 340 nm for rutin, according to the absorption maxima of analyzed compounds, where each compound was identified by its retention time. The temperature of the column was maintained at 35 °C. All the prepared solutions were sonicated for 5 min to degas the entrapped air bubbles.

**Validation method**

Validation of an analytical procedure is the process of demonstrating that analytical procedure is suitable for their intended use and that they support the identity, strength, quality, purity, and potency of the sample. Results obtained from a method validation can be used to judge the reliability of results.

There are various parameters to validate the reproducibility of a method, such as the effectiveness, linearity, specificity, accuracy, precision, repeatability, the limit of detection (LOD), and the limit of quantification (LOQ).

The effectiveness of HPLC method was detected with the standard solutions of gallic acid and rutin. In this study different proportions of acetonitrile or methanol with 1.0% aqueous acetic acid were used to achieve the best resolution.

Linearity was determined using different known concentrations of gallic acid and rutin standard solutions, 5.0, 10.0 20.0, 30.0, 40.0, 50.0, 60.0, and 70.0 µg/mL, which were injected individually in triplicate to the HPLC system and calibration curve was obtained by plotting the peak area versus the concentration for each one where the square of correlation coefficient (R2) is indicative of the measure of linearity.

Specificity was carried out by running a procedure blank, standard, and sample solutions, then a comparison for the obtained chromatograms was carried out.

Accuracy is a measure of closeness of results obtained by a method to true value. It was determined by calculating the recovery, where known concentration (30.0 µg/mL) of gallic acid or rutin was injected in a triplicate, then recoveries were determined by comparing results of the actual concentration and amounts recovered, equation 1.

$$Recovery\% = [Experimental\ amount/Actual\ amount] \times 100 \quad (1)$$

Precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed condition, where it refers to the degree of proximity of the results expressible as relative standard deviation (% RSD) of the retention time and the peak area and it measures the reproducibility of the whole analytical method. Precision was determined by studying the repeatability. The intra-day precision was determined by injecting 30.0 µg/mL of standard gallic acid or rutin three times within one day into HPLC system, whereas

the inter-day precision was checked for three consecutive days. The mean and relative standard deviation % RSD were calculated.

## Results and discussion

### *Spectrophotometric determination of total phenolic and flavonoid contents*

The constituents under investigation were also estimated by microplate reader "Fluostar Omega", a standard calibration curve for gallic acid was constructed in the range 7.8-500.0 µg/mL, Fig. 1. The amount of the total phenolic compounds present in the solvent extracts of Moringa Oliefera leaves was found to be 61.80 ± 2.2 µg/mL equivalents to gallic acid in 70% methanolic extract, and it was found to be 52.50 ± 3.0 µg/mL in 70% ethanolic extract.

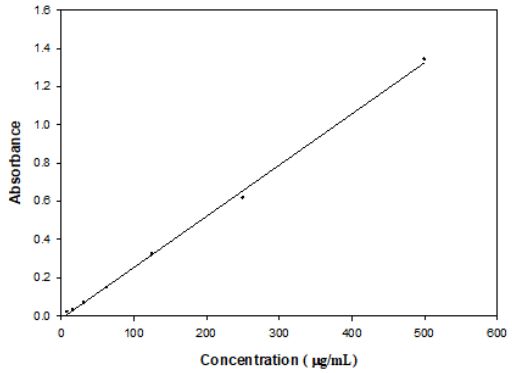


**Fig.1. Spectrophotometric calibration graph for standard gallic acid solution**

Moreover, a standard calibration curve for rutin was constructed in the range 50.0-1000.0 µg/mL, Fig. 2. The amount of the total flavonoid compounds (rutin equivalent) present in the solvent extracts of Moringa Oliefera leaves was found to be 83.70 ± 7.0 µg/mL equivalent to rutin in 70% methanolic_extract, and it was found to be 78.20 ± 2.8 µg/mL in 70% ethanolic extract. Comparing the obtained results with that previously published in India [4], which reported that the amount of total phenolic and flavonoid compounds present in the methanol extract of the leaves of *Moringa oleifera* estimated by Visible-Spectroscopy was found to be 8.0 and 27.0 µg/mL, respectively. The later reflects that the Moringa Oliefera species cultivated in Egypt is of high quality.

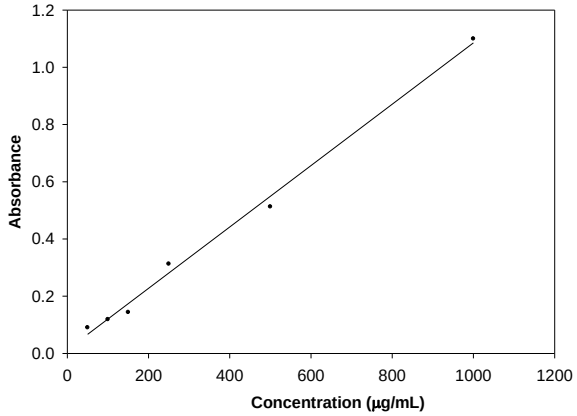


**Fig.2. Spectrophotometric calibration graph for standard rutin solution.**

## HPLC method for determination of gallic acid and rutin

### *Optimization of HPLC conditions*

Under the optimized conditions, the suitable mobile phase for the estimation of gallic acid was tested. Various trials were carried out and series of solutions were studied with different ratios (v/v) of acetonitrile, 10.0, 20.0, 30.0, and 40.0% acetonitrile in 1.0% aqueous acetic acid, where the injected concentration of gallic acid was 30.0 µg/mL. The best resolution was observed in case of using 10% acetonitrile:1.0% aqueous acetic acid as a mobile phase with a flow rate of 1.0 mL/min, and at wavelength of 280 nm. In case of rutin; a series of solutions was studied with different ratios (v/v) of methanol, 30.0, 40.0, 50.0, and 60.0% methanol in 1.0% aqueous acetic acid, utilizing 30.0 µg/mL rutin solution. The mobile phase selected was 60% methanol:1.0% aqueous acetic acid with a flow rate of 1.0 mL/min, at wavelength 335 nm.

Using the above chromatographic conditions, it was possible to separate gallic acid and rutin by injection of their corresponding standards in their individual solutions, Figs. 3, and 4. The developed optimized

method resulted in the elution of gallic acid and rutin at a retention time of 5.45 and 4.50 min, respectively.

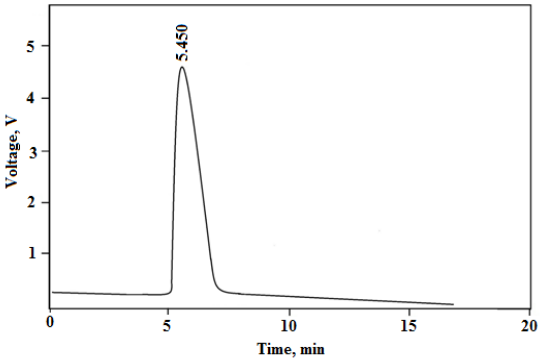


**Fig. 3. HPLC chromatogram of 30.0 µg/mL gallic acid standard solution**

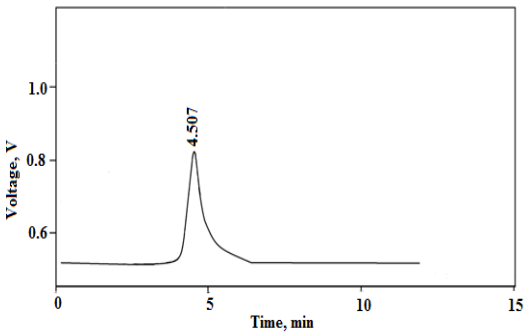


**Fig. 4. HPLC chromatogram of 30.0 µg/mL rutin standard solution**

**Method validation**

The method was validated according to the International Conference on Harmonization (ICH) guidelines [21]. Linearity was evaluated by the peak area against concentration of gallic acid and rutin standard solutions, Figs. 5, and 6. The method was found to be linear within the ranges of 5.0-40.0 and 5.0-60.0 µg/mL for gallic acid and rutin, respectively. This was confirmed by good fitting of the curve.

The correlation coefficients ($R^2$) of the present graphs were 0.999 and 0.997 for gallic acid and rutin, respectively, Table 1. These values indicate a good linearity of the graphs compared with the minimum acceptable correlation coefficient, 0.990 [22]. Specificity was studied by comparing the chromatogram obtained from blank, standard, and sample solutions of gallic acid and rutin standard solutions. It was found that the retention times were 5.42 and 4.32 min for gallic and rutin sample solutions respectively.

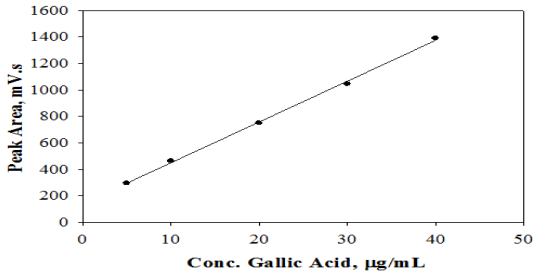


**Fig. 5. HPLC calibration curve of gallic acid standard solution**

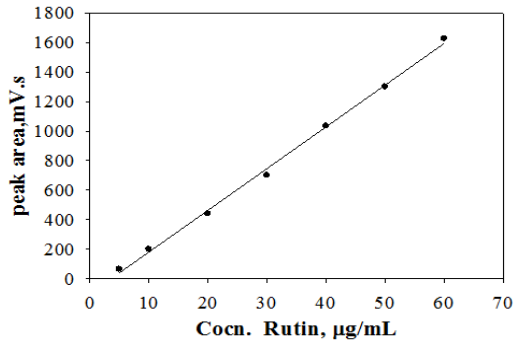


**Fig. 6. HPLC calibration curve of rutin standard solution**

Table 1. HPLC conditions for gallic acid and rutin standard solutions.

| Parameters | Gallic acid | Rutin |
|---|---|---|
| Conc. Range (µg/mL) | 5.0-40.0 | 5.0-60.0 |
| $R_t$ (min) | 5.45 | 4.50 |
| Slope [mV.s /(µg/mL] | 30.8 | 28.3 |
| Intercept (mV.s) | 142.07 | -102.30 |
| $r^2$ | 0.999 | 0.997 |
| LOD (µg/mL) | 0.13 | 0.36 |
| LOQ (µg/mL) | 0.43 | 1.19 |
| Repeatability | 1.39 | 1.47 |
| Ruggedness | RSD< 2.0 | RSD< 2.0 |

$r^2$: Correlation coefficient, $R_t$: Retention time

Recovery was used to assess the accuracy of the developed method. The average percent recoveries for intra-day conditions for gallic acid and rutin solutions were found to be 97.00 - 99.66% ± 0.41 and 96.40 - 99.20% ± 0.43, respectively. These values show the high accuracy of the present method. In addition, this method demonstrated acceptable precision with

relative standard deviation (RSD%) values lower than 2.0%. For inter-day conditions, the average percent recoveries for gallic acid and rutin solutions were found to be 86.70 - 89.00% ± 0.61 and 95.00 -96.40% ± 0.31, respectively. The data pertaining to repeatability (intra-) and intermediate (inter-day) precision was summarized in table 2, which indicates that the proposed procedure is precise.

Limit of detection (LOD): the detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated under the stated experimental conditions.

Limit of quantification (LOQ): the quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with accuracy.

LOD and LOQ were determined based on the signal-to-noise ratio response. They were calculated using the following formula: LOD = 3 б/S and LOQ = 10 б/S

Where б: standard deviation of response (peak area) and S: slope of the calibration curve. LOD and LOQ values were found to be 0.13, 0.43 and 0.36, 1.19 µg/mL for gallic acid and rutin, respectively. These low values indicate that the method provides adequate sensitivity.

**Identification and quantification of gallic acid and rutin in *Moringa oleifera***

The developed HPLC method was utilized to analyze gallic acid and rutin in two solvent extracts, methanol and ethanol, of *Moringa oleifera* leaves. It was found that, methanol, ethanol and their mixtures with water extracts revealed the presence of gallic acid and rutin peaks at retention time, 5.42 min, Fig. 7, and at 4.32 min, Fig 8, respectively. It was realized that the concentration of gallic acid and rutin was changed in the extracts with changing the solvent : water percentage, Table 3.

**Table 2**. Intra-day and inter-day precision of gallic acid and rutin standard solutions.

| Standard | Injected (µg/mL) | Measured (µg/mL) | Mean (µg/mL) | SD | RSD % | Recovery % |
|---|---|---|---|---|---|---|
| **Intra-day** | | | | | | |
| **Gallic acid** | 30.0 | 29.1 | | | | 97.00 |
| | | 29.6 | 29.54 | 0.41 | 1.39 | 98.66 |
| | | 29.9 | | | | 99.66 |
| **Rutin** | 30.0 | 28.9 | | | | 96.40 |
| | | 29.7 | 29.31 | 0.43 | 1.47 | 99.20 |
| | | 29.3 | | | | 97.70 |
| **Inter-day** | | | | | | |
| **Gallic acid** | 30.0 | 26.0 | 26.64 | 0.61 | 2.30 | 86.70 |
| | | 27.2 | | | | 90.70 |
| | | 26.7 | | | | 89.00 |
| **Rutin** | 30.0 | 28.5 | | | | 95.00 |
| | | 28.3 | 28.60 | 0.31 | 1.10 | 94.40 |
| | | 28.9 | | | | 96.40 |

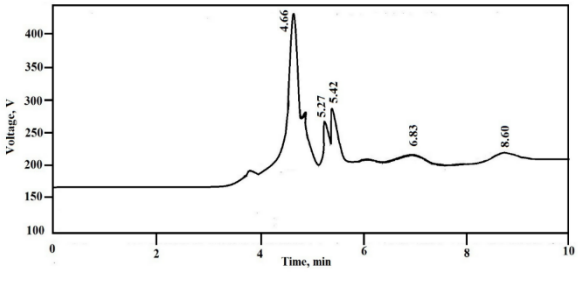


**Fig. 7. HPLC chromatogram represents gallic acid in 50% methanol extract of *Moringa Oliefera* leaves.**

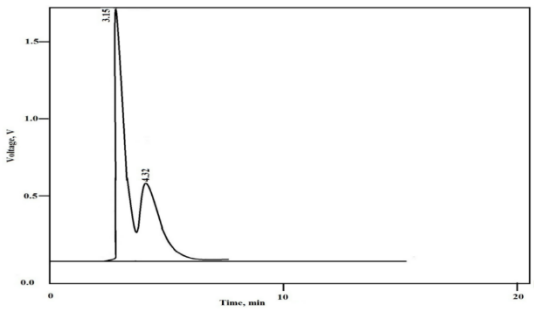


**Fig. 8. HPLC chromatogram of rutin in 100% methanol extract of *Moringa Oliefera* leaves.**

**Table 3.** The values of peak area and concentration of gallic acid and rutin in different percentage of solvent extracts of *Moringa oleifera* leaves

| | Methanol Extraction | | | | Ethanol Extraction | | | |
|---|---|---|---|---|---|---|---|---|
| | **100%** | **70%** | **50%** | **30%** | **100%** | **70%** | **50%** | **30%** |
| **Gallic acid** | | | | | | | | |
| **Concentration (µg/mL)** | 30.00 | 22.75 | 51.20 | 12.20 | 25.31 | 27.35 | 30.26 | 11.50 |
| **Peak area (mV.s)** | 1074.86 | 843.26 | 1596.30 | 509.50 | 928.92 | 989.5 | 1081.14 | 493.90 |
| **Rutin** | | | | | | | | |
| **Concentration (µg/mL)** | 60.00 | 51.63 | 46.30 | - | 59.93 | 45.50 | 37.70 | - |
| **Peak area (mV.s)** | 1600.00 | 1355.14 | 1237.37 | - | 1596.00 | 1189.00 | 958.62 | - |

**Effect of solvent concentration on the extraction efficiency gallic acid**

The values of peak area and concentration of gallic acid in different percentage of ethanol or methanol: water extracts were reported in Table 3. The results showed that, the peak area and consequently the gallic acid concentration is significantly change with changing the ethanol or methanol percentage. The data reveal that the best extraction for gallic acid is 50% methanol or ethanol. By decreasing the percentage of solvent from 100 to 50%, the concentration of gallic acid increases from 30.0 to 51.2 µg/mL, and from 25.31 to 30.26 µg/mL in case of methanol and ethanol extracts, respectively. However, on decreasing the percentage of solvent below 50%, the gallic acid content decreases to 12.20 and 11.50 µg/mL, for methanol and ethanol extracts, respectively, Table 3.

**Effect of solvent concentration on the extraction efficiency rutin**

The best extraction of rutin was found in 100% methanol or ethanol, 60.0 and 59.93 mg/mL, respectively. This concentration decreases to 46.3 µg/mL and from 37.70 µg/mL by decreasing the percentage of solvent percentage to 50% for both solvents, respectively. From the above discussion, we can conclude that the highest extraction efficiency for gallic acid was in 50% solvent, while for rutin was in 100% solvent.

**Comparative study**

The major bioactive compounds; gallic acid and rutin, which expressed strong antioxidant activity [5,9] were identified in *Moringa oleifera* leaves grown in Egypt, contrary to studies in India and Ghana, which reported the primary compounds as chlorogenic acid and rutin [7, 23]. The variation may possibly be the consequence of different plantation regions or genetic diversity of *Moringa oleifera*.

In Southern Tunisia, Bennour et.al [19] reported some phenolic compounds including gallic acid and rutin from the leaves of *Moringa oleifera* by high-performance liquid chromatography coupled with photodiode array and mass spectrometry detection (HPLC-PDA-ESI/MS). The analysis was performed using linear gradient elution of 0.2% acetic acid in 95% water and 5% methanol (solvent A), and 0.2% acetic acid in 50% water and 50% acetonitrile (solvent B). The flow rate of the mobile phase was 0.5 mL/min and absorbance at 280 nm wavelength and on-line ultraviolet (UV) spectra from 190 to 800 nm were recorded. The results showed that the retention time of gallic acid observed at 7.95 min [19], while the present work showed that the retention time of gallic acid was detected at 5.45 min. On the other hand, the retention time of rutin was observed at 59.2 min [19], while in the present study, it was detected at 4.5 min. These results indicate that our new developed method is time and solvents saving.

**Conclusion**

The present work involved development validation of an HPLC method for quantitative estimation of gallic acid and rutin which act as the main bioactive components in two different solvent extracts of Egyptian *Moringa oleifera* leaves. The established HPLC assay showed good separation of the compounds and linear, sensitive, accurate, and reproducible results. Therefore, the method is suitable for the determination of gallic acid and rutin in various alcoholic extracts of *Moringa oleifera* with shorter run time < 6 min and high precision (RSD < 2.0). The presence of significant amount of respective bioactive components in different percentage of ethanol and methanol water extracts and the highest concentration

was obtained from 50% solvent: water extracts for gallic acid, while the highest concentration of rutin was obtained from 100% solvent. This variation of the quantity determined is based on the polarity of the solvent used for the extraction process. The phenols and flavonoids present in *Moringa oleifera* may be responsible for their therapeutic effectiveness against various diseases and may protect our cells against oxidative damage.

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