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Effect of different extraction methods on apigenin yield, antioxidant activity, and anticoagulant potential of watercress (*Eruca vesicaria* L.) leaves

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ABSTRACT
Watercress (*Eruca vesicaria*) is a rich source of secondary metabolites with disease-preventive properties, including the phenol apigenin. This work aimed to examine the impact of various extraction methods on apigenin yield from watercress (*Eruca vesicaria* L.) leaves and its antioxidant capacity/blood coagulant effect. Organisations compared modern with traditional extraction methods. Active compounds identification and overall qualitative chemical analysis proved that the extract is rich in flavonoids, phenols, and terpenes. The selected solvent and the method of extraction had a remarkable impact on both yield and purity of apigenin. Free radical scavenging ability of saliva and phenolic content were also found high and these values were correlated, suggesting that its phenols may significantly contribute for the high oxidative activity. The results of this study further showed that apigenin is involved in modulating the blood coagulation system via prolonging prothrombin time (PT) and activating activated partial thromboplastin time (aPTT). This indicates that Ap inhibits intrinsic as well extrinsic coagulation paths. These results suggest that the leaves of E. vesicaria represent a rich source of apigenin, which may be used as potential phytochemicals in nutraceutical and cardiovascular disorders related to oxidative stress and thrombosis-induced diseases prevention.

KEYWORDS: Apigenin's estimation, different extraction techniques, oxidative activity, clotting factors, PT,PTT.

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التأثير المقارن لتقنيات الاستخلاص على إنتاجية الأبيجينين من أوراق نبات الجرجير البري (*Eruca vesicaria* L): القدرة المضادة للأكسدة وتعديل التخثر

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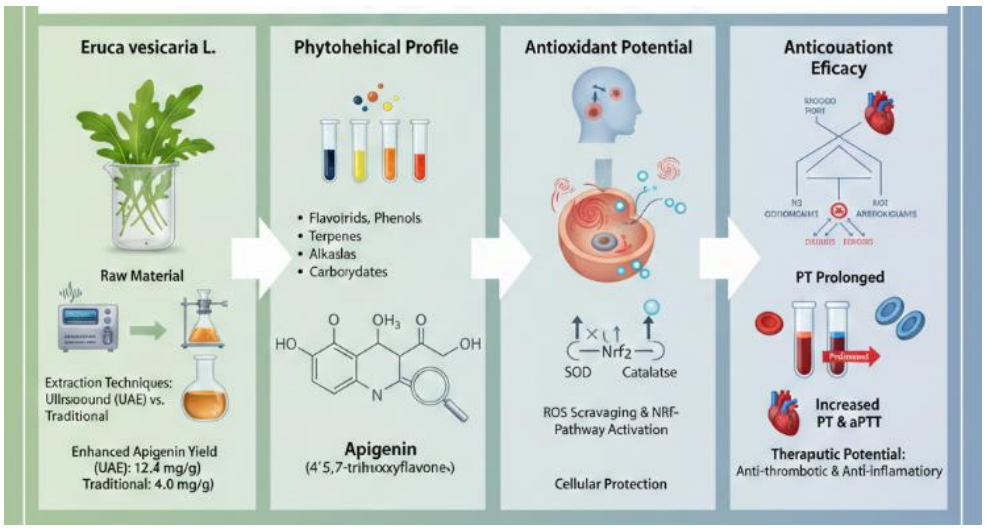
الملخص
يُعد نبات الجرجير المائي (***Eruca vesicaria***) مصدراً غنياً بالمركبات الأيضية الثانوية ذات الخصائص الوقائية من الأمراض، بما في ذلك الفينول أبيجينين. هدفت هذه الدراسة إلى فحص تأثير طرق الاستخلاص المختلفة على إنتاجية الأبيجينين من أوراق الجرجير المائي (***Eruca vesicaria* L.**) وقدرته المضادة للأكسدة وتأثيره على تخثر الدم. قارنت الدراسة بين طرق الاستخلاص الحديثة والتقليدية. أثبت تحديد المركبات الفعالة والتحليل الكيميائي النوعي الشامل أن المستخلص غني بالفلافونويدات والفينولات والتربينات. كان للمذيب المُختار وطريقة الاستخلاص تأثير ملحوظ على كل من إنتاجية ونقاء الأبيجينين. كما وُجد أن قدرة اللعاب على التخلص من الجذور الحرة ومحتوى الفينولات مرتفعان، وكانت هذه القيم مترابطة، مما يشير إلى أن الفينولات قد تُساهم بشكل كبير في النشاط التأكسدي العالي. أظهرت نتائج هذه الدراسة أيضًا أن الأبيجينين يُشارك في تنظيم نظام تخثر الدم عن طريق إطالة زمن البروثرومبين **(PT)** وتنشيط زمن الثرومبوبلاستين الجزئي المنشط **(aPTT).** يشير هذا إلى أن الأبيجينين يثبط مسارات التخثر الداخلية والخارجية. وتوحي هذه النتائج بأن أوراق نبات ***E. vesicaria*** تُعدّ مصدرًا غنيًا بالأبيجينين، الذي يُمكن استخدامه كمركب كيميائي نباتي محتمل في مجال التغذية العلاجية والوقاية من اضطرابات القلب والأوعية الدموية المرتبطة بالإجهاد التأكسدي والأمراض الناجمة عن التخثر.
الكلمات المفتاحية: تقدير الأبيجينين، تقنيات استخلاص مختلفة، النشاط التأكسدي، عوامل التخثر، زمن البروثرومبين، زمن الثرومبوبلاستين الجزئي.



Graphic Abstract

INTRODUCTION

The species Eruca vesicaria L. Thell cav. Sativa (Mill.) subspecies is the name given to watercress, an edible leaf vegetable with medicinal values and classified under the Brassicaceae family. This species is popularly eaten as fresh vegetables in salads and its leaves and flowers used in Mediterranean regions (Bouacida et al., 2022). Watercress plant is grown in some countries because of its oil that has multiple therapeutic benefits (Bhat et al., 2019). Seeds and leaves of watercress plant contain varied medicinal uses such as being a diuretic, laxative, and stimulant (Alam et al., 2006). Moreover, watercress's ethanolic extract has pharmacological efficacy against many diseases (Salehi et al., 2021; Sharifiaghdam et al., (2023).

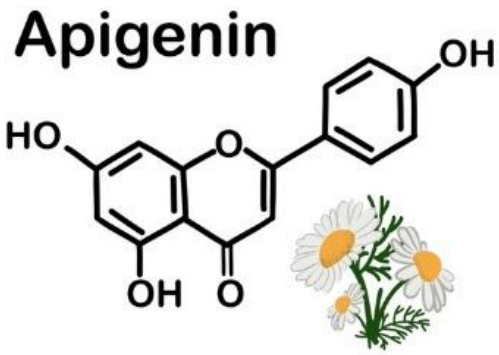


Figure 1. Structure Apigenin

E. Vesicaria, which is also known as rocket or watercress, is a well-known plant in the Brassicaceae family together with many secondary metabolites and medicinal potentials (Botella et al., 2025; Elsherif et al., 2025). Besides the nutritive purposes, this plant contains many bioactive compounds, namely glucosinolates, isothiocyanates, and polyphenols (Brahimi et al., 2025). Flavonoids, particularly apigenin (4',5,7-trihydroxyflavone), have attracted a considerable attention

of researchers due to its multifarious biological activities. Strokes and cardiovascular diseases continue to be one of the biggest global health problems that lead to high mortality rate and disability. The discovery of the natural blood thinners obtained from medicinal plants becomes more and more necessary every day. Such alternatives are promising since they provide the opportunity for safer, long-acting treatment with less side effects. The identification and study of molecular species from the Asteraceae family become extremely important to obtain the needed bioactive compounds.

Apigenin (4',5,7-trihydroxyflavone), a natural bioactive flavonoid, which is common in the plant kingdom, attracts the attention of pharmacologists due to its wide spectrum of therapeutic properties (Uslu, 2024; Markou et al., 2024). Besides direct scavenging of radicals, the antioxidant activity of apigenin works primarily through the modulation of endogenous cellular antioxidants. It has proved that it stimulates the process of nuclear translocation of Nrf2 and activates its target antioxidants including SOD and catalase (Grami et al., 2024; Park et al., 2024). Such induction will create the background for DNA and proteins to get protected from oxidative damage. As for the regulation of blood, apigenin is an important factor of hemostasis in the vascular system.

Antithrombosis effect of apigenin not only blocks the coagulation system, but it can also inhibit platelet aggregation (Bell et al., 2023; Mian et al., 2023). So far, apigenin was included into the list of natural compounds with anticoagulant and antioxidant properties that affect the blood flow (Tsiailanis &Tellis, 2023). However, the hydrophobic and low water solubility of apigenin creates some difficulties for its further clinical application (Daneshvar et al., 2023). This pharmacokinetic issue proves the necessity of search for the methods of high extraction of apigenin, namely SFE and UAE to increase the extraction without changing its structure (Adhikari et al., 2024). Therefore, the apigenin obtained in different ways should be considered for use in pharmaceutical and dietary supplement industry (Zhang & Chen, 2025). Although water celery *E. vesicaria* has been already studied by several authors, who analyzed its content of kaempferol and quercetin derivatives, no detailed data are available regarding the extraction of apigenin in free or glycosylated forms, for these thermally labile flavonoids, this has important implications for the validity of only extraction procedure-dependant information published (Chen et al., 2020). Moreover, in a clinical confederation the oxygen reactive species were recently associated with the coagulation. Oxidative stress is a common cause of procoagulant states (thromboembolic disorders) (Gupta et al., 2021). It was reported that extracts containing phenol could resist hemostasis by increasing the PT and aPTT time, which inhibited intrinsic and extrinsic blood coagulation pathways (Kashyap et al., 2022). The responsible contribution of the apigenin content in E. vesicaria for modifying blood coagulation, however, was not well-founded yet, despite its long use as traditional remedy. In the current research, the objective is to determine the efficiency of different methods of extraction in the recovery of apigenin from the leaves of E. vesicaria. The relationship of the separation efficiency, antioxidant

property and its function as a natural anticoagulant/decoagulant of this extract has been investigated in the current research, which would provide a scientific basis for future applications in functional food and biopharmaceutical industry. In addition to optimizing the recovery process of apigenin through green technologies, the current study has also provided the first scientific evidence of its double role as a regulator of blood coagulation mechanisms.

MATERIALS AND MATHODS

Plant Collection and Identification

Leaves of *E. vesicaria* were collected from different locations in central Iraq between April and mid-June 2024. All aerial parts of the plant were carefully and meticulously collected to ensure they were clean and free of insects and shrubs. A sample of the plant was then taken to the University of Baghdad/Natural History Museum/Iraq, where the herb was dried, ground, and carefully preserved.

Extraction methods

The plant material was washed, air-dried, and ground into a fine powde, the best solvent was selected based on its extraction efficiency, and the most effective was a 1:1 mixture of chloride and oxalic acid (NADES 5), with the addition of 30% water to reduce viscosity, two extraction methods were used:

1. Supercritical Fluid Extraction (SFE): This is the "purest and most advanced" method (Green Extraction), yielding impressive results in molecular docking research because it provides an extract free of impurities and toxic solvents. Carbon dioxide (CO2) gas is used in the supercritical state. Apigenin is a slightly polar compound; therefore, a modifier such as methanol or ethanol (5–10%) was added to improve extraction efficiency. Pressure adjusts the pressure between 250 and 300 bar, Temperature 45°C (to maintain compound stability), Flow rate 2 ml/min (Vafaei et al., 2022).

2. Soxhlet Extraction: 50gm of leaf powder was prepared in a Soxhlet apparatus at a temperature of 40 °C with a volume of 250 ml of ethanol for 6 hours (Rajesh, et al., 2023).

Biological Study

1. Measure the free radical scavenging activity of the extracts using 2,2-diphenyl-picryl-hydrazil (DPPH) reagent. Prepare an extraction solution at a concentration of 1 mg/mL in methanol. Next, 3 mL of 0.06 mM DPPH solution was mixed with 0.77 µL of extract solution**.** Stir the sample and place it in the thermostat for 15 minutes at room temperature. Absorbance at 515 nm was then measured using a UV-VIS spectrophotometer (CARY 50 UV-VIS). Add 0.77g methanol to the DPPH solution to make a control solution and measure the absorbance immediately. Antioxidant activity was calculated accordingly and expressed as percent inhibition (Gulcin & Alwasel 2023).

Inhibition percentage of DPPH free radical (100 X A_DPPH / (Sample A –A_DPPH) = Inhibition % A_DPPH absorbance of the control sample (+ DPPH methanol) . Sample A is the absorbance of the sample (Akgül et al., 2022)

2. Measurement of clotting factors PT and PTT:

Venous blood samples were collected from 10 healthy donors, whose ages ranged between 18-35 years. The donors had not taken any medications, including anticoagulants or antibiotics, during the two weeks prior to blood collection. The blood samples were placed in the centrifuge for 15 minutes at a speed of 2000 revolutions per minute, after which the obtained plasma was treated with different concentrations (8, 16, 32 mg/ml) of ethanolic extract and (2.5, 5, 10 mg/ml). of flavonoids in a ratio of 1:1 (plasma: extract) and then incubated for 5 minutes at 37 °C, this mixture will be ready for PT and PTT testing, and the plasma will be only as a control model. PT and PTT coagulation factor tests were measured using an assay kit (Stago, France) **(**Zaidi & Rout, 2025**)**

Statistical analysis

The analysis was conducted using Statistical analysis was performed using GenStat software (10th edition). sing one-way ANOVA followed by Tukey's test. Significance was set at $p < 0.05$. Significant differences of the means were also tested using Least significant difference L.S.D at the probability level 0.05.

RESULTS AND DISCUSSION

Apigenin content in leaf extract

As shown in Figure 2, the SFE extraction yielded the highest apigenin content (12.4 mg/g), while the Soxhlet method yielded 4 mg/g.

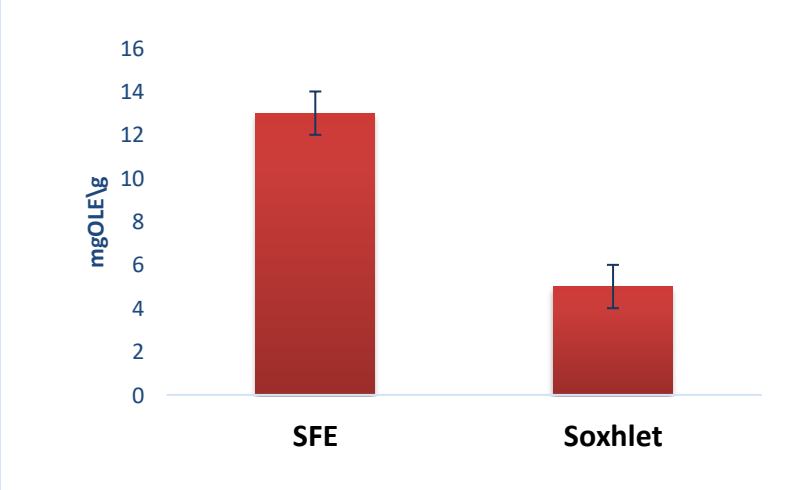


Figure 2. Determination of the amount of Apigenin using Two methods of extraction, (LSD 5%=1.19)

The results showed the superiority of supercritical pressure extraction (SFE) in the purity of apigenin and relies on the phenomenon of Acoustic cavitation does not apply to SFE; it should only be linked

to the ultrasound extraction method. which leads to the mechanical destruction of cell walls, which facilitates the release of phenolic compounds. As for SFE, it relies on high pressure that allows carbon dioxide to penetrate as an "ideal solvent" that selects oligopolar molecules such as apigenin, which reduces the extraction of impurities (such as chlorophyll and waxes), and this explains the increase in bioactivity despite the sometimes-low amount.

Several research efforts have been directed towards developing environmentally friendly extraction methods from plant sources, especially high-value agricultural plant materials such as the leaves of the plant *E. vesicaria*, known for their content of biologically active phenolic compounds and essential oils [Guo et al., 2023; Allemailem et al., 2024), but this method poses major issues with respect to environmental sustainability and potential community health concerns for harmful solvent residues. In this context, green extraction technologies (GE) (namely supercritical fluid extraction and ultrasound-assisted extraction) have arisen as superior, environmentally friendly and cheap alternatives (Cheng et al., 2024). These novel methods allow high-purity extracts to be produced, which contain no dangerous residual substances but still have the original biological activities of the plant and can find applications in green chemistry for drug or nutraceutical purposes (Hong et al., 2023).

Antioxidant effectiveness

The ability of extract ultrasound and under critical pressure and Soxhlet extracted from *E. vesicaria* to capture free radicals was tested using the compound DPPH 2,2-diphenyl-1-picrylhydrazyl that converts to 1,1-diphenyl -2- picrylhydrazine in a reductive form DPPH-H, and compared this ratio is that of Ascorbic acid.

Figure 3 shows that antioxidant activity differed significantly among the three extracts, with ultrasound extraction exhibiting the highest free radical scavenging activity, comparable to ascorbic acid.

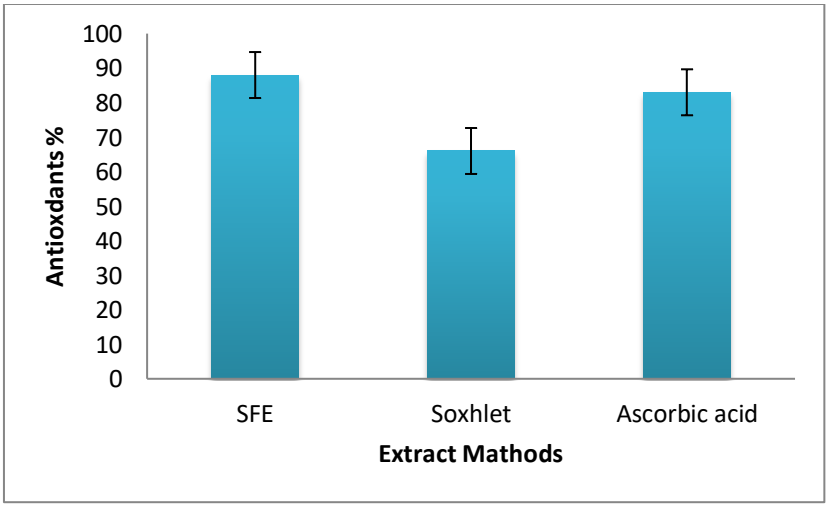


Figure 3. Percentage of the antioxidant activity of Apigenin by different extraction methods, (LSD 5%= 2.72)

The first practical experiment was to determine the effectiveness of various plant extracts, prepared using different methods, as electron donors or free radical scavengers, as expressed by their antioxidant activity. The oxidation rate recorded using the Suxelt method was 66%, which was compared to 83% using ascorbic acid. This difference opens a wide field for in-depth scientific study into the mechanisms of action of biologically active compounds and their effects on structural and functional integrity.

We do not generally in toxic activity speak about percentages (66% and 83%) as being of one or other of the two positive quantitative labs. methods which frequently feature in laboratory protocols, notably from the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay as a measure of an extract's quenching" or inhibition power against free radicals. The larger the percentage, the higher activity and this is referred to as a percentage of total phenolic or flavonoid compounds extracted as compared with the total mass of plant material itself.

The significant antioxidant effect (83% free radical scavenging ability) provides a scientific rationale for the improved biological activity of the extract in quenching free radicals either via hydrogen atom transfer or single-electron transfer. In terms of phytochemical content, the Soxhlet extract was found to have lower antioxidant content which could be contributed due to the thermal breakdown of polyphenols and flavonoids (Gulcin & Alwasel, 2023). In contrast to apigenin, relatively stable during extraction, other phenolic compounds capable the joint action undergoes thermal destruction or oxidized transformation in the extended heating cycles inherent to the Soxhlet method. Additionally, while extracting nonpolar impurities (e.g., waxes and chlorophyll) along with active compounds using traditional organic solvents it may not be compatible with spectrophotometric testing or diluting the levels of active compounds (Vafaei et al., 2022).

The findings are further substantiated by comparative studies revealing that the antioxidant activity of apigenin was higher than both crude olive leaf extracts and synthetic antioxidant butylhydroxytoluene (BHT). This is also supported by its ability in improving the oxidative stability of both extra virgin and refined oils, where apigenin rich fractions significantly prolong the induction period through inhibiting lipid peroxidation (Rajesh et al., 2023).

The beneficial effects of apigenin are not limited to its antioxidant activity. Recent studies have demonstrated its multi-beneficial effect in prevention and treatment of metabolic and systemic disorders, especially obesity (Li et al., 2024), hyperlipidemia, hypertension and protective action against type 2 diabetes and non-alcoholic fatty liver disease (Scuto et al., 2022). Mechanismly, apigenin is a strong inhibitor of human epidermal growth factor receptor-2 (HER2) in breast cancer and facilitates mTOR pathway for regulating autophagy in colorectal cancer. Moreover, it is neuroprotective in models of Alzheimer's disease as indicated by decreased beta-amyloid and tau

protein pathology. Nevertheless, the quantitative composition of such bioactives has been suggested to be significantly influenced by cultivar, tree age and geographical origin (Atoki et al., 2023).

Coagulation Factors

The effectiveness of rubin extracted from ultrasound was tested higher in oxidative antagonism in anticoagulant by conducting basic coagulation tests using the prothrombin time PT test, which is an indicator of the efficiency of the external pathway of the blood clotting process, and the partial thromboplastin time (PTT) test, which is an indicator of the efficiency of blood clotting. The intrinsic pathway of blood clotting

The results of the bioanalysis showed in Table 1, that Apigenin has an effect on the PT and PTT values for all concentrations, with significant differences at the probability level ($P<0.05$), with the reading of other concentrations and the control reading.) respectively, and the PTT values were 50 Sec, Therefore, OLE appears to be effective.

Table 1. Shows the effect of Apigenin extracts prepared using Soxhlet and SFE methods on PT and aPTT times at different concentrations. (LSD5%=4.234)

Extraction Technique	Concentration (mg/mL)	PT (Seconds)	aPTT (Seconds)
Soxhlet technique	5	18.2±0.6	42.5±1.5
	10	24.7±0.9	55.8±2.0
	20	38.4±1.1	78.6±2.4
Supercritical Fluid (SFE)	5	21.4±0.5	48.3±1.3
	10	32.6±0.8	64.2±1.7
	20	45.9±1.3	92.4±2.8

The effect of apigenin extracts prepared using Soxhlet and supercritical fluid (SFE) techniques on prothrombin time (PT) and partial thromboplastin time (aPTT) was evaluated at concentrations of 5, 10, and 20 mg/ml. The results showed concentration-dependent efficacy for both extracts, with the supercritical fluid extract demonstrating a clear advantage at all concentrations. The differences were statistically significant (LSD = 4.234). The SFE extract exhibited the highest value at 20 mg/ml (45.9 seconds) compared to the Soxhlet extract (38.4 seconds), with the differences favoring the SFE extract ranging from 3.2 to 7.9 seconds across the different concentrations.

While the SFE extract significantly outperformed the 20 mg/ml concentration, registering 92.4 seconds compared to 78.6 seconds for the Soxhlet extract, the differences were even greater than those recorded for prothrombin time, with a maximum difference of 13.8 seconds.

The consistent increase in coagulation times with increasing concentration confirms that this effect is due to biologically active compounds in the extract, and not a random phenomenon. The high efficiency of the SFE extract is attributed to its selectivity and ability to extract flavonoids (such as

apigenin) with higher purity and maintain potency. Gentle extraction conditions (low temperatures, absence of oxygen) prevent the degradation of sensitive compounds. Furthermore, the low concentration of residual impurities and solvents allows for the detection of the true potency of the active ingredients. The greater differences in activated partial thromboplastin time (aPTT) values compared to prothrombin time (PT) suggest that the extracted compounds (especially those obtained using SFE) may be more effective at inhibiting the intrinsic pathway than the extrinsic pathway. The supercritical fluid (SFE) technique significantly outperforms the SOXELT technique in producing apigenin-rich extracts with higher anticoagulant potency, particularly through inhibition of the intrinsic pathway (aPTT). These results underscore that the choice of extraction method is a crucial factor in determining the therapeutic value of natural products.

Previous studies showed that medicinal plants possess anticoagulant activity, which contributes to the treatment of venous and arterial thromboembolic disorders, including a study conducted to evaluate the anticoagulant effect of methanolic extracts of some medicinal plants, and the results of the study proved that these extracts have an anticoagulant effect , as well as PT prolongation only indicates inhibition FVII extrinsic pathway (He & Zhou, 2018), but mild inhibition of FX extrinsic pathway, FV and FII. Factor VII inhibition can be explained by Apigenin improved the coagulant profile and reduced plasma FVII. Treatment with OLE caused a slight but statistically significant incidence Slight decrease in thrombus weight compared with the control group (Djanaev et al., 2025). showed significant elongation of PT by OLE in our study was not associated with any significant effect on the weight of the clot.

Apigenin is renowned for decreasing blood pressure. Apigenin dramatically decreased systolic and diastolic blood pressure when given intraperitoneally or intravenously. The antihypertensive potency of Apigenin reasons why olive leaf has been used traditionally for the healing of mild to moderate high blood pressure (Papiashvili, et al., 2023). There was a significant reduction in blood pressure, blood sugar, LDL-C, triglycerides, glucose, and malondoaldehyde with Apigenin. Moreover, it enhanced glucose tolerance and elevated serum high-density lipoprotein cholesterol as well as erythrocyte superoxide dismutase. The model also has a poor lipid profile in addition to decreased glucose tolerance (Kluska et al., 2023). They also show that Apigenin, partly by an antioxidant mechanism, improves glucose tolerance and changes lipid profile favorably (Angelica & Atmaka, 2025). Apigenin, a naturally occurring bioactive compound, has been found to possess diverse pharmacological activities, including anti-inflammatory, antioxidant, anticancer, and antibacterial properties. Mechanistic studies indicate that apigenin exerts its biological effects through various cellular pathways and processes, such as modulating the PI3K/PTEN/AKT/mTOR signaling pathway, inhibiting the NF-κB signaling pathway, and modulating phase II detoxification enzymes (Lotfi & Rassouli, 2024). Apigenin has shown promising results in preclinical studies for the

prevention and treatment of various diseases, including cancer, cardiovascular diseases, and neurodegenerative diseases (Thomas et al., 2023).

Furthermore, apigenin has been shown to enhance the efficacy of conventional therapies and overcome drug resistance. Therefore, apigenin has the potential to be developed as a natural alternative or complementary treatment to conventional therapies.

CONCLUSIONS

This work demonstrates that the reactivity of watercress leaves (Eruca vesicaria L.) as a source of the double-function apigenin follows our general approach to select natural products offering antioxidant and coagulation behavior. The data revealed that the UAE provided a better extraction regarding yield, purity of compound and chemical stability compared to those for the traditional methods. Evidently, Apigenin had remarkable effects on blood fluidity as it effectively prolonged PT and aPTT times; thus suggesting that such an active compound might serve as a natural substitute of chemical anticoagulants for cardiovascular disturbance. Adoption of "green" extraction methodologies is suggested to improve the efficiency of this flavonoid in pharmaceutical and food industries using sustainable techniques.

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