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ESTIMATION OF ANTIOXIDANT AND ANTITHROMBOTIC EFFECTS OF CRUDE OLEUROPEIN EXTRACTED FROM *OLEA EUROPAEA* USING THREE DIFFERENT TECHNIQUES

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Thromboembolic diseases are becoming more common and leading causes of death and morbidity worldwide. Oleuropein, the phenolic compound found in olive leaves, is well known for its medicinal benefits. This work aims to determine the effectiveness of the oleuropein as anticoagulants by conducting basic coagulation tests using the prothrombin time (PT) and the partial thromboplastin time (PTT). Oleuropein was extracted using three techniques. The ultra-sonication induced the highest yield (20±1.0 mg/g), followed by the pressurized liquid (12.43±1.03 mg/g), while the soxhlet method afforded the lowest yield (4±1.0 mg/g). The DPPH (2,2-Diphenyl-1-picrylhydrazyl) test showed that ultrasonically-extracted oleuropein inhibited DPPH radicals effectively with an inhibition rate of 85 ± 2.04%. Oleuropein was tested at 10, 15, and 20 mg/ml for anticoagulant effect. At 20 mg/ml, PT increased by 22 ± 2.52 sec. and the PTT by 50 ± 2.69 sec. In silico analysis showed that oleuropein has the potential to prevent thrombosis and platelet clotting by binding to active sites on the protease-activated receptor 1 (PRP1) and Glycoprotein Ib alpha (GPIbα). It also prevents clot formation through interactions involving GPIbα and von Willebrand factor (VWF) by binding to over 11 active sites on PRP1 and 4 active sites on GPIbα. This study suggests that oleuropein may counteract blood coagulability and protect against blood-clotting diseases.

Keywords: *Oleuropein Estimation; Extraction Techniques; Antioxidant Activity; Clotting Factors; Molecular Docking*

INTRODUCTION

Olive's species belong to the olive family (*Oleaceae*), many species of this family are used in industrial products such as food, cosmetics, and medicines. Because it contains varying amounts of tri-glycerol as well as small amounts of free fatty acids, pigments, aromatic compounds, phenols, and other substances, olive oil is considered one of the most important components of the Mediterranean diet[1]. Olives (oil, fruit, and leaves) have been examined for their pharmacological characteristics due to their phenolic content, resulting in valuable components for both medicine and a healthy diet[2]. All parts of the olive plant contain phenolic compounds, but the nature and concentration of these compounds vary according to the type of tissue. The most predominant phenolic compound in olive cultivars is oleuropein, which can be found in amounts up to 140 mg/kg of dry young olive leaves, and 60 to 90 mg/kg of dry mature leaves[3]. Caffeic acid, verbascoside, oleuropein, luteolin-7-O-glucoside, rutin, apigenin-7-O-glucoside, and luteolin-4'-O-glucoside were identified in olive leaf extract[4].

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Oleuropein is a glycosylated seco-iridoid and consists of three structural subunits

hydroxytyrosol, elenolic acid, and a glucose molecule **(Fig. 1)**[5]. Oleuropein consumption has been linked to a variety of health benefits, including protection against heart disease[6], improved fat metabolism, and decreased obesity-related diseases[7]. Oleuropein, found in olive oil, has antioxidant, anti-inflammatory, anti-cancer, anti-angiogenic, and neuroprotective properties[8-10].

Thromboembolic conditions are dangerous and could be fatal. Thrombotic diseases continue to be a leading cause of disability and mortality worldwide, despite the affordability of antithrombotic medications for both the prevention and management of arterial and venous thrombosis. Consequently, there is still a need for improved therapies for dealing with these disorders[11]

Thrombosis is a condition that occurs when a blood clot forms in a vein, which can be dangerous if left untreated. There are certain risk factors that increase the likelihood of developing thrombosis, such as prolonged immobility, injury or trauma to a vein, cancer or cancer treatments, certain medical conditions that affect blood clotting, hormonal changes, obesity and smoking. Symptoms of thrombosis include swelling, pain or tenderness in the affected area, which may feel like cramping or a pulled muscle, warmth or redness in the affected leg, and enlarged veins that are visible just under the skin's surface. In some cases, thrombosis can cause no symptoms or mild symptoms, which can make it difficult to detect. However, it's crucial to seek medical attention immediately if any of these symptoms are observed because thrombosis can lead to serious complications such as pulmonary embolism[12].

Some reviews showed that the majority of researches on the olive plant's antithrombotic effect focused on olive oil. Both in vitro and in vivo experimental evidence of olive oil's ability to prevent thrombosis and they hypothesized that decreased fibrinogen concentrations and impaired platelet-vessel wall interactions might mediate this effect. Studies have also demonstrated that taking nutritional supplements containing olive oil has improved blood lipid levels, decreased blood clotting in blood vessels, and activated platelets in people with high blood cholesterol[13]. The aims of this study are to compare between different extraction methods of oleuropein, measure antioxidant activity of the different obtained extract, as well as measure the anti-coagulant properties of the extracts by in vitro and in silico methods.

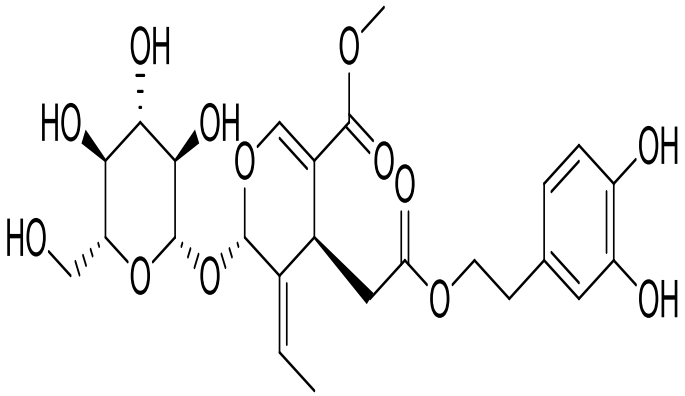

Fig. 1: Structure of oleuropein[14].

MATERIAL AND METHODS

The chemicals used in this study were obtained from Sigma Aldrich St. Louis, MO, USA. Oleuropein standard (purity by HPLC, ≥95%) was from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). All solutions were prepared using ultrapure or reverse osmosis water from Millipore RIOS 5 purification system (Bedford, MA, USA). Coagulant reagents from Stago, France, and vacuum blood tubes from Italy, Arzergrande Vacutest. The plant materials (specify) were collected from …… tree grown at … .A specimen (number 241N) was kept at …….

During the fruiting season (spring and summer 2021), olive leaves are collected in the afternoon to ensure that the plant leaves are fully exposed to sunlight. Only healthy and tender parts were selected, not including diseased or damaged parts. They are also carefully sorted to ensure they are clean and free of insects or debris. The olive leaves were dried, ground, and stored at low temperatures until needed. All donors (18–35 years) of human blood samples gave their informed consent for inclusion before they participated in the study. The study protocol was approved by the Ethics Committee of Aswan University, Egypt, with IRP No. 846/10/22.

Extraction methods

To optimize the oleuropein extraction efficiency and minimize the processing loss of the phenolic in olive leaves, three distinct approaches were employed: conventional soxhlet extraction (SE) and nonconventional techniques, such as pressurized liquid extraction (PLE) and extraction by ultra-sonication (USE).

a)Pressurized liquid extraction (PLE)

It is a method of extracting components from solid or semi-solid samples by liquid solvents (ethanol / water solutions; 50 and 70%). To complete the extraction of oleuropein, distilled water was used under high pressure of 121 pascal (Pa) and high temperatures of 100℃ for 20 minutes, followed by filtering and purification[15,16].

b)Extraction by ultra-sonication (USE)

Deep Eutectic solvent (DES) is prepared and created by combining glycerol, glycine, and water in a molar ratio of Gli: Gly: HO = 7:1:9 at temperatures ranging from 80°C – 90°C. Addition of 1.5 g crushed olive leaves (particle size, 2.0 mm to 3.0 mm thick) to the container of an Erlenmeyer flask near the midpoint. After that, 10 mL of DES was added. The reaction was then carried out in an ultrasonic bath at 40 kHz, for one hour at a temperature of 50℃. The mixture was then centrifuged at 6,000 rpm the mixture was filtered. After discarding the residue, the extract was frozen in anticipation of further HPLC examination[17,18]

c) Soxhlet Extraction (SE)

250 ml of ethanol was used with 50 g of leaf powder in a Soxhlet apparatus over a 6-hour period at 40°C[19,20]

Oleuropein quantitative and qualitative analysis using HPLC

A binary pump, an automated sampler, and a diode array detector were used in the HPLC system to find the quantity and concentration of oleuropein in olive leaf extracts. The system was run on Agilent HPLC 2D Chem-Station SW software. The oleuropein was separated using a C18 column that also served as a C18 analytical protection column. With the help of two mobile phases, A (1% CH3COOH) and B (100% acetonitrile), separation was carried out in gradient mode. Oleuropein signals were observed at a wavelength of 280 nm. By comparing the peak UV-vis spectra and retention times of the extracts with an oleuropein standard, the oleuropein in the extracts was identified and quantified using calibration curves. Oleuropein concentrations were calculated as mg/g of dry olive leaves[21].

***In Vitro* study**

a) Evaluation of the antioxidant activity of *OleaOlea europaea* leaves extracts obtained by various extraction techniques

The antioxidant activity of the three extracts produced by the three different extraction techniques (PLE, USE and SE) for *Olea europaea* leaves was compared in vitro using DPPH (2, 2'-diphenyl-1-picryl-hydrazyl

hydrate according to Andrejč et al.[16] Prepare 1 mg/mL of *Olea europaea* leaves extract in methanol. Then, 3 mL of 0.06 mM in methanol of the freshly prepared DPPH reagent were added to 0.77µL of each tested extract in a bottle and incubated in dark at room temperature for 15 min. A volume of 0.77µL of ascorbic acid as reference solution in methanol. After that, the absorbance was measured using a UV-vis spectrophotometer [(CARY 50 UV-VIS), Australia] at 515 nm. The antioxidant activity was calculated as % inhibition.

Inhibition % = [(Average absorbance of blank – Average absorbance of the test) /Average Absorbance of blank] × 100

Where: blank is (DPPH + methanol)

b)Estimation of the antithrombotic activity of *OleaOlea europaea* leaves extracts obtained by various extraction techniques

From 10 healthy donors (18 – 35 years old) venous blood samples were taken using 3.2% tri-sodium citrate vacuum blood tubes (Arzergrande, Vacutest, Italy). During 14 days before giving blood, these donors avoided taking any medications like anticoagulants, antibiotics, or vitamins. After centrifuging the blood samples for 15 minutes at 2000 rpm, the PT and PTT values were acquired according to Dub & Dugani (2013)[13], by using coagulant kits (Diagnostica Stago, France). Water was used as a negative control, and heparin (1.25 mg/ml) was used as a positive control.

Prothrombin time (PT) measurement

The citrated plasma that had already been collected in analysis tubes was incubated at 37°C for two minutes. Then, 0.1 ml of plasma was mixed with (10, 15, and 20 mg/ml) of the oleuropein extract, which was then diluted into 50% (1:1 (v/v) with distilled water before being incubated at 37°C for the recommended 15 minutes. Following the incubation, 0.2 ml of thromboplastin reagent that had been pre-incubated for 15 minutes at 37°C was added, and the clotting time was noted (until fibrin filaments were observed). The clotting time, measured in seconds (s), represents the outcomes (normal rate, generally ranging from 12 to 15 seconds)[13].

Activated partial thromboplastin time (aPTT) measurement

The citrated plasma that had already been collected in analysis tubes was incubated at 37°C for two minutes. Next, 0.1 ml of plasma was mixed with 1ml of oleuropein extract at three varying concentrations (10, 15, and 20 mg/ml), which was then diluted with distilled water to 50% (1:1 (v/v)) before being incubated at 37°C for 15 min. Following the incubation, 0.1 ml of aPTT reagent that had been pre-incubated for 5 min. at 37°C was added, and the process of coagulation proceeded with the addition of 0.1 ml of pre-warmed calcium chloride solution. PTT normally ranges from 30 - 40 sec[13].

***In Silico* Study**

The Auto-Dock Vina program was utilized to perform molecular docking analysis in order to investigate the potential activity of oleuropein against PAR1 and GPIb-α target site, which play a role in regulating coagulation response factors. The target proteins, specifically Protein ID: 3vw7-3pmh, were sourced from the Protein Data Bank (PDB). The structures of both the target protein and oleuropein were prepared and subjected to energy minimization using the Merck molecular force field 94 (MMFF94). Subsequently, molecular docking was carried out, resulting in the generation of twenty poses. The most favorable orientations were then selected, and the affinity scores and root-mean-square deviation (RMSD) values were documented. The 3D orientation was generated using Discovery Studio 2016 visualizer software[22].

Statistical analysis

Statistical analysis was performed using Genstat-Tenth Edition Version-10.3.0.0. Furthermore, the significance of differences in means was assessed using the least significant difference (L.S.D) test at a probability level of 0.05[23]

RESULTS AND DISCUSSION

Results

HPLC determination of the oleuropein content of *Olea europaea* leaf extracts produced by various extraction techniques.

By using High-performance liquid chromatography (HPLC), and by comparison with the oleuropein standard (OS) (purity by HPLC, ≥95%) was from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA; the findings in **Fig. 2** displayed that the ultrasonic extraction (USE) method proved to be the most effective in extracting the phenolic compound oleuropein, yielding a concentration of 20± 1.0^{b} mg/ml, followed by the pressurized liquid extraction (PLE) method 12.43± 1.03^{c} mg/ml, and Soxhlet extraction (SE) method resulted in a lower concentration of 4± 1.0^{d} mg/ml. The findings align with the research conducted by Vural et al. (2020)[24], which demonstrated that ultrasonic extraction using deep eutectic solvent yielded the greatest concentration relatively 45 mg/ml. These variations in oleuropein content can be attributed to the specific extraction techniques employed and the choice of solvents.

The antioxidant potential effect of the *Olea europaea* leaf extract produced using different extraction techniques

The current study conducted a thorough assessment of the antioxidant activity of olive leaf extracts using three distinct methods (USE, PLE, and Soxhlet). The study measured the extracts' ability to scavenge free radicals using the 2,2-diphenyl-1-picrylhydrazyl compound (DPPH). Compounds that effectively scavenge free radicals convert DPPH to reduced form (DPPH-H), specifically1,1-diphenyl-2-picrylhydrazine. To compare the extracts' inhibition rates, we also included ascorbic acid in our evaluation. The findings, as illustrated in **Fig. 3**, reveal a significant difference ($p < 0.05$) in the antioxidant activity of the three extracts described in descending manner (85 ± 2.04^{b} % for USE, 52 ± 2.6^{c} % for PLE, and 40 ± 3.01^{d}% for Soxhlet) in comparison to ascorbic acid 35 ± 1.02^{a} %. Specifically, the extract obtained through ultrasonic extraction demonstrated the highest inhibition rate when compared to the other techniques and ascorbic acid.

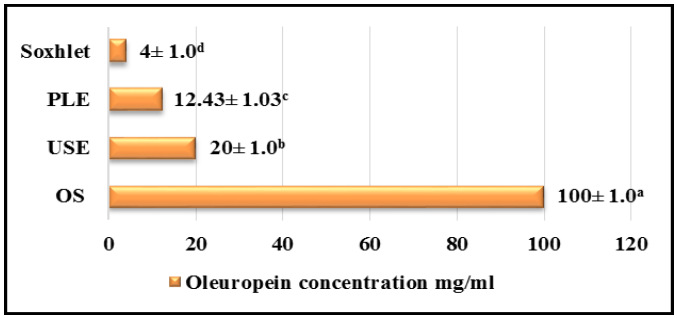

Fig. 2: HPLC Determination of the amount of Oleuropein in the *OleaOlea europaea* leaf extracts using three different methods of extraction. The **Fig.** represents the average (mean) ± standard deviation for each extraction method (LSD 5% = 0.12).

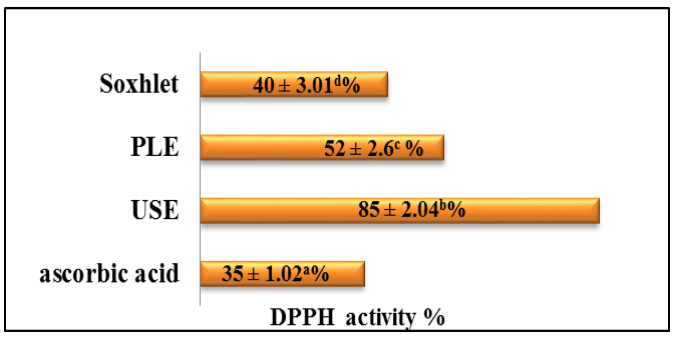

Fig. 3: The *OleaOlea europaea* leaf extracts antioxidant activity (DPPH) expressed in percentage, by using different extraction methods. The **Fig.** represents the average (mean) ± standard deviation for each extraction method (LSD 5%= 3.22).

The antithrombotic effect of the *Olea europaea* leaf extract produced using different extraction techniques

Fig. 4 shows that Oleuropein content in the *OleaOlea europaea* leaf extracts has an influence on PT and aPTT values at all concentrations examined, with significant differences ($p < 0.05$) when compared to the controls. Oleuropein extract at concentrations of 10 mg/ml, 15 mg/ml, and 20 mg/ml significantly extended the periods of PT. The periods of PT for the different concentrations were 16 ± 2.05^{b}, 18±2.07^{b}, and 22 ± 2.52^{c} sec. respectively. The negative control, which received water, had a PT period of 15 ± 2.02^{a} sec. The differences in PT periods were statistically significant ($p<0.05$). When heparin was used as a positive control, the PT was considerably extended to 30±2.79^{d} sec. compared to the other groups ($p < 0.001$).

Fig. 4, also reports the observed effects of oleuropein (in *Olea europaea* leaf extracts) different doses (10, 15, and 20 mg/ml) on aPTT values. Each dose showed a significant increase ($p<0.05$) in aPTT values as compared to the negative control group that was treated with water (40±1.75^{a} sec). The respective values obtained for aPTT were 39±1.75^{a} sec, 45±2.57^{b} sec, and 50±2.69^{c} sec. Additionally, heparin directed at a dose of 1.25 mg/ml also significantly enhanced aPTT ($p<0.05$) as compared to the negative control, with a time period of 75±3.30^{d}. These prolonged PT and aPTT changes indicate that oleuropein may influence the extrinsic coagulation pathway and possess potential antithrombotic capacities.

***In silico* study of Oleuropein effect on PAR1 and GPIb-α receptors**

The current study employed a computer-based chemistry approach to examine Oleuropein's potential against two target sites; glycoprotein Ib-α (GPIb-α), a high-affinity platelet receptor of thrombin, and protease-activated receptor-1 (PAR1), a primary mediator of human platelet activation by thrombin. Firstly, target proteins were downloaded from the Protein Data Bank (protein ID: 3vw7-3pmh). The structures of both the target proteins and Oleuropein were prepared, and energy was minimized using an MMFF94 force field. Molecular docking was then performed, and the most favorable orientations were selected from the twenty generated poses. **Table (1)** contains affinity scores and root-mean-square deviation (RMSD) values gathered during this process.

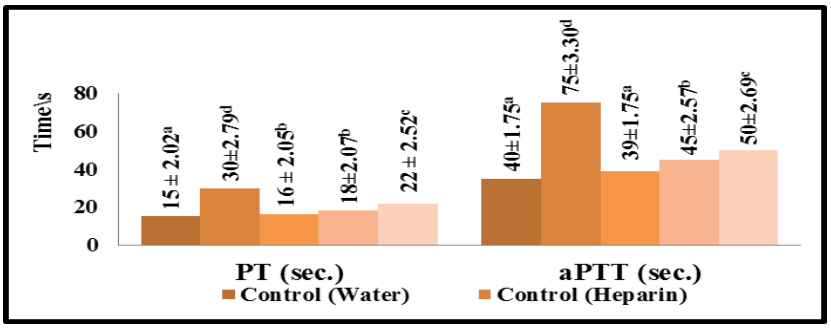

Fig. 4: Antithrombotic effect of different Oleuropein concentrations present in the *Olea europaea* leaf extracts. The **Fig.** represents the average (mean) ± standard deviation for each extraction method (LSD5%=0.234).

Table 1: Molecular docking analysis of Oleuropein against PAR1 and GPIbα target sites**.**

Targets	Tested compounds	RMSD value (Å)	Docking (Affinity) score (kcal/mol)	Interactions	
				H.B	Pi – interaction
PAR1	Oleuropein	1.65	-10.03	3	11
GPIbα	Oleuropein	1.55	-8.01	7	4

H.B.: Hydrogen Bond
RMSD: Root-Mean-Square Deviation

Oleuropein has the ability to bind the two distinct target sites, PAR1 and GPIbα, each exhibiting varying levels of energy binding. When bound to PAR1, Oleuropein creates an energy binding level of -10.03 kcal/mol through a remarkable eleven hydrophobic interactions with specific amino acids, including Leu332, Leu333, His336, Tyr353, Leu237, Tyr183, Phe271, Leu262, His255, and Leu258. Moreover, Oleuropein also forms three hydrogen bonds with bond lengths of 1.71, 2.66, and 2.34 Å between Leu258 and Tyr350. The visually illustrate **Fig. (5 & 6)** represented significant interactions .On the other hand, when bound to the GPIbα target site, Oleuropein demonstrates an energy binding level of -8.01 kcal/mol. It achieves this through four hydrophobic interactions with Leu99, Ile174, Tyr60A, and Trp60D, as well as seven hydrogen bonds with bond lengths of 2.61, 2.52, 2.83, 2.10, 2.67, 3.06, and 2.61 Å with Cys191, Gly193, His57, Ser195, Trp96, and Trp60A. **Fig.6** provides a visual representation of these interactions.

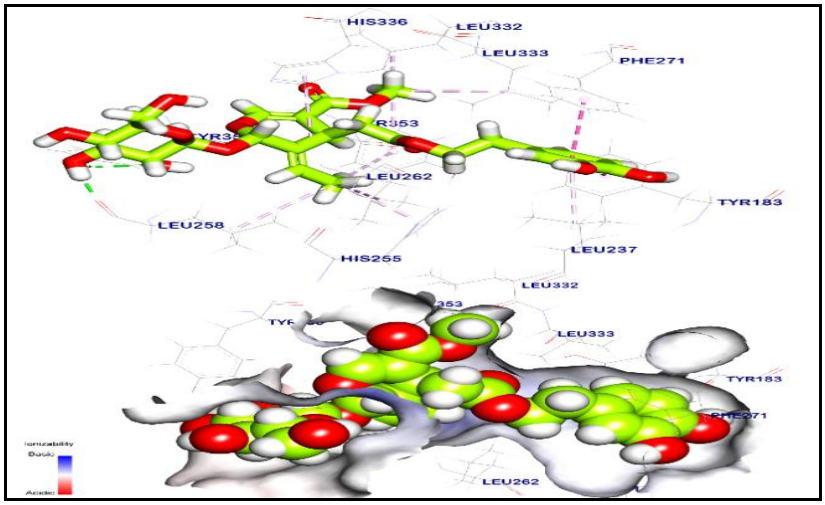

Fig. 5: 3D orientation and surface mapping of Oleuropein against PAR-1 target site.

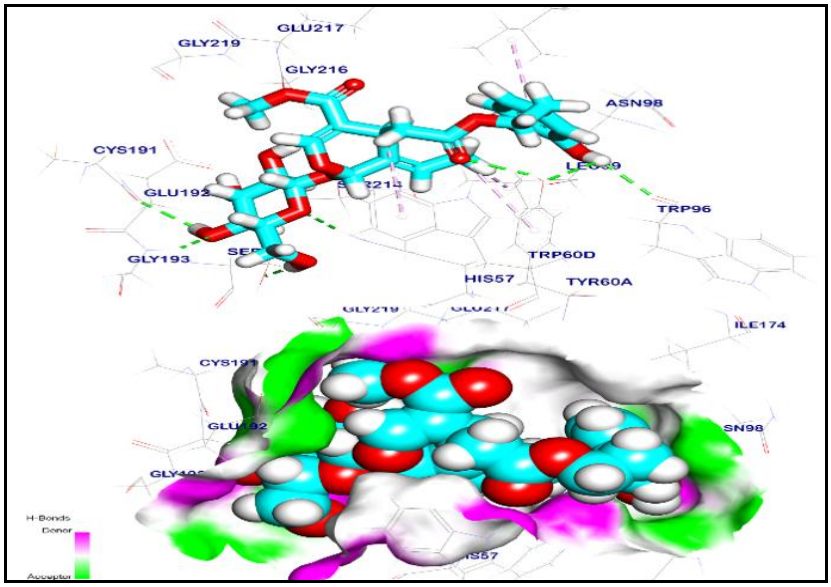

Fig. 6: 3D orientation and surface mapping of Oleuropein against GPIb-α target site.

Discussion

Oleuropein, a phenolic compound that is highly abundant in leaves of *Olea europaea*, has gained significant attention in research due to its valuable biological properties. Extraction of olive leaves can be done by conventional methods (such as Soxhlet extraction) and non-conventional methods (such as extraction by ultra-sonication, and pressurized liquid extraction). Identification of oleuropein content in olive leaves extract, primarily through chromatographic techniques (such as HPLC), allows for its utilization as an anticoagulant agent. Numerous studies have been conducted on various extraction methods, including those applicable to plants in general and specifically to olive leaves, which are valued for their economic significance as beneficial oils[25]. In many industrial processes, organic solvents are commonly employed to extract olive leaf components[26]. However, the use of such solvents presents significant risks to both human health and the environment. Consequently, an alternative method that shows great assurance in terms of both effectiveness and cost-efficiency, while also being environment friendly, is the utilization of supercritical and ultrasonic fluid extraction methods to obtain high-quality extracts without any residual organic solvents[27].

Ultrasonic extraction (USE) is a promising technique for utilizing olive leaves in a bio-refinery because it reduces extraction time through the induction of cavitation, which accelerates chemical reactions. Several studies have successfully used USE to extract the best oleuropein content in olive leaves, resulting in a significant reduction in solvent volume. Additionally, USE promotes plant material dilation and hydration, leading to increased swelling and solvent penetration. For instance, Zun-Qiu et al. (2015)[28], achieved a purity of 13.52% oleuropein using USE with an 80% methanol-water solution. Additionally, Lama-Muñoz et al. (2019)[29], compared and improved the USE extraction of oleuropein from olive leaves to the traditional Soxhlet extraction method, and USE provided the best results. Del Mar-Contreras et al. (2020)[30], obtained a high level of oleuropein using specific operational parameters. All these previous studies were matched with our results which demonstrated that USE is the best technique used for getting the best *Olea europaea* leaf extracts with respectable amounts of oleuropein (12.43± 1.03^{c} mg/ml).

Pressurized fluid extraction (PLE) is a technique that utilizes pressures and temperatures to rapidly extract various compounds from plants. It reduces solvent consumption and enables extraction at high temperatures. Oleuropein extraction yield from olive leaves was maximized using pressured liquid extraction[31]. PLE is an alternative technique to super-critical fluid extraction (SCFE) for extracting olive leaves' phenolic compounds. PLE using an array of ethanol and water, a pressure of 10.3 MPa, a temperature of 60°C, and an extraction time of 110 min. yielded the highest total yield of 30.91%. The PLE extract obtained using ethanol at 60°C had the most oleuropein content, at 73.65 mg/g of extract[15]. According to the current results, PLE is considerably the best second technique for releasing oleuropein content into the *Olea europaea* leaf extract by yield of 12.43± 1.03^{c} mg/ml.

Soxhlet extraction (SE) is a commonly employed method in laboratory settings, although it has disadvantages such as the potential degradation of thermos-labile compounds and the consumption of significant amounts of water and energy. However, the efficiency of extraction can be enhanced by combining solvents with water. In particular, Yateem and Al-Rimawi, (2014)[32], demonstrated that a mixture of 80% ethanol and 20% acetonitrile has been found to be the most effective for extracting oleuropein. Furthermore, increasing the extraction temperature has been shown to increase the oleuropein content, while raising the pH level has been found to reduce the extraction yield. In comparison to cold extraction, Soxhlet extraction has demonstrated greater efficiency in extracting oleuropein. Among conventional techniques, Soxhlet extraction has exhibited the highest recovery rate, reaching 62%. Various combinations of solvents have been tested, and it has been determined that ethanol: water (80:20) is the optimal choice for Soxhlet extraction. Additionally, the emerging technique has shown even higher extraction yields, ranging from 80% to 95%, in contrast to other conventional methods[33]. Based on the current results, Soxhlet extraction is considered

the conventional method for extracting oleuropein. However, when compared to other techniques such as USE and PLE, it is the least effective method resulting in a lower concentration of oleuropein (4 ± 1.0^{d} mg/ml) in the leaf extract.

Oleuropein exhibits a higher level of antioxidant activity suggesting its potential in preventing oxidation stress. In the current study, Oleuropein content found in the *Olea europaea* leaf extracts surpasses butylated hydroxyl-toluene (BHT) in terms of potency (85 ± 2.04^{b} % for USE, 52 ± 2.6^{c} % for PLE, and 40 ± 3.01^{d} % for Soxhlet), highlighting its potential applications in nutrition and human health[34]. The addition of oleuropein-rich extracts to both virgin and refined olive oils enhances their oxidative stability, as evidenced by prolonged induction periods[35]. As a result, the study set out to investigate the effectiveness of oleuropein in inhibiting oxidative stress and preventing blood clotting using ultrasound. They conducted basic coagulation tests, such as prothrombin time (PT) and partial thromboplastin time (aPTT) tests.

The aPTT test measures the effectiveness of the intrinsic blood clotting route, while the PT test measures the effectiveness of the external blood clotting system. Numerous antithrombotic agents can be used to control and treat thrombotic disorders, but a lot of them are expensive and toxic. Therefore, it is necessary to search for safe, affordable natural products from different natural sources. Several studies evaluate the anticoagulant effect of methanolic extracts of medicinal plants, for example, those performed on selected Egyptian plants to highlight their influence on the coagulation curves of the prothrombin time (PT) and activated partial thromboplastin time (aPTT) tests[36]. Some of these plants showed interesting outcomes that required a more detailed evaluation of their anticoagulant activity as the effect of *Hibiscus* calyx extract on PT. In addition, extracts from the aerial parts of clover and *Pimpinella anismus* fruits have been shown to affect aPTT. Also oleuropein also showed the strongest anticoagulant activity of the two intrinsic and extrinsic pathways 44.77 ± 0.25 and 15.84 ± 0.12, respectively[35], which is in matching with our results obtained by ultrasonic extraction (50 ± 2.69^{c} sec. and 22 ± 2.52^{c} sec. at concentration of 20 mg/ml of *Olea europaea* leaf extract).

Furthermore, Dub and Dugani, (2013)[13], which evaluated the effect of the ethanolic extract derived from olive leaves on its anticoagulant properties in rabbits, as an *in vivo* model. They demonstrated that orally administered of the olive leaves extract significantly prolongs PT against deep vein thrombosis (DVT). This can be attributed to the existence of the oleuropein compound present in the extract, which is consistent with the results obtained. Because of PT and PTT were responsible for measuring the activity of factors II, V, VII, X, and fibrinogen, investigating the influence of oleuropein on these factors is valuable. Future research will be required to assess the impact of crude oleuropein on liver function.

Computational (in silico) methods are frequently utilized during drug discovery or design phases for virtual biological screening. This technique has led to a greater understanding of targeted sites and the identification of compounds as inhibitors or activators. Platelets express various receptors to respond to different stimulants. The two most prevalent surface receptors on platelets are PAR1 and GPIb-α, which have several functions, including thrombopoiesis, which in turn affects PT and PTT blood clotting time[37]

There are four members in the PRP protein family, namely PAR 1-4. The most extensively researched one is the protease-activated receptor 1 (PAR1). This receptor is activated by thrombin, a coagulation protease, through a cleavage of its N-terminal exo-domain. This cleavage creates a fresh N-terminus that remains attached to the receptor and acts as a ligand referred to as a tethered ligand (TL), then it initiates conformational changes in the receptor, causing G-protein activation at the end of signaling cascades[38]. According to Coughlin, (2000)[39], prothrombin plays a crucial role in the process of blood clotting. It prompts the activation of another protein called thrombin, which then triggers the thrombin PAR1 cascade. This cascade ultimately results in the activation of a PAR1 protein that causes platelet aggregation, the process by which platelets group together to form a clot. Consequently, in the presence *in silico* study, oleuropein has the ability to block

or inhibit the PAR1 receptor, thereby halting the entire thrombin PAR1 cascade. This, in turn, stops the activation of PAR1 that drives platelet aggregation, ultimately preventing the formation of blood clots.

Moving on, another important player in the formation of blood clots, GPIbα, or Glycoprotein Ib alpha, is a notable contributor to the development of blood clots. Its main function is to signal and engage with other factors that lead to clotting. Specifically, GPIbα binds to VWF (von Willebrand factor), which regulates the formation of fibrin, an essential component of blood clots. Additionally, GPIbα can be activated by VWF, resulting in activities that promote the coagulation process in platelets. In a study conducted by Bury et al. (2019)[40], it was observed that by inhibiting GPIbα with oleuropein, its interactions and signaling pathways can be disrupted. This obstruction of GPIbα leads to the loss of platelet adhesion to surfaces, thereby impeding the initial stages of clot formation where platelets aggregate at the site of injury. The findings are further supported by results from molecular docking analysis. It is important to understand the interplay between GPIbα, PRP1, and thrombin. GPIbα acts as thrombin's high-affinity platelet receptor, and its binding triggers thrombin's cleavage and activation of PAR1, its low-affinity platelet receptor. Furthermore, GPIb-IX-mediated signaling collaborates with PAR1 signaling[41].

Recent animal studies indicate that the usage of oral thrombin inhibitors leads to a slight rise in acute coronary syndromes, which is linked to the interaction between GPIbα and thrombin[42]. In summary, both inhibiting the PAR1 receptor and blocking GPIbα are approaches that can prevent the formation of blood clots through different mechanisms. Oleuropein stops the activation of platelets through the thrombin PAR1 cascade, while the latter prevents platelet adhesion and initial clot formation through interactions involving GPIbα and VWF.

Conclusion

The current study hypothesized that thrombin inhibitors could have both anticoagulant and antiplatelet effects at the same time. To study this theory, we used molecular docking to analyse the impact of oleuropein on PRP1 and GPIbα receptors. Oleuropein, the most important phenolic compound found in olive leaves, which extracted by the highest yield usingultra-sonication of *Olea europaea* leaves. The findings indicated that oleuropein-rich extracts acts as a highly selective and direct thrombin inhibitor, providing a new mechanism to compete with systemic drugs like heparin. As computationally observed, by binding to over 11 active sites on PRP1 and 4 active sites on GPIbα, oleuropein has the potential to prevent thrombin-mediated thrombosis and platelet clotting. Both *in vitro* and *in silico* studies highlight the potential of oleuropein to have simultaneous anticoagulant and antiplatelet effects by blocking thrombin's active site.

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تقدير التأثيرات المضادة للأكسدة والمضادة للتخثر لخام الأوليوروبين المستخرج من *Olea europaea* باستخدام ثلاث تقنيات مختلفة

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أصبحت أمراض الجلطات أكثر شيوعًا ومن المسببات الرئيسية للوفاة في جميع أنحاء العالم. الأوليوروبين، المركب الفينولي الموجود في أوراق الزيتون، معروف بفوائده الطبية المختلفة. يهدف هذا العمل إلى تحديد فعالية الأوليوروبين كمضاد للتخثرعن طريق إجراء اختبارات التخثر الأساسية باستخدام زمن البروثرومبين (PT) وزمن الثرومبوبلاستين الجزئي (aPTT). تم استخلاص الأوليوروبين الخام باستخدام ثلاث تقنيات حيث كانت الموجات فوق الصوتية الأعلى إنتاجاً (٢٠±١.٠) ملجم/جرام، يليه الاستخلاص تحت ضغط (١٢.٤٣±١.٠٣)ملجم/جرام، في حين أن طريقة السوكسليت حققت أدنى إنتاج (٤±١.٠) ملجم/جرام. أظهر اختبار DPPH(2,2-Diphenyl-1-picrylhydrazyl) أن الأوليوروبين المستخرج بالموجات فوق الصوتية يثبط DPPH بشكل فعال بمعدل تثبيط قدره (٨٥ ± ٢.٠٤%). تم اختبار الأوليوروبين عند ١٠ و١٥ و٢٠ ملجم/مل من أجل التأثير المضاد للتخثر. عند ٢٠ مجم/مل، زاد PT بمقدار (٢٢ ± ٢.٥٢) ثانية، وaPTT بمقدار(٥٠ ± ٢.٦٩) ثانية. وفقا لنتائج السيليكو، فإن الأوليوروبين لديه القدرة على منع تجلط الدم وتخثر الصفائح الدموية عن طريق الارتباط بالمواقع النشطة على مستقبل البروتياز المنشط ١ (PRP1) والبروتين السكري (GPIbα). كما أنه يمنع تكوين الجلطة من خلال التفاعلات التي تتضمن GPIbα وعامل von Willebrand (VWF) من خلال الارتباط بأكثر من ١١ موقعًا نشطا على PRP1 وأربعة مواقع نشطة على GPIbα. تشير الدراسة إلى أن الأوليوروبين قد يساهم بشكل فعال في علاج أمراض تخثر الدم.