

# Determination of Quercetin in Iraqi Green Walnut Husk via Green Ionic Liquid-Based Digestion Extraction and Gel Filtration Procedures-Spectrophotometry and Microfluidic Paper-Based Analytical Platform Detection

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## Abstract

This study was conducted to extract, isolate and quantify Quercetin from Iraqi green walnut husk (GWH) using a novel ionic liquid-based digestion extraction and gel filtration procedure coupled with visible spectrophotometry detection and compared with a novel microfluidic paper-based analytical device ( $\mu$ PADs) aiming for an economic, and green method. 1-butyl-3-methylimidazolium chloride (BMIMCl) aqueous solution was used as a green solvent alternative to conventional organic solvents due to their impressive solvation properties. The results showed that 100 mg of plant husk required 15 mL of BMIMCl solvent, and 30 minutes of digestion at 75° C, which involves a simpler, and greener extraction method compared with other extraction methods. Under optimal conditions, Quercetin was isolated using a gel filtration technique, and further investigations were conducted using high-performance liquid chromatography and Fourier-transmitted infrared to confirm the isolation of Quercetin. Aluminum chloride assay was selected to determine the concentration of Quercetin giving a linearity of 10-100 mg L<sup>-1</sup> and correlation coefficient of 0.9369 and 0.7917 for spectrophotometric and  $\mu$ PADs methods, respectively. The antioxidant activity of isolated Quercetin showed good antioxidant activity and the ANOVA test was used to compare  $\mu$ PADs method with the spectrophotometric method and the results showed that there was no statistically significant difference between the two methods. In conclusion, BMIMCl solvent provides an excellent safe and suitable solvent for the extraction of phytochemicals from botanical drugs. In addition, the  $\mu$ PADs platform has been approved for its superiority against traditional methods for analytical purposes due to its green characteristic.

**Keywords:** Antioxidant, Green extraction, Green walnut husk, Ionic liquids, Microfluidic paper-based analytical device, Quercetin.

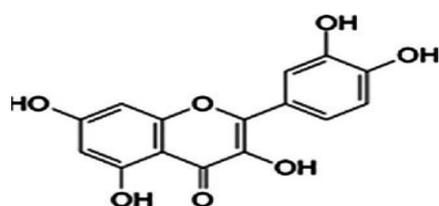
## Introduction

Botanical drugs (medicinal plants) have a major impact on humankind's health due to the natural presence of phytochemicals that possess various therapeutic activities in addition to their role in plants<sup>1</sup>. The walnut tree (*Juglans regia* L.) which is a member of the *Juglandaceae* family is

considered a major source of various phytochemicals with antioxidant and antimicrobial potential, as well as anti-histaminic, anti-nociceptive, anti-asthmatic, anti-ulcer, immunomodulatory, anti-diabetic, hepatoprotective, anti-fertility, anti-inflammatory, central nervous system stimulant, lipolytic, wound

healing, insecticidal and larvicidal properties, and many others, all of which have a positive impact on human health<sup>2</sup>.

Quercetin (3,3',4',5,7-pentahydroxyflavanone, or 3,3',4',5,7-pentahydroxy-2-phenylchromen-4-one)<sup>3</sup> is classified as a flavonol which is a group of plant phytochemicals known as flavonoids that have the same flavone backbone (a three-ringed molecule with hydroxyl [OH] groups attached)<sup>4</sup>, which is identified as a total of thirteen flavonoid compounds found in walnut husks<sup>5</sup> Fig 1. Anti-inflammatory, antioxidant, and antitumor properties make Quercetin widely researched as a chemopreventive agent<sup>6</sup>. The extraction of Quercetin is usually done using organic solvents such as ethanol, methanol and ethyl acetate<sup>7</sup> but these solvents suffer from being flammable and have low level of extraction selectivity. Therefore, there was a need for alternative (sustainable) solvents that can replace current organic solvents. Ionic liquids such as 1-butyl-3-methylimidazolium chloride (BMIMCl) which is an organic salt that is liquid at room temperature or close to it, have long been of interest and can be recognized as an alternative solvent and effective separation solvent due to its capability of higher extraction efficiency, environmentally friendly because of its capacity to be recycled and lower solvent loss during the extraction process<sup>8</sup>.



**Figure 1. Chemical structure of Quercetin.**

## Materials and Methods

### Plant Sample Preparation and Extraction

In August 2022, raw green walnut husks (GWH) were purchased from a local market in Sulaymaniyah Governorate, Iraq. The GWH was rinsed under running water to remove dust and other contaminants, separated from the kernel, cut into small pieces using a knife, dried and ground into a fine powder using an electric mixer, and stored in sealed plastic bags for further use. Three different solvents, ethanol (Sigma - Aldrich / Germany), deionized water, and ethyl acetate (Sigma - Aldrich /

Germany) were used for GWH phytochemicals extraction using Soxhlet apparatus and these solvents were compared with the green aqueous 1-butyl-3-methylimidazolium chloride (BMIMCl) (Shanghai Maclean Biochemical Company/ China) solvent (2 % wt/v) prepared by dissolving 2 g of BMIMCl in 100 mL of deionized water. 10 mg of GWH powdered samples were loaded into a thimble and put inside the Soxhlet apparatus. Then, 100 ml of the four different solvents were added individually to the Soxhlet apparatus and heated at 78 °C with ethanol and ethyl acetate and 100 °C with deionized water and BMIMCl for twelve hours. The mixture was

Various analytical techniques were used for the determination of Quercetin, whilst these techniques provide high sensitivity, they require sophisticated instruments as well as abundant use of solvents and energy, trained personnel, and laborious protocols<sup>9</sup>. Recently, the development of miniaturized systems using paper so-called microfluidic paper-based analytical devices ( $\mu$ PAD) as the medium for analytical detection allows the construction of accurate, portable, easy-to-use, low-cost, time-saving, disposable, and real-time detection systems that fulfil the criteria for green analytical chemistry<sup>10</sup>. The fundamental principle that paper microfluidics lies on is the paper basic material which is cellulose that is considered naturally hydrophilic and allows fluids penetration within its fibre matrix. Paper devices consist of hydrophilic zones surrounded with hydrophobic barriers to create micron-scale capillary channels on paper which enable reagents reaction with the analyte zone<sup>11</sup>. For the detection, simple smartphone, digital cameras, and scanners can be used and the recorded intensity of the colour processed via computer-based image analysis software is used as a function for the quantitation of analyte concentration<sup>12</sup>. Here, we investigated an environmentally-friendly method for the extraction of the phytochemical compound Quercetin from a waste agricultural (Iraqi green walnut husk) using green ionic liquid (BMIMCl) solvent. We have determined the concentration and antioxidant activity of the isolated Quercetin using traditional UV/vis spectroscopy and then compared it with a new method known as a microfluidic paper-based analytical device ( $\mu$ PAD) that is affordable and accessible to be taken outside centralized laboratories.

filtered using Whatman Grade No. 18 filter paper before being vacuum-dried at 40°C. Then, the extraction yield of four extracts were calculated depending on the below Eq. 1<sup>13</sup>.

$$\text{Yield \%} = \frac{\text{Weight of dried crude extract (g)}}{\text{Weight of dried plant sample taken (g)}} \times 100 \dots\dots\dots \text{Eq.1}$$

### Extraction Methods

Four different methods were used to extract phytochemicals from GWH powered samples including maceration (1 g of the GWH powdered samples were placed in an Erlenmeyer flask, 10 ml of BMIMCl solvent was added. Samples were left to macerate for 24 hours at room temperature, then filtration was performed)<sup>14</sup>, ultrasound –assisted extraction (UAE) (10 ml of BMIMCl solvent was added to 1 g of GHW powered samples in the Erlenmeyer flask, and placed inside the ultrasonic bath (Elmasonic S10H) and treated with ultrasound at 25°C, the bath power was 280 W with 50/60 Hz frequency. After extraction, filtration was done)<sup>15</sup>, and digestion extraction method (the BMIMCl solvent was poured into a clean Erlenmeyer flask followed by GHW powdered samples and the mixture was placed in a water bath at a temperature about 50 °C)<sup>16</sup>. After filtration, the extracted samples were put into an oven set at 50 °C overnight to evaporate any trace amounts of solvent left. Soxhlet apparatus was used to compare the efficiency of other extraction methods.

### Optimization of the Operational Conditions

In order to optimize the extraction process, the factors including extraction time (5 ,15 ,30 ,60 ,90 minute), extraction temperature (25 ,50 ,75 ,100°C), solid - liquid ratio (0.5:5 ,0.5:10 ,0.5:15,0.5:20,0.5:40 g/ml ), liquid-solid ratio (0.5:15, 1:15, 1.5:15 g/mL) , pH (2,3,5,7,9 ) were studied using a single factor experiments. When one factor is studied, the other factors are constant<sup>17</sup>.

### Qualitative Analysis of GWH Extract Phytochemicals

Standard procedures were used to qualitatively determine the existence of phytochemicals such as Flavonoids, Steroids, Terpenoids, Saponins, Tannins, Phenolic compounds, and Carbohydrates in

GWH-BMIMCl extract<sup>18</sup> using traditional methods and  $\mu$ PAD platform for comparison.

### Isolation and Quantification of Quercetin (Q)

#### Gel-filtration Chromatography for Isolating Quercetin

The gel was prepared by dissolving Sephacryl S-200 in hexane (Sigma Aldrich) and packing it in a (48x1.6) cm glass column<sup>19</sup>. After packing, several column volumes of hexane (25 mL) were passed through the column to remove any air bubbles and to test the column homogeneity. The column was loaded with a 5 mL volume of standard Quercetin at a concentration of 20 mg L<sup>-1</sup> in ethanol and at a flow rate of 1 mL/30 min. Fractions of 1 mL were collected as the sample elutes from the column. Then, 5 mL of the GHW-BMIMCl extract was loaded into the glass column at a flow rate of 1 mL/30 min, and fractions of 1 mL were collected. Elution curves were obtained by measuring the absorbance of elution samples at  $\lambda_{\text{max}} = 415$  nm using a spectrophotometer.

#### FTIR Measurements

The isolated GWH- BMIMCl extract was dried in an electric oven at 40 °C for eight hours, and converted into a powder form. FTIR spectra were recorded in the range of 400-4000 cm<sup>-1</sup> using Bruker ALPHA II FTIR Spectrometer.

#### HPLC-Analysis

HPLC analysis was performed using a Shimadzu CTO-20A HPLC column oven Series. Separation was performed using a MACHEREY -NAGEL Germany C18 column (4.6 mm x 250 mm i.d., 5  $\mu$ m)<sup>20</sup>. The mobile phase consisted of methanol and a Buffer solution of 2% orthophosphoric acid at a flow rate of 0.8 ml min<sup>-1</sup>. The mobile stage was programmed respectively in a linear fashion; the gradient was as follows: 0-5 min (90 % methanol with 10 % Buffer solution); 5-10 minutes (80% methanol with 20% buffer solution); 10-15 minutes (60% methanol with 40% buffer solution); 15-20 minutes (40% methanol with 60% buffer solution); 20-25 minutes (20% methanol with 80% buffer solution)<sup>21</sup>. The multi-wavelength detector was detected at  $\lambda_{\text{max}} = 374$  nm. The injection volume was 5  $\mu$ L for each of the sample solutions. The column temperature was maintained at 50 °C.

## Determination of Quercetin Concentration Using Aluminum Chloride Assay

To estimate the concentration of isolated Quercetin, Aluminum chloride colorimetric assay<sup>22</sup> was used and the detection was done by two different techniques including a conventional spectrophotometer: The  $\text{AlCl}_3$  reagent (prepared by dissolving 1 gram of  $\text{AlCl}_3$  (BDH / England) with 10 ml of deionized water) was employed to calculate the concentration of isolated Quercetin from GWH-BMIMCl extract. Various concentrations (10, 20, 40, 60, 80, and 100  $\text{mg L}^{-1}$ ) of standard Quercetin were added to 2 mL of green BMIMCl. Followed by the addition of 0.15 of Sodium nitrate (1  $\text{mol L}^{-1}$ ), mixed for 5 minutes, 0.15 mL of  $\text{AlCl}_3$  (10% w/v), 1 ml of NaOH (BDH / England) (1  $\text{mol L}^{-1}$ ), the final volume was made to 5 ml using BMIMCl and the tubes were kept in the dark place for 30 minutes. The absorbance was measured at  $\lambda_{\text{max}}=510$  nm against reagent blank. The second detection method was  $\mu\text{PADs}$ : the fabrication of the paper was done according to Peters *et al*<sup>23</sup> procedure, Five microliters of different concentration of standard Quercetin (10, 20, 40, 60, 80, and 100  $\text{mg L}^{-1}$ ) and isolated Quercetin were added into the detection circular reaction zone using a micropipette, individually. Then, twenty microliters of BMIMCl (2 %), fifteen microliters of  $\text{NaNO}_2$ , fifteen microliters of  $\text{AlCl}_3$ , and one hundred microliters of NaOH were added to the detection circular reaction zone and stored in darkness for 30 minutes. After the dryness of the detection circular reaction zone at room temperature, the intensities were recorded against reagent blank via photographs taken by a Samsung note 9 and Image J software program according to Dawson procedure<sup>24</sup>.

## Ferric Reducing Antioxidant Power (FRAP)

## Results and Discussion

### Selection of Optimum Extraction Conditions

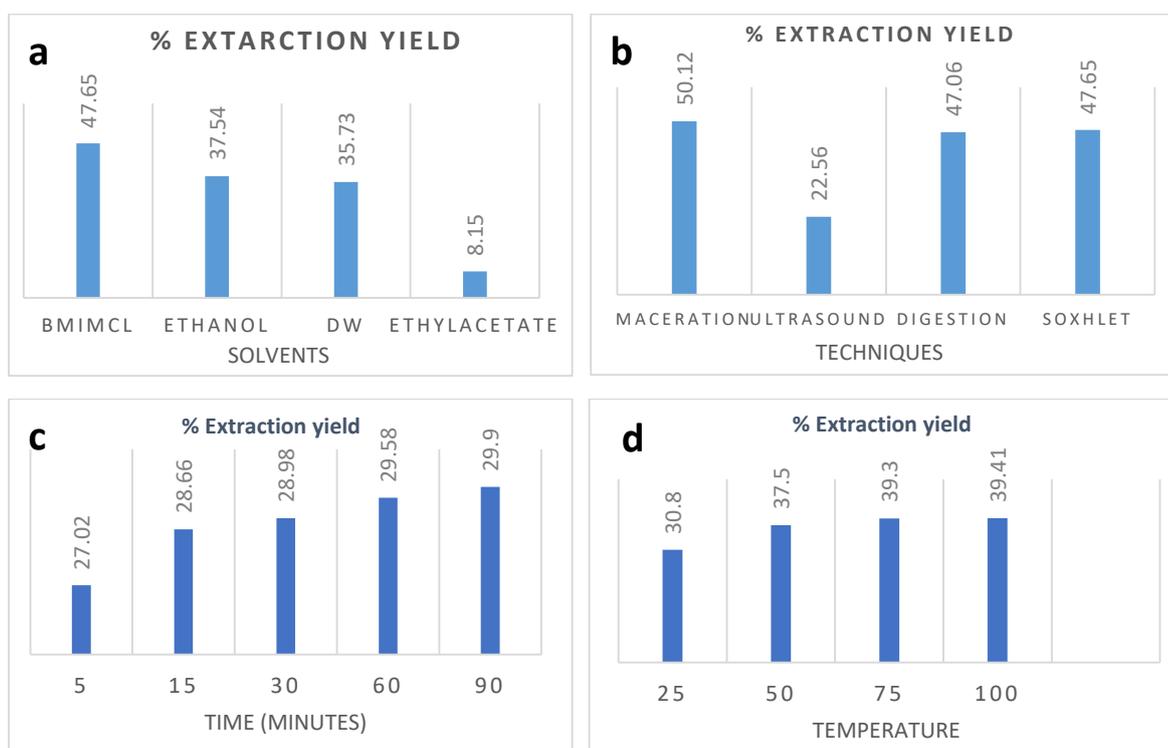
Due to the unique ionic liquid solvents properties for swelling and dissolving of phytochemicals that can lead to a high extraction yield percentage of valuable phytochemicals in GWH which is mainly due to the basic structure of ionic liquid solvent that can avoid the cellulosic matrix dissolution and at the same time allow the target compounds to be extracted<sup>26</sup>, green BMIMCl solvent was used and compared with three different solvents including deionized water, ethanol,

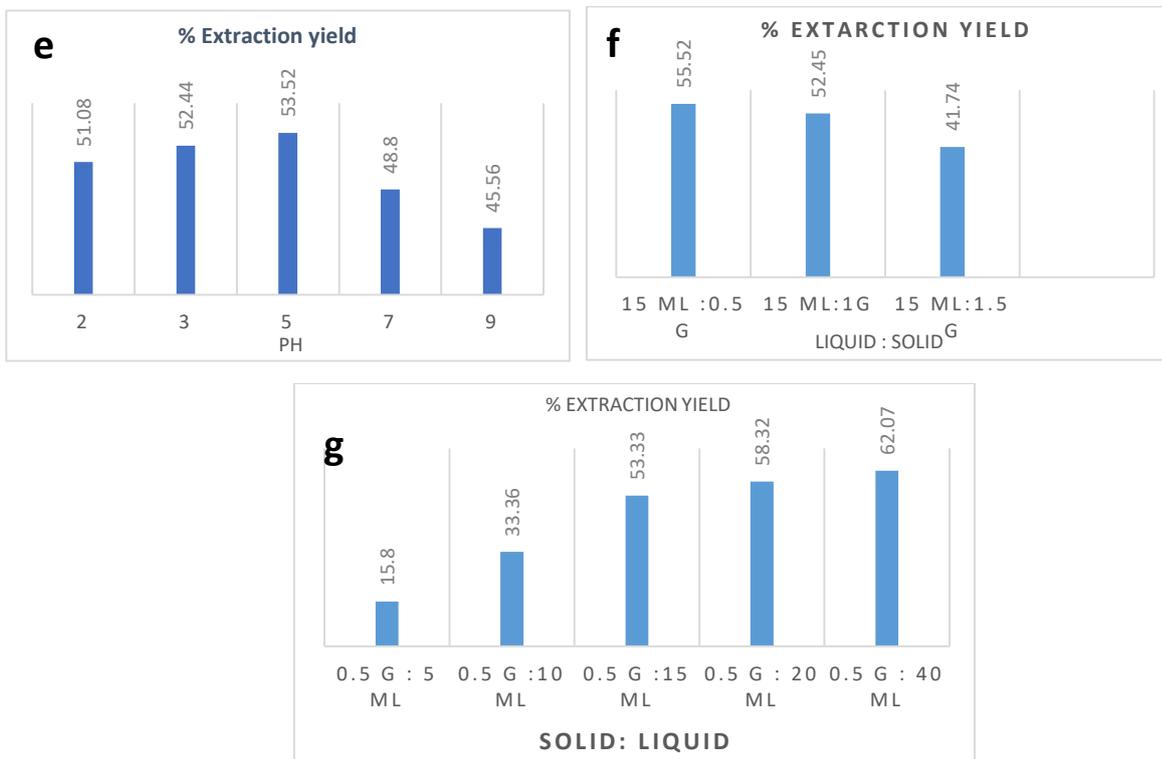
Reducing antioxidant power of the isolated extract was tested using a method described by Schmitt *et al.*<sup>25</sup> and the detection was done by two different techniques (spectrophotometric and microfluidic paper-based analytical device ( $\mu\text{PADs}$ )). Spectrophotometric procedure: Vitamin C (1000  $\text{mg L}^{-1}$ ) was prepared by dissolving 0.05 gram of Vitamin C with deionized water in 50 mL volumetric flask. Various concentrations (200, 400, 600, and 800  $\text{mg L}^{-1}$ ) of standard Vitamin C and isolated Quercetin were added to 0.4 mL phosphate buffer (0.2 M, pH 6.6), and 0.5 mL potassium ferricyanide (10  $\text{mg L}^{-1}$ ), individually. The mixtures were incubated for 20 minutes at 50°C followed by the addition of 0.1 mL of tri chloro acetic acid (100  $\text{mg L}^{-1}$ ). Afterwards, deionized water and 0.1% (w/v) ferric chloride were added to the mixtures. After 10 minutes, the absorbance was measured at  $\lambda_{\text{max}}=700$  nm against reagent blank. The second detection method was  $\mu\text{PADs}$ : the fabrication of the paper was done according to Peters *et al*<sup>23</sup> procedure. Five microliters of different concentrations of standard Vitamin C (200, 400, 600, and 800  $\text{mg L}^{-1}$ ) and isolated Quercetin were added into the detection circular reaction zone using a micropipette, individually. Then, twenty microliters of phosphate buffer (0.2 M, pH 6.6), and twenty-five microliters of potassium ferricyanide (10  $\text{mg L}^{-1}$ ) were added to the detection circular reaction zone and stored for 20 minutes at 50°C, individually followed by the addition of twenty-five microliters of tri chloro acetic acid (100  $\text{mg L}^{-1}$ ). Afterward, five microliters of deionized water, and five microliters of 0.1% (w/v) ferric chloride were added and kept for 10 minutes. After the dryness of the detection circular reaction zone at room temperature, the intensities were recorded against reagent blank via photographs taken by Samsung note 9 and Image J software program.

and ethyl acetate using the Soxhlet apparatus to extract the GWH phytochemicals. As depicted in Fig. 2a, the BMIMCl gave an extraction yield of up to 47.65 % which was higher than those accomplished with the other solvent (37.54 for ethanol, 35.73 for deionized water, and 8.15 for ethyl acetate). Afterwards, the influence of four different extraction techniques was investigated as can be seen in Fig. 2b. Despite the high extraction yield obtained by the maceration (50.12 %) and Soxhlet (47.65 %), the digestion extraction technique (47.06 %) was

chosen. Maceration is considered a simple, cheap and easy-to-use technique for extraction but the need for a long time and the consumption of large quantities of solvent lead to the elimination of this technique<sup>27</sup>. On the other hand, Soxhlet technique suffers from the special equipment need that adds complexity alongside continuous supply of tap water for at least 24 hours. Therefore, the digestion extraction technique was adopted for GWH phytochemicals extraction. Time is one of the key factors which influence any process in terms of cost economics as well as process scale up. A linear increase was observed as the reaction time increase from 5 minutes to 15 minutes with an extraction yield of 27.02% to 28.66%, as shown in Fig. 2c. This may be due to an increase in intermolecular exposure time and disruption of intermolecular forces<sup>28</sup>. Prolonged exposure beyond 15 min had a negligible effect on the extraction yield. Thus, the extraction time of 15 minutes was chosen in subsequent experiments. The effect of extraction temperature 25-100 °C on the extraction yield was examined showing the increase of extraction yield from (30.8% to 39.3 %) as revealed in Fig. 2d, with the temperature rising above 75 °C, no discernible change in the extraction product was seen when the temperature increased above 75 °C, which is attributed to a decrease in the viscosity of the solvent, that encourages diffusion<sup>29</sup>,

<sup>30</sup>. Hence, the extraction temperature of 75°C was chosen in subsequent experiments. Fig. 2e gave the pH influence in the range 2–9 with an increase in the extraction yield up to pH 5. Further increase in pH leads to a decrease in extraction yield due to its aggregation and interactions of monomeric and polymeric subunits of the extracts<sup>28</sup>. The increase in GWH plant weight in a constant solvent volume leads to a decrease in extraction yield as can be seen in Fig. 2f from 55.52% to 41.74% and this decrease may be to the decrease in the solubility of the solids due to the effect of increasing the amount of plant with a constant volume of the solvent and thus decreasing the contact surface area between the plant matrix and the solvent<sup>31</sup>. Therefore, the ratio of 15 mL of solvent to 0.5 gram of GWH plant weight was chosen for further experiments. The effect of changing the solvent volume with constant weight of GWH plant on the extraction yield was investigated. The extraction yield increased from (15.8% to 62.07%), as shown in Fig. 2g; however, the increase in the extracted product is slight with an increase in the solvent volume above 20 mL. The first rise in extraction may be due to the improved solubility of the solids as a result of the extended solvent interaction with the solids. Therefore, the ratio of 0.5 g of GWH: 20 ml of solvent was chosen in subsequent experiments.





**Figure 2. Extraction yield percentage of the optimized extraction system parameters. (a) extraction solvent (BMIMCl, ethanol, deionized water, ethyl acetate), b) extraction techniques (maceration, ultrasound, digestion, and Soxhlet), c) extraction time ranging from (5-90) minutes, d) temperature (25, 50, 75, 100) °C, e) pH ranging from (2-9), f) liquid to solid (15 mL : 0.5 g, 15 mL : 1 g, 15 mL : 1.5 g), g) solid to liquid ratio (0.5 g : 5 mL, 0.5 g : 10 mL, 0.5 g : 15 mL, 0.5 g : 20 mL, 0.5 g : 40 mL).**

### Phytochemical Screening Methods

Using traditional qualitative phytochemicals analysis procedures, the phytochemical chemicals found in the GWH- BMIMCl extract was examined. The results in Table.1 showed the presence of Phenols, Tannins, Terpenoids, Saponins, Glycosides, Steroids and Flavonoids compounds, this is due to the effectiveness of the BMIMCl ionic liquid solvent and the extraction method in the solubility of the phytochemicals presented in the GWH<sup>32</sup>. Aiming for a greener procedures for screening the GWH-BMIMCl phytochemicals,  $\mu$ PAD platform was used

and the results showed positive results for Phenols, Tannins, Saponins, Steroids and Flavonoids compounds with the exception of Terpenoids, and Glycosides due to the harsh conditions involves in the procedures such as the use of concentrated acid and boiling. Despite these obstacles, the  $\mu$ PAD platform can be considered as an alternative to traditional qualitative chemical analysis procedures where  $\mu$ PAD share the same end goal, to produce a platform which can function easier, cheaper, portable, disposable, greener, and just as well as the conventional methods currently available.

**Table 1. Qualitative phytochemical screening of GWH- BMIMCl using standard procedures and  $\mu$ PAD platform.**

Phytochemicals	Reagents	Remarks	GWH- BMIMCl extract using traditional procedures.	GWH-BMIMCl extract using $\mu$ PAD platform
Phenols	Sodium carbonate and Folin Ciocalteu's reagent	The appearance of blue/green color.		
Tannins	Ferric Chloride	The appearance of brownish-green color.		
Terpenoids	Chloroform and H <sub>2</sub> SO <sub>4</sub>	The appearance of a reddish-brown color.		
Saponins	boiling water	The appearance of foam.		
Glycosides	Benedict reagent	The appearance of reddish-brown precipitate.		
Steroids	Acetic anhydride and H <sub>2</sub> SO <sub>4</sub>	The appearance of thin blue to green circle color.		
Flavonoids	Lead acetate	The appearance of yellow precipitate.		
	H <sub>2</sub> SO <sub>4</sub>	The appearance of yellow color.		

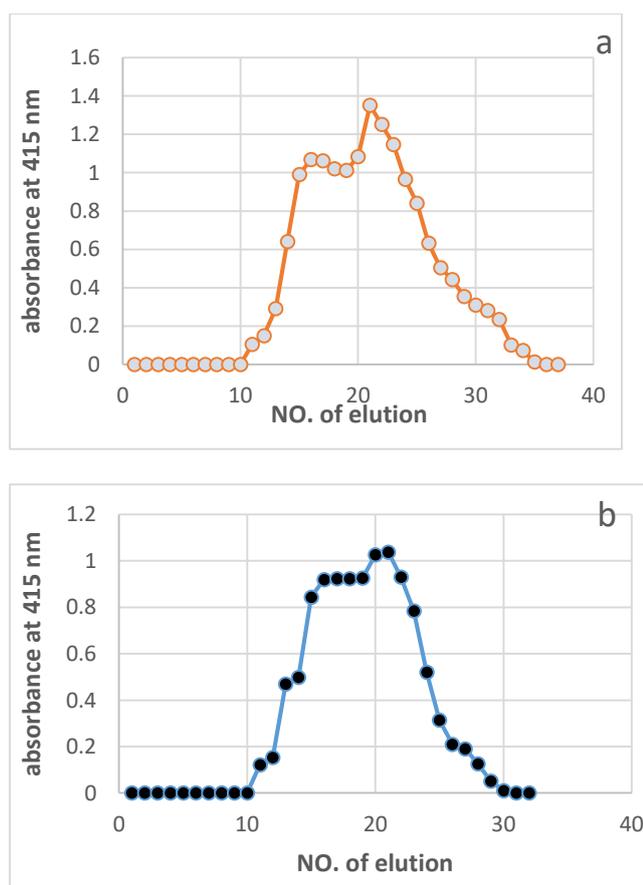
## Isolation and Quantification of Quercetin

### Separation Column

To isolate Quercetin from GWH-BMIMCl extract, a gel filtration technique was employed using a

Sephacryl S-200 packed bed, followed by measuring the absorbance at  $\lambda_{\text{max}} = 415 \text{ nm}$  for each fraction. The elution sample No. 11 indicated the first appearance of Quercetin through the column. The highest absorption of Quercetin in GWH-BMIMCl

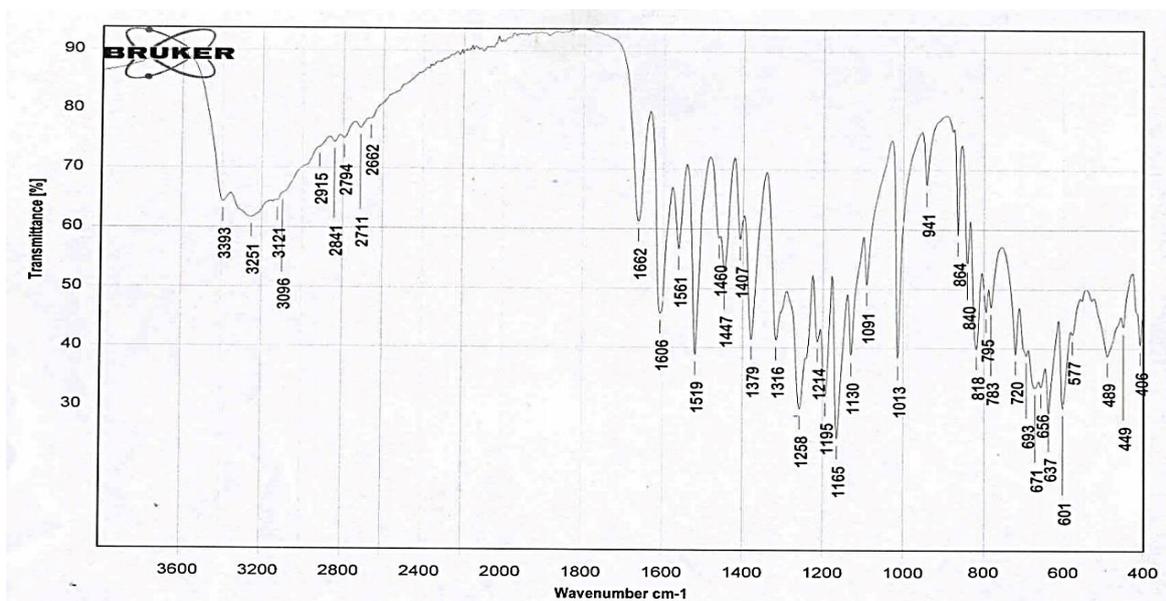
extract was at 1.352 in elution sample No. 21, and elution sample No. 35 indicated the completion of the purification process, as shown in Fig. 3a. The total number of elution samples collected during the purification process was thirty-seven. In order to know the accuracy of the results obtained from the purification of Quercetin in the GWH-BMIMCl extract, the standard Quercetin was loaded on a Sephacryl S-200 column following the same procedure for the extract. The elution sample No. 11 recorded the first appearance of standard Quercetin and the elution sample No. 21 recorded the highest absorbance (1.03) of the standard Quercetin, while elution sample No. 30 indicated the completion of the purification process. The total number of elution samples collected for standard Quercetin was thirty-two as shown in Fig. 3 b.



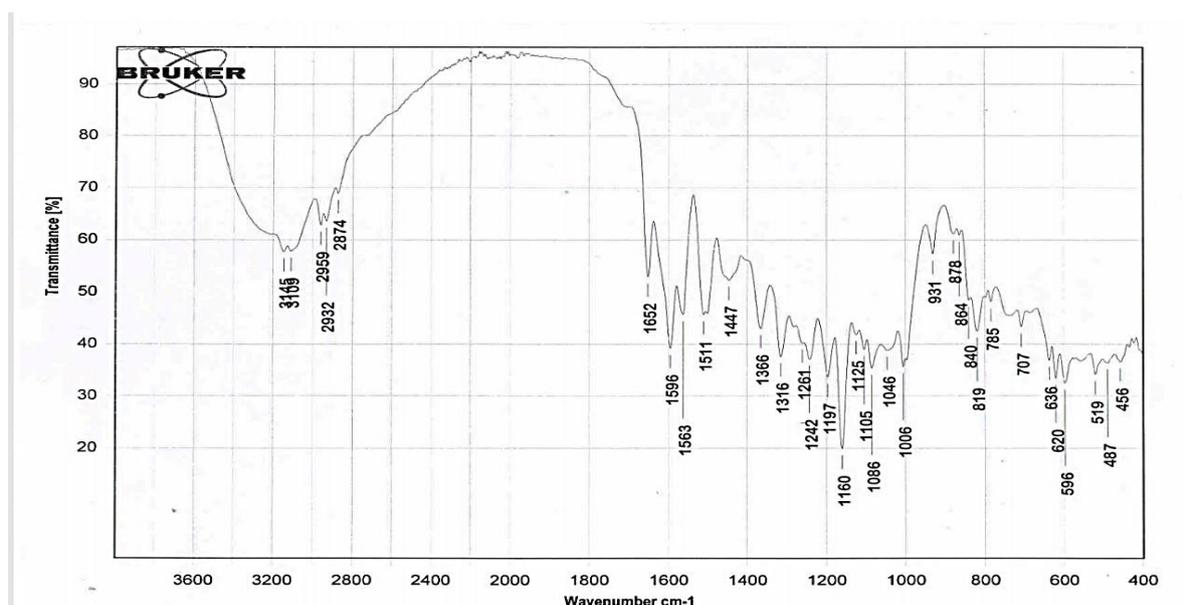
**Figure 3. Elution profile (a) elution volume of GWH-BMIMCl extract (b) elution volume of standard Quercetin.**

### Interpretation of FTIR Spectra

The FTIR spectrum of standard Quercetin and isolated Quercetin is presented in Fig. 4a,b, where its characteristic bands were detected. Within the standard Quercetin, OH groups were detectable at 3393 and 3251  $\text{cm}^{-1}$  while the OH-bending of the phenol function was detectable at 1379  $\text{cm}^{-1}$ . The expansion absorption of C = O aryl ketonic appeared at 1662  $\text{cm}^{-1}$ . C = C aromatic ring expansion bands were detectable at 1606, 1561 and 1519  $\text{cm}^{-1}$ . The plane C-H bending band in the aromatic hydrocarbon appeared at 1316  $\text{cm}^{-1}$ , out-of-plane bending bands were evident at 941, 818, 671 and 601  $\text{cm}^{-1}$ . Bands at 1258, 1195 and 1165  $\text{cm}^{-1}$  that are attributed to the C-O stretching of the aryl ether ring, C-O stretch in phenol, C-CO-C stretching and bending in ketones, respectively were recorded<sup>33</sup>. As for the isolated Quercetin, the OH groups were detectable at 3145 and 3109  $\text{cm}^{-1}$ , while the OH-bending of the phenol function was detectable at 1366  $\text{cm}^{-1}$ . The expansion absorption apparent at 1652  $\text{cm}^{-1}$  was for C = O aryl ketonic. C = C aromatic ring expansion bands were presented at 1596, 1563 and 1511  $\text{cm}^{-1}$ . It was possible to detect the C-H bending band in the aromatic hydrocarbon at 1316  $\text{cm}^{-1}$ , out-of-plane bending bands were evident at 931, 819, 671 and 636  $\text{cm}^{-1}$ . Bands in 1261, 1197 and 1160  $\text{cm}^{-1}$  which is attributed to the C-O stretching of the aryl ether ring, C-O stretching in phenol, and C-CO-C stretching and bending in ketones, respectively were observed. The FTIR spectrum of standard Quercetin was compared with the FTIR spectrum of isolated Quercetin and the results showed that isolated Quercetin was highly compatible with standard Quercetin.



A



B

Figure 4. FTIR spectrum of a) standard Quercetin, and b) isolated Quercetin.

### Qualitative Detection of Quercetin Using the HPLC Method

The chromatogram of the injected standard Quercetin ( $20 \text{ mg L}^{-1}$ ) showed the presence of a peak at a retention time of 2.6 minute that belongs to Quercetin as shown in Fig. 5a. A similar peak at the same retention time 2.5 minute as can be seen in Fig. 5b appeared after the injection of isolated Quercetin from GWH-BMIMCl extract<sup>34</sup>. The results of the isolated Quercetin showed a higher absorbance peak

(5.8) from the Quercetin standard absorbance (5.2), which confirms the presence of Quercetin in the isolated GWH-BMIMCl extract at a concentration higher than  $20 \text{ mg mL}^{-1}$ <sup>31</sup>. The isolated Quercetin peak width broadens due to an increase in concentration compared to standard Quercetin, where the higher the concentration of the material, the longer it takes to travel through the column, resulting in a broadening of the bandwidth<sup>35</sup>. The retention time of both the standard Quercetin and the isolated Quercetin from GWH-BMIMCl extract is

approximately similar indicating the presence of Quercetin in the isolated extract of GWH-BMIMCl.

### Determination of Quercetin (Total Phenolic Compound)

Quercetin as a member of flavonoid family was determined using aluminum chloride colorimetric assay depending on the formation of a stable complex between aluminum and keto groups in C-4 atoms and a hydroxyl groups at C-3 and C-5<sup>36</sup> using spectrophotometric and  $\mu$ PADs methods. The results presented in Fig. 6 a,b showed that the concentration of Quercetin isolated from GWH-BMIMCl extract using  $\mu$ PAD ( $19 \text{ mg mL}^{-1}$ ) was not substantially different from that of traditional spectrophotometric method ( $23.16 \text{ mg mL}^{-1}$ ). By comparing the two methods statically using ANOVA program (at 95% confidence level) for six samples, the results in Table 2 revealed that  $F_{\text{tab}} = 4.964$  is significantly greater than  $F_{\text{Stat}} = 0.204$ . Consequently, there was no statistically significant difference between the two methods. Furthermore, comparing the results from the spectrophotometric and  $\mu$ PAD methods revealed a good correlation between the two methods ( $r = 0.825$ ).

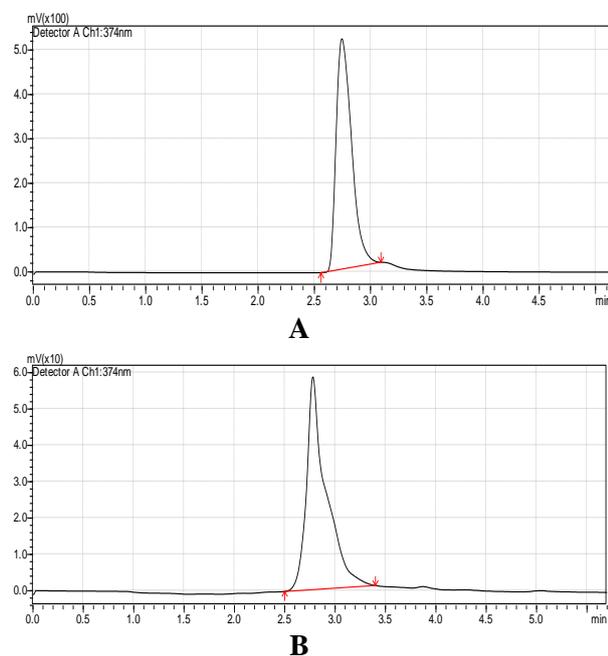


Figure 5. Chromatogram of a) standard Quercetin solution at a concentration of  $20 \text{ mg L}^{-1}$ , b) chromatogram of isolated Quercetin from GWH-BMIMCl extract.

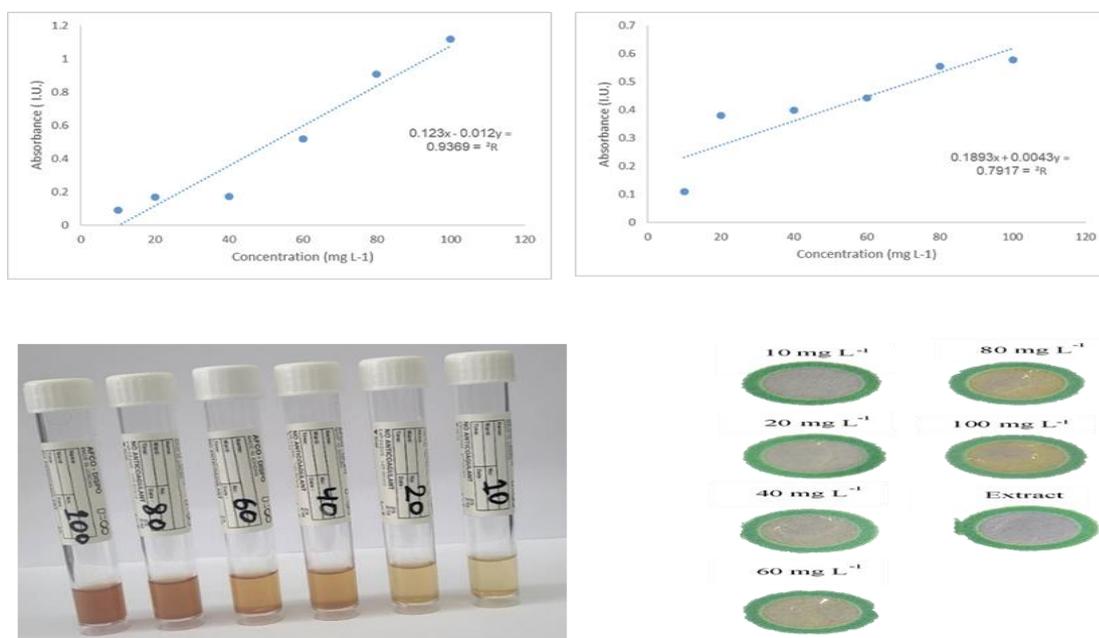


Figure 6. (a) Linear calibration graph for spectrophotometric determination of Quercetin concentration in the range between  $10\text{-}100 \text{ mg L}^{-1}$ , (b) calibration graph for  $\mu$ PADs determination of Quercetin concentration ( $10\text{-}100 \text{ mg L}^{-1}$ ), (c) an image of serial concentration of Quercetin in a volumetric flask, (d) an image of the paper microfluidic device with serial standard concentrations of Quercetin spotted inside the sensing zone.

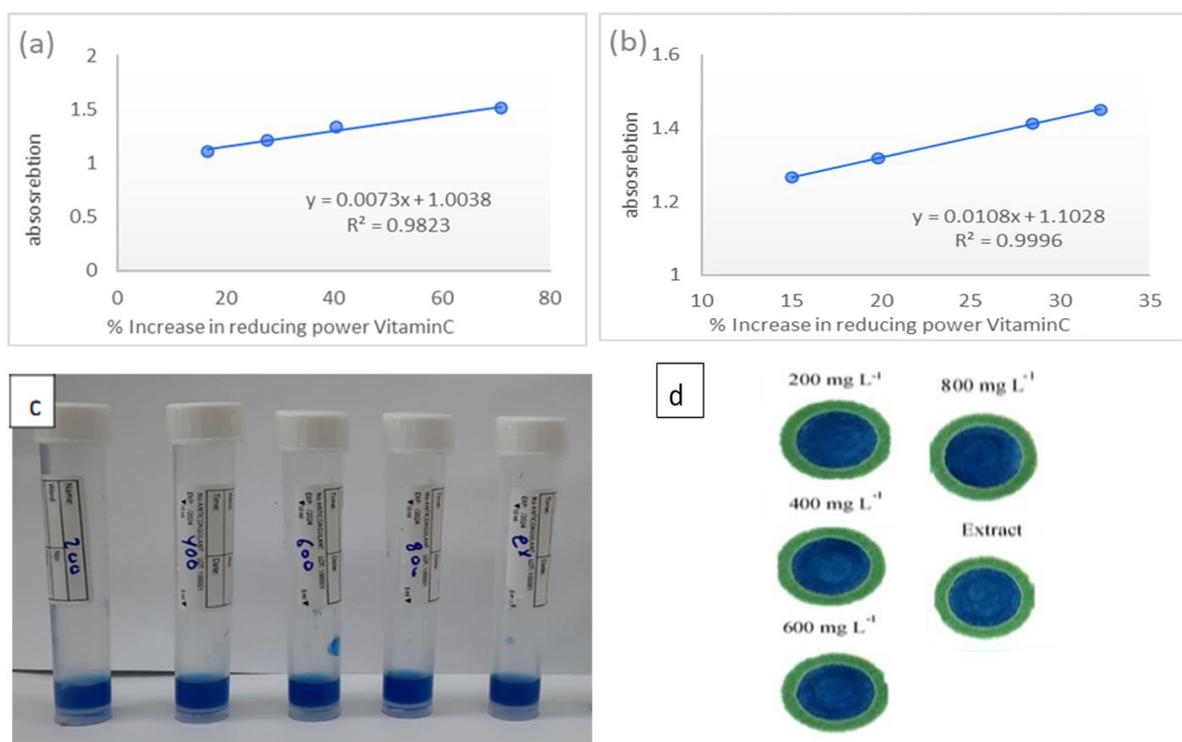
**Table 2. Statistical analysis using ANOVA program and correlation between spectrophotometric and  $\mu$ PADs methods.**

Source of variation	Sum of Squares	df	Mean Square	F stat	F tab	Method	Spector	$\mu$ PADs
Between Groups	0.022	1	0.022	0.204	4.964	Spector	1	0.825
Within Groups	1.076	10	0.108			$\mu$ PADs	0.825	1
Total	1.098	11						

### Reducing Power

Antioxidant activity was estimated using the reducing power assay which depends on the potential reduction of Quercetin that enables the reaction with potassium ferricyanide to form potassium ferrocyanide which reacts with ferric chloride to form a complex that is measurable at  $\lambda_{\max}=700 \text{ nm}$ <sup>37</sup> using spectrophotometric and  $\mu$ PADs methods. The results shown in Fig. 7 demonstrate the percentage increase in the reducing power of Quercetin using

$\mu$ PAD method was not statistically different from that of traditional Spectrophotometric. By comparing the two methods statically using the ANOVA program (at 95% confidence level) for six samples, the results in Table 3 revealed that  $F_{\text{tab}}= 5.987$  is significantly greater than  $F_{\text{stat}} = 1.470$ . As a result, there was no statistically significant difference between the two methods. Furthermore, comparing the results from the spectrophotometric and  $\mu$ PAD methods revealed a very good correlation between the two methods ( $r = 0.935$ ).



**Figure 7. (a) Linear calibration graph for spectrophotometric determination of Quercetin concentration in the range between (200-800) mg L<sup>-1</sup>, (b) Calibration graph for  $\mu$ PADs determination of Quercetin concentration (200-800) mg L<sup>-1</sup>, (c) an image of serial concentration of Quercetin in a volumetric flask, (d) an image of the paper microfluidic device with serial standard concentrations of Quercetin spotted inside the sensing zone.**

**Table 3. Statistical analysis using ANOVA program and correlation between spectrophotometric and  $\mu$ PADs methods.**

Source of variation	Sum of Squares	df	Mean Square	F stat	F tab	Method	Spector	$\mu$ PADs
Between Groups	451.546	1	451.546	1.470	5.987	Spector	1	0.935
Within Groups	1843.438	6	307.240			$\mu$ PADs	0.935	1
Total	2294.984	7						

## Conclusion

In this work, we have employed a green ionic liquid (BMIMCl) solvent to extract the antioxidant phytochemical compound Quercetin from the Iraqi green walnut husk to meet the requirements of an environment-friendly extraction procedure using a digestion method under optimized conditions including a solid-liquid ratio of 0.5 g: 15 ml, pH = 5, and incubation time of 15 minutes at 75 °C. The isolation of Quercetin from the extract was successfully done using a gel filtration technique and was confirmed using HPLC and FTIR techniques. Two methods were proposed for the quantitative determination of the concentration and antioxidant

activity of isolated Quercetin using a spectrophotometric method and compared with new paper-based microfluidic analytical devices ( $\mu$ PADs). The paper-based testing platform presents an easy, rapid, inexpensive, efficient and environmentally friendly treatment that could be used for quantitative and qualitative analysis of phytochemicals in herbal medicines. This platform introduces the idea of reducing the analytical activities' impact on the environment by offering an alternative *in situ* technique that can be used outside traditional laboratories.

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## Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been

included with the necessary permission for republication, which is attached to the manuscript.

- Ethical Clearance: The project was approved by the local ethical committee in University of Mustansiriyah.

## Authors' Contribution Statement

M. A. M. carried out the practical part of research manuscript. J. O. A. and Z. N. N. planned the conception, designed the research, supervised the project with providing support and giving the critical

feedback during writing the manuscript. All authors discussed the results and contributed to the final manuscript.

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## تقدير الكيورستين في قشور الجوز الأخضر العراقي باستخدام طريقة الاستخلاص المبنية على الهضم والترشيح بالجل باستخدام السوائل الايونية وتقديرها باستخدام المطيافية الضوئية وتقنية السوائل المايكروية الورقية

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### الخلاصة

في هذه الدراسة تم استخلاص، عزل وتقدير الكيورستين الموجود في قشور الجوز العراقي باستخدام طريقة جديدة تعتمد على الاستخلاص بطريقة الهضم المبنية على استخدام السوائل الايونية و طريقة الترشيح بالجل للفصل المقترنة مع المطيافية الضوئية للتقدير ومن ثم مقارنة مع تقنية جديدة تسمى تقنية السوائل المايكروية الورقية مستهدفة بناء طريقة اقتصادية، صديقة للبيئة وخضراء. ولقد تم اختيار 1 بيوتيل-3-مئيل ايمادزوليم كلورايد كمذيب اخضر بديل للمذيبات التقليدية بسبب خصائصه الذوبانية. ولقد اظهرت النتائج ان 100 ملغرام من القشور تحتاج الى 15 مل من المذيب و 30 دقيقة من الهضم عند درجة 75°C كظروف مثلى لاعطاء افضل ناتج للمستخلص بطريقة الهضم مقارنة مع طرق الاستخلاص الاخرى. تحت هذه الظروف المثلى تم عزل الكيورستين باستخدام طريقة الترشيح بالجل وتم التأكد من المركب المعزول باستخدام تقنية كروماتوغرافيا السائل عالية الاداء و اشعة فورية تحت الحمراء. تم تقدير الكيورستين المعزول من المستخلص لونيا ( طريقة كلوريد الالمنيوم) باستخدام طريقتي المطيافية الضوئية و تقنية السوائل المايكروية الورقية معطيا الخطية لمنحنيات المعايرة لكلتا الطريقتين بين 10-100 ملليغرام / لتر مع معامل ارتباط 0.9369 و 0.7917 لكلتا الطريقتين. ومن ثم تم تقدير الفعالية المضادة للاكسدة باستخدام طريقة قوة الاختزال للمركب المعزول مظهرة فعالية مضادة للاكسدة جيدة و بين تحليل التباين (ANOVA) عدم وجود فرق كبير بين تقنية المطيافية الضوئية وتقنية السوائل المايكروية الورقية. كخلاصة يمكن اعتبار المذيب الايوني BMIMCL مذيبا امنا ومناسبا لاستخلاص المركبات النباتية ذات الخواص الطبية وكذلك فان تقنية السوائل المايكروية الورقية اثبتت مقدرتها على التحليل بكفاءة مساوية الى طرق التحليل التقليدية بالاضافة الى خصائصها الخضراء.

**الكلمات المفتاحية:** مضادات الأكسدة، الاستخلاص الأخضر، قشر الجوز الأخضر، السوائل الأيونية، جهاز التحليل الورقي ميكروفلويديك، الكيورستين.