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Determination of Betanin, Apigenin, Catechin, and Caffeic Acid Levels in Iraqi Beetroot (*Beta vulgaris L.*) Extracts Using Various Extraction Methods

Malik K. Wadi and Ibtehaj M. Hakeem*

College of Agricultural Engineering Sciences, University of Baghdad, Iraq

*Corresponding author: Email: ibtehaj@coagri.uobaghdad.edu.iq

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ABSTRACT
Beetroot is renowned for its rich content of natural pigments and antioxidants, particularly betalains and flavonoids, which are linked to various health benefits. Due to these compounds, beetroot is increasingly viewed as a valuable natural alternative to artificial food additives. This study aimed to quantify the levels of betanin- a key pigment in beetroot- and certain flavonoid compounds in beetroot extracts using six different extraction methods. The extraction solvents included water, alcohol, three concentrations of citric acid (1%, 0.5%, and 0.2%), ascorbic acid, and a combination of citric and ascorbic acids. High-performance liquid chromatography (HPLC) was utilized to determine the concentrations of betanin and flavonoids in each extract. The analysis revealed that both betanin and flavonoid levels varied depending on the extraction method, with betanin consistently being the most prevalent compound across the samples. These results highlight the importance of choosing the right extraction solvent to optimize the recovery of desired compounds, whether betanin or specific flavonoids. Ultimately, this research supports the use of beetroot as a source of beneficial natural additives and emphasizes the necessity of careful selection of extraction techniques to maximize yield and effectiveness.
Keywords: Betanin, Flavonoids, Apigenin, Catechin, Caffeic acid

INTRODUCTION
Food coloring is essential for enhancing the visual appeal of foods and beverages, which significantly influences consumer preference and acceptance. While natural colorants exist, the food industry increasingly relies on synthetic dyes due to their lower production costs, brighter and more consistent colors, and greater stability over time. However, many products on the market contain unauthorized synthetic dyes or excessive amounts of approved ones. This misuse raises serious health concerns, including the risk of genetic mutations, cancer, lowered hemoglobin levels, and allergic reactions. These potential dangers underscore the need for strict regulation of artificial color additives to ensure consumer safety [1-3].
Beetroot is highly regarded for its numerous health benefits, largely due to its abundance of essential nutrients such as vitamins, minerals, phenolic compounds, carotenoids, nitrates, vitamin C, and betalains. These elements play a significant role in its status as a functional food. Betalains, the pigments that give beetroot its vibrant colors, come in two primary forms: betacyanins, which produce a red-violet hue, and betaxanthins, which create yellow-orange shades. These pigments are often used as natural food colorants because they are safe, stable, non-toxic, and devoid of carcinogenic or harmful effects. Consequently, beetroot has become a valuable ingredient in the food industry, frequently used as a natural dye or additive in products such as ice cream, yogurt, and a variety of other foods [4]. Also the Iraqi beetroot tubers have inhibitory activity against the alpha-amylase, antioxidant and antibacterial activity was reported, so that it can be considered a medicinal plant [5]. In recent years, researchers have increasingly focused on plant-based sources for food additives and natural colorants, particularly using environmentally friendly extraction techniques. Among these sources, beetroot has emerged as a standout option due to its dual benefits: it promotes health while also serving as an effective natural coloring agent [6]. In this study we aimed to use seven cold extraction methods to beetroot extraction. for each method, the extract was investigated for betanin and some of flavonoids compounds by high performance liquid chromatography (HPLC).

MATERIAL AND METHODS

Chemicals
Methanol, Phosphorsäure, Ameisensäure und Acetonitril (CDH, Indien). Standards für Betanin, Rutin, Apigenin, Gallussäure, Catechin und Kaffeesäure (Sigma Aldrich).

Samples and Extraction
Seven extraction methods for beetroot, as shown in the table below and described in our previous study [7], were investigated using HPLC to quantify Betanin, Rutin, Apigenin, Gallic acid, Catechin, and Caffeic acid. In each method, 50 grams of fresh beetroot were soaked in 100 mL of the corresponding solvent, blended, and then filtered. The supernatant was analyzed for the six flavonoids.

Table 1 Beetroot extraction methods

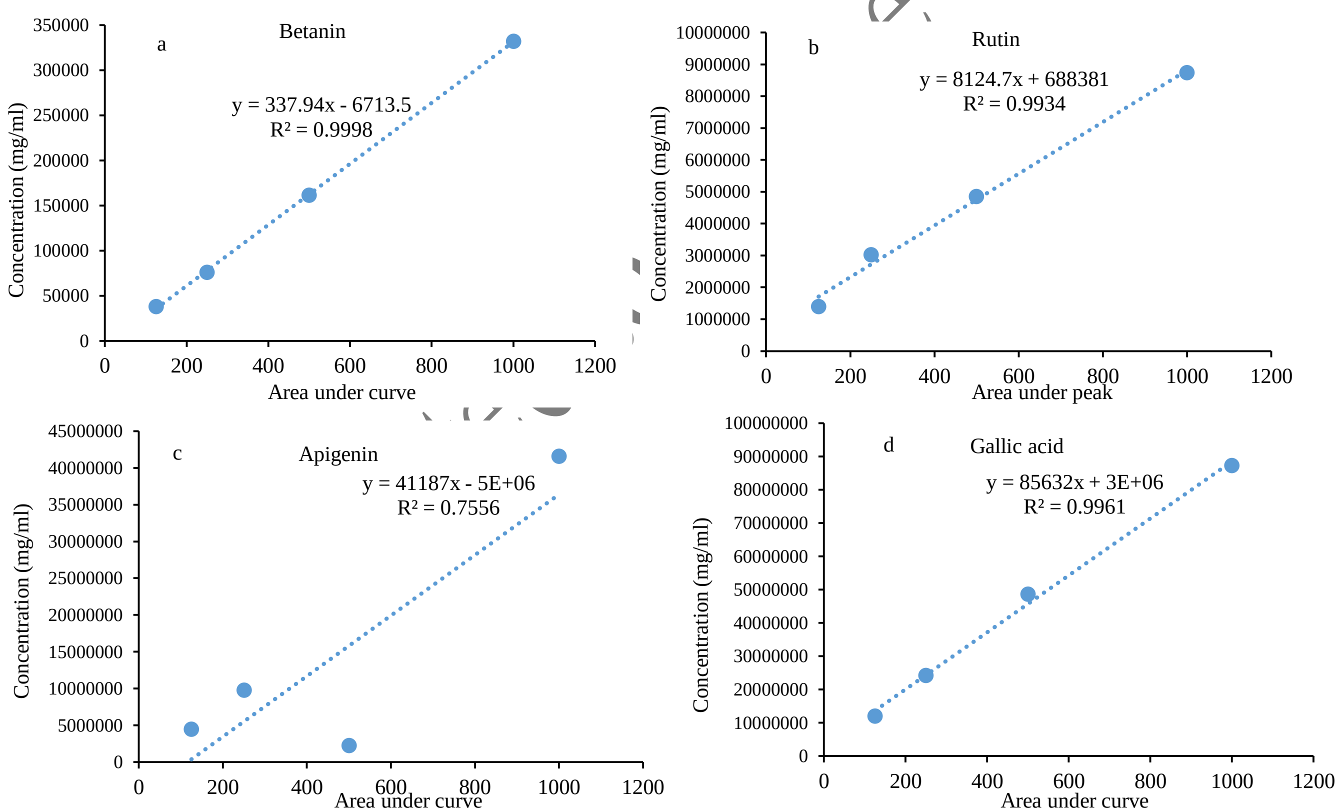
Sample ID	Extraction method
Sample 1	Water extraction
Sample 2	Alcoholic extraction
Sample 3	1% Citric acid
Sample 4	0.5 % Citric acid
Sample 5	0.2 % Citric acid
Sample 6	1 % Ascorbic acid
Sample 7	0.5 % Citric acid and 0.1 % Ascorbic acid

HPLC Methods

1. **Betanin:** Column: C18, 4.6 × 150 mm, 5 µm particle size. Mobile Phase: 80% water + 20% acetonitrile with 0.1% phosphoric acid (isocratic). Flow Rate: 0.8 mL/min. Detection Wavelength: 480 nm (for betanin absorption). Injection Volume: 20 µL [8]**.**
2. **Rutin:** Column: C18, 4.6 × 150 mm, 5 µm particle size. Mobile Phase: 80% water with 0.1% phosphoric acid, 20% acetonitrile (isocratic). Flow Rate: 1.0 mL/min. UV Detection Wavelength: 354 nm. Injection Volume: 20 µL [9].
3. **Apigenin:** Column: C18, 4.6 × 150 mm, 5 µm particle size. Mobile Phase: 60% water + 0.1% phosphoric acid, 40% acetonitrile (isocratic). Flow Rate: 1.0 mL/min. UV Detection Wavelength: 340 nm. Injection Volume: 20 µL [10]**.**
4. **Gallic Acid:** Column: C18, 4.6 × 150 mm, 5 µm particle size. Mobile Phase: 90% water (with 0.1% phosphoric acid), 10% methanol (isocratic). Flow Rate: 1.0 mL/min. UV Detection Wavelength: 270 nm. Injection Volume: 20 µL [11].
5. **Catechin:** Column: C18, 4.6 × 150 mm, 5 µm particle size. Mobile Phase: 90% water (with 0.1% phosphoric acid), 10% acetonitrile (isocratic) or gradient elution. Flow Rate: 1.0 mL/min. UV Detection Wavelength: 280 nm. Injection Volume: 20 µL [12].
6. **Caffeic Acid:** Mobile phase: 0.1% formic acid in water (A) and acetonitrile (B). Gradient elution: Start with 90% A and 10% B, then gradually increase B to 50% over 30 minutes. Flow rate: 1.0 mL/min. UV detection: 320 nm [13].

Standard Curve Plotting

For the six extracts, standard concentrations of 125, 250, 500, and 1000 mg/L of each flavonoid were analyzed using HPLC. The area under the peaks was plotted against concentration. Figure 1 shows the standard curve for betanin and other phenolic compounds.



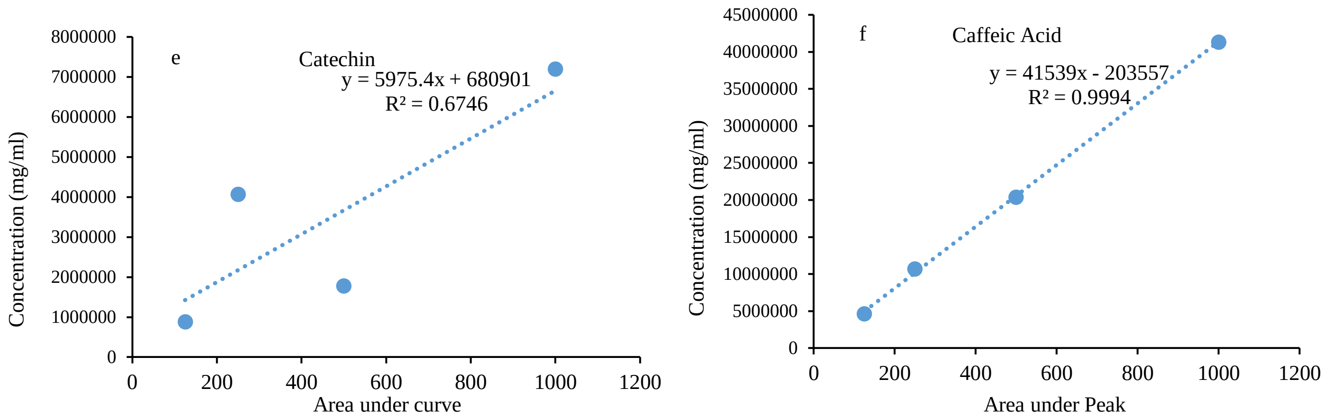


Fig. 1 Standard curves for (a): Betanin, (b): Rutin, (c): Apigenin, (d): Gallic Acid, (e): Catechin, and (f): Caffeic Acid

RESULTS AND DISCUSSION

Betanin is a natural pigment found in various fruits and vegetables, particularly known for enhancing the red color of products like beetroot. Table 2 and Figure 2 present the concentration of betanin extracted from beetroot using seven different methods. The highest yield was achieved with a sample extracted using a combination of 0.5% citric acid and 0.1% ascorbic acid, followed closely by the sample extracted with 0.2% citric acid. In contrast, the sample extracted with 0.5% citric acid alone yielded the lowest amount of betanin. These differences in extraction efficiency are likely influenced by variations in the pH of the extraction environment. This observation is supported by research conducted by *Noelia López* and colleagues, who also investigated how temperature impacts both the yield and stability of betanin during extraction [14].

Table 2 Betalin concentration (mg/L) in different extraction methods.

Extraction method	Betanin
Water extraction	628.39
Alcoholic extraction	116.77
Citric acid 1%	166.04
Citric acid 0.5 %	88.14
Citric acid 0.2 %	1375.59
Ascorbic acid 1 %	189.81
Citric acid 0.5 % and Ascorbic acid 0.1 %	5735.26

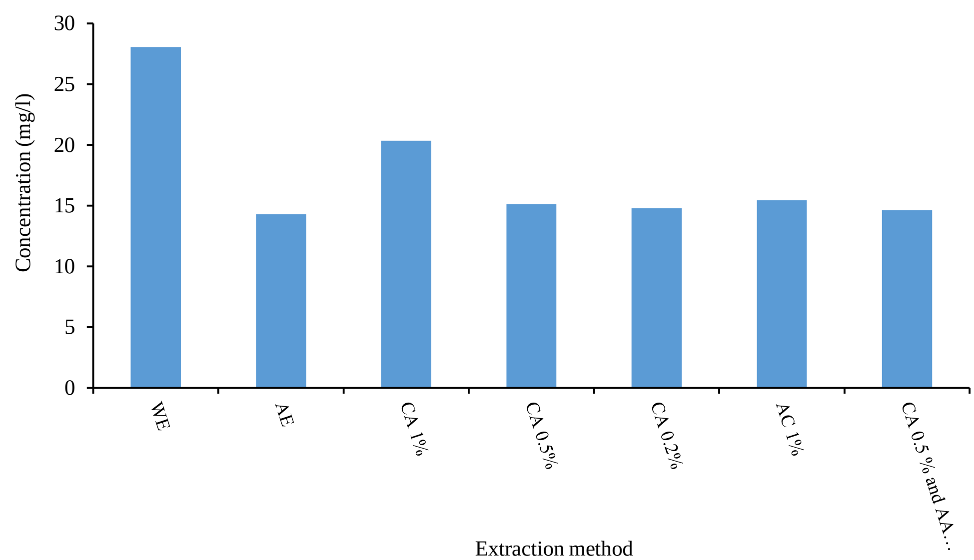


Fig. 2 Betalin levels in different extraction methods. Water extraction (WE), Alcoholic extraction (AE), Citric acid 1% (CA 1%), Citric acid 0.5 % (CA 0.5%), Citric acid 0.2 % (CA 0.2%), Ascorbic acid 1 % (AC 1%), Citric acid 0.5 % and Ascorbic acid 0.1 % (CA 0.5 % and AA 0.5 %)

The results other flavonoid (Rutin, Apigenin, Gallic acid, Catechin and Caffeic acid) which investigated in beetroot were illustrated in table (3) and figure (3) and explained in the following paragraphs.

Rutin (30,40,5,7-tetrahydroxy-flavone-3-rutinoside) is a flavonol glycoside, which has been reported to present clinically relevant functions, potentially beneficial in preventing diseases and protecting genome stability [15]. Rutin compound was identified in different Iraqi plants in different proportions and by the HPLC method [16]. The extraction method utilizing 0.5% citric acid combined with 0.1% ascorbic acid yielded the highest amount of rutin. Water extraction ranked second in yield, whereas using 0.5% citric acid alone produced the lowest rutin content. These differences in extraction efficiency are likely attributed to variations in the acidity of the extraction medium and potential changes in solvent polarity [17].

Apigenin, chemically known as 4′, 5, 7-trihydroxyflavone, has been utilized by humans in the form of plant extracts to treat various disorders and inflammatory conditions, prior to its identification as a core compound [18]. Among the various extraction methods tested, water extraction yielded the highest amount of apigenin, while alcoholic extraction did not recover any measurable quantity of this compound. These findings indicate that acidic extraction methods are less effective than water-based extraction for isolating apigenin [19].

Gallic acid (3,4,5-trihydroxybenzoic acid) is well soluble in ethanol and it is commonly known that a pure Gallic acid is a colorless, crystalline powder. Besides the fact that it is soluble in water, it can be also dissolved in alcohol, ether, and glycerol [20]. Table 3 shows that the highest yields of gallic acid were obtained using a water-alcohol mixture as the solvent, consistent with previous studies. Conversely, the use of citric acid in the extraction medium negatively affected the yield of gallic acid; higher concentrations of citric acid resulted in decreased amounts extracted. Furthermore, earlier research has identified gallic acid in methanolic extracts of Iraqi soybean seeds through HPLC analysis, highlighting its antibacterial and antifungal properties against various microorganisms [21].

Catechin, a type offlavan-3-ol, is a secondary metabolitethat provides antioxidant benefits in plants. The results indicated that the extraction method using 0.5% citric acid and 0.1% ascorbic acid yielded the highest amount of catechin, while the 0.5% citric acid method alone produced the lowest quantity. Additionally, catechin has been identified as one of the components found in beetroot [22]. Catechin was reported that has toxic properties which invested as anti-cancer agent and with selective cytotoxicity [23]. In this study, our results were correlated with another anti-cancer study that used similar extraction methods. The extraction method using 0.5% citric acid and 0.1% ascorbic acid yielded the highest levels of catechin, which demonstrated the greatest anti-cancer activity [24-26].

Caffeic acid is a type of polyphenol, a class of micronutrients known for their antioxidant properties. As shown in table (3), the results of caffeic acid was highest when the water was the solvent of extraction where the extraction was lowest when Citric acid 0.5 % was used as a solvent of extraction. caffeic acid is one of beetroot component and other plants, this fact was studied well [27]. In this study we investigate the effect of different extraction mediums.

Table 3 The other flavonoids concentration (mg/L) in different extraction methods**.**

Extraction method	Rutin	Apigenin	Gallic acid	Catechin	Caffeic acid
Water extraction	88.63	28.06	6.55	175.89	21.23
Alcoholic extraction	71.91	ND	16.06	163.03	20.39
Citric acid 1%	109.73	20.36	0.62	120.52	14.13
Citric acid 0.5 %	20.39	15.14	1.54	60.54	7.95
Citric acid 0.2 %	35.11	14.80	4.75	98.47	16.57
Ascorbic acid 1 %	157.05	15.46	8.03	147.55	10.11
Citric acid 0.5 % and Ascorbic acid 0.1 %	117.06	14.63	3.50	177.92	10.08

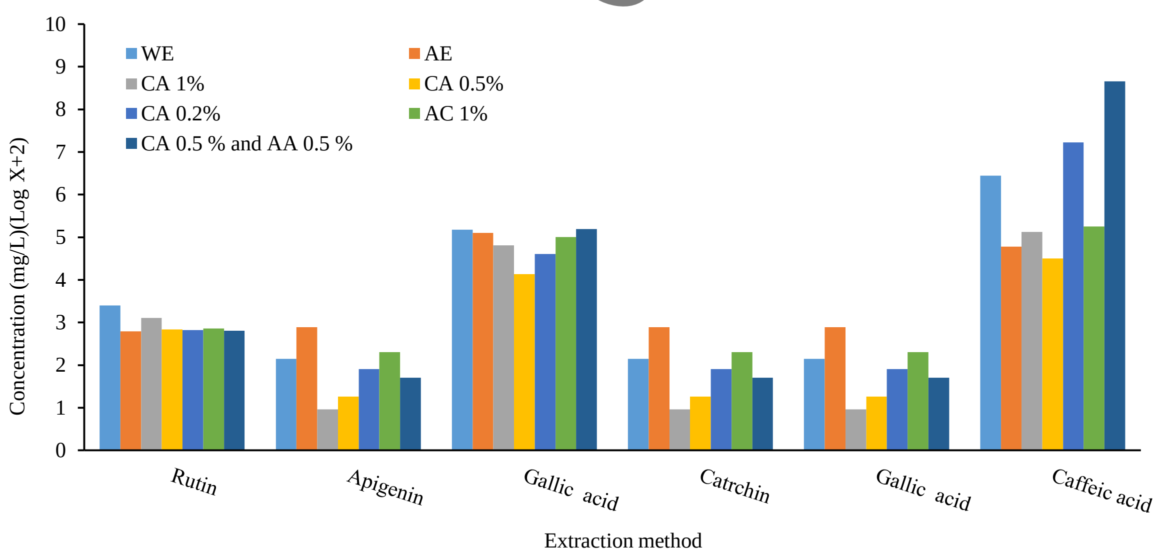


Table 3 The other flavonoids Levels in different extraction methods. Water extraction (WE), Alcoholic extraction (AE), Citric acid 1% (CA 1%), Citric acid 0.5 % (CA 0.5%), Citric acid 0.2 % (CA 0.2%), Ascorbic acid 1 % (AC 1%), Citric acid 0.5 % and Ascorbic acid 0.1 % (CA 0.5 % and AA 0.5 %)

The findings indicated that beetroot extracts have significantly higher levels of betanin than other flavonoids. As a naturally occurring pigment, betanin serves as a viable alternative to synthetic food colorants. In addition to its coloring properties, betanin is known for its antioxidant and anticancer effects. Utilizing raw beetroot extract as a food dye may also offer extra health benefits, as it contains other flavonoids with similar protective properties. The strong antioxidant capacity of beetroot extracts can be attributed to the high concentration of phenolic groups in betanin and the presence of flavonoids, both rich in free hydroxyl groups that effectively neutralize free radicals [28].

The differences in the amounts of a specific compound extracted using various methods can often be attributed to the compound's unique characteristics, including its molecular structure, functional groups, ionization tendencies, and solubility in different solvents. Each extraction technique functions within a distinct pH environment, which can affect these properties. Furthermore, slight changes in solvent polarity—whether among different aqueous solutions or in comparison to alcohol—may also contribute to these variations.

CONCLUSION

This study shows that beetroot is a rich source of betanin, making its extracts ideal for use as natural food colorants or additives that enhance food quality. Furthermore, the presence of various flavonoids—some identified in our research—boosts the nutritional and health benefits of beetroot extracts. Additionally, the extraction method chosen significantly affects the yield of these beneficial compounds, enabling the selection of the most effective technique based on the specific compound of interest.

REFERENCES

1. Dey S., Nagababu B.H. Applications of food color and bio-preservatives in the food and its effect on the human health. Food Chemistry Advances. 2022; 1: 100019.

2. Roy S. Membrane-associated RING ubiquitin ligase RNF-121 and advancement of cancer. Cellular, Molecular and Biomedical Reports. 2025; 5(2): 80-90.

3. Valizadehkakhki E., Mohammadi Zonoz F., Saljooghi A.S., Matin M., Ahmadi Koopaee Z.S. Toxicity comparison of two nanocarriers, magnetic mesoporous silica and polyoxometalate, using in vitro methods in PC-3 cells. Cellular, Molecular and Biomedical Reports. 2025; 5(3): 187-197.

4. Chhikara N., Kushwaha K., Sharma P., Gat Y., Panghal A. Bioactive compounds of beetroot and utilization in food processing industry: A critical review. Food Chemistry. 2019; 272: 192-200.

5. Hussein S., Laluf A., Ahmed Y., Ahmed B., Iyad A. Determination of inhibition activity of α-amylase enzyme, antioxidant activity, antibacterial activity and phenolic compounds by using some medical plants. Iraqi Journal of Agricultural Sciences. 2020; 51(1).

6. Borjan D., Šeregelj V., Andrejč D.C., Pezo L., Šaponjac V.T., Knez Ž., Vulić J., Marevci M.K. Green techniques for preparation of red beetroot extracts with enhanced biological potential. Antioxidants. 2022; 11(5): 805.

7. Wadi M.K., Hakeem I.M. Antioxidants and Anticancer Activity of Beetroot (Beta vulgaris L.) Extract. Journal of Medicinal plants and By-Products. 2024.

8. Sandate-Flores L., Rodríguez-Hernández D.V., Rostro-Alanis M., Melchor-Martínez E.M., Brambila-Paz C., Sosa-Hernández J.E., Parra-Saldívar R., Rodríguez-Rodríguez J., Iqbal H.M. Evaluation of three methods for betanin quantification in fruits from cacti. MethodsX. 2022; 9: 101746.

9. Fang Q., Wu P., Yang L. Separation and determination of puerarin by high performance liquid chromatography. Yao xue xue bao= Acta pharmaceutica Sinica. 1983; 18(9): 695-699.

10. Farhan M.M., Hassawi D.S., Ibraheem N.K. Extraction, fractionation, and seperatione of cynaroside and scolymoside from Cynara scolymus L. grown in Iraq. The Iraqi Journal of Agricultural Science. 2023; 54(2): 421-430.

11. Singh M.P., Gupta A., Sisodia S.S. Qualitative analysis of gallic acid by HPLC method in different extracts of Terminalia bellerica Roxb. fruit. FABAD Journal of Pharmaceutical Sciences. 2019; 44(2): 101-106.

12. Li D., Martini N., Wu Z., Wen J. Development of an isocratic HPLC method for catechin quantification and its application to formulation studies. Fitoterapia. 2012; 83(7): 1267-1274.

13. Woottisin N., Kongkiatpaiboon S., Sukprasert S., Sathirakul K. Development and validation of stability indicating HPLC method for determination of caffeic acid, vitexin and rosmarinic acid in Thunbergia laurifolia leaf extract. Pharmacognosy Journal. 2020; 12(3).

14. López N., Puértolas E., Condón S., Raso J., Alvarez I. Enhancement of the extraction of betanine from red beetroot by pulsed electric fields. Journal of Food Engineering. 2009; 90(1): 60-66.

15. Sharma S., Ali A., Ali J., Sahni J.K., Baboota S. Rutin: therapeutic potential and recent advances in drug delivery. Expert opinion on investigational drugs. 2013; 22(8): 1063-1079.

16. Al-Abide N. Morphological and chemical characteristics of two species belong to Alysseae and Lepidieae tribes spread in northern Iraq. Iraqi Journal of Agricultural Sciences. 2022; 53(4): 911-921.

17. Gullon B., Lú-Chau T.A., Moreira M.T., Lema J.M., Eibes G. Rutin: A review on extraction, identification and purification methods, biological activities and approaches to enhance its bioavailability. Trends in food science & technology. 2017; 67: 220-235.

18. Ali F., Rahul, Naz F., Jyoti S., Siddique Y.H. Health functionality of apigenin: A review. International Journal of Food Properties. 2017; 20(6): 1197-1238.

19. Cvetanović A., Švarc-Gajić J., Gašić U., Tešić Ž., Zengin G., Zeković Z., Đurović S. Isolation of apigenin from subcritical water extracts: Optimization of the process. The Journal of Supercritical Fluids. 2017; 120: 32-42.

20. Sarvamangala D., Babu C., Sowjanya N. Production of gallic acid-a short review. Int. J. Sci. Res. Methodol. 2015; 4: 125-132.

21. Alsaloom A.N. Testing and evaluation of bioactive compounds in soybean. Iraqi Journal of Agricultural Sciences. 2023; 54(1): 85-92.

22. Georgiev V.G., Weber J., Kneschke E.-M., Denev P.N., Bley T., Pavlov A.I. Antioxidant activity and phenolic content of betalain extracts from intact plants and hairy root cultures of the red beetroot Beta vulgaris cv. Detroit dark red. Plant foods for human nutrition. 2010; 65: 105-111.

23. Khattab S.A., Hussien W.F., Raafat N., Ahmed Alaa El-Din E. Effects of catechin hydrate in benzo [a] pyrene-induced lung toxicity: Roles of oxidative stress, apoptosis, and DNA damage. Toxicology Mechanisms and Methods. 2021; 31(6): 467-475.

24. Wadi M.K., Hakeem I.M. Antioxidants and Anticancer Activity of Beetroot (Beta vulgaris L.) Extract. Journal of Medicinal plants and By-Products. 2024; In Press.

25. Ramezani S.R., Mojra A., Tafazzoli-Shadpour M. Investigating the effects of substrate stiffness on half-maximal inhibitory concentration of chemical anticancer drugs, cell viability and migration of cell lines. Cell Mol Biomed Rep. 2025; 5(3): 141-147.

26. Reddy K.T.K., Reddy A.S. Recent Breakthroughs in Drug Delivery Systems for Targeted Cancer Therapy: An Overview. Cell Mol Biomed Rep. 2025; 5(1): 13-27.

27. Ye J.-C., Hsiao M.-W., Hsieh C.-H., Wu W.-C., Hung Y.-C., Chang W.-C. Analysis of caffeic acid extraction from Ocimum gratissimum Linn. by high performance liquid chromatography and its effects on a cervical cancer cell line. Taiwanese Journal of Obstetrics and Gynecology. 2010; 49(3): 266-271.

28. Abid S., Abdula A.M., Al Marjani M., Abdulhameed Q. Synthesis, antimicrobial, antioxidant and docking study of some novel 3, 5-disubstituted-4, 5-dihydro-1H-pyrazoles incorporating imine moiety. Egyptian Journal of Chemistry. 2019; 62(4): 739-749.