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Research Article

Hemostatic Properties of Green Hydrogel Synthesized from Chitosan/Gelatin Polymers and Hydroalcoholic Extract of *Juglans Regia L*: An In-Silico and In-Vitro Study

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ABSTRACT

Objectives: Quercetin-3-O-rhamnoside, an effective antioxidant, exists in walnut leaves and is supposed to maintain the structural integrity of the Band 3 protein of red blood cells (RBCs). In this context, an attempt was made to explore the in-silico and in-vitro hemostatic effects of chitosan/gelatin hydrogels containing hydro-alcoholic extract of Iranian walnut leaves (*Juglans regia L.*), rich in quercetin-3-O-rhamnoside.

Methods: In-silico molecular docking predicted the potential interaction between quercetin-3-O-rhamnoside and Band 3 protein. Hydroalcoholic extraction (1:1 water: 70% ethanol) of walnut leaves was performed in a Soxhlet extractor. The hemostatic activity from various extract concentrations (0%, 2.5%, 5%, 10%, and 20% v/v) was assayed using the prothrombin time test on citrated blood samples. Gelatin/Chitosan hydrogels (3:1 and 2:1) loaded with the hemostatic concentrations were prepared by the casting method, and their adhesion ability to RBCs was tested by measuring the concentration of free hemoglobin in comparison with controls such as sterile gauze and free-hydrogel positive controls.

Results: Quercetin-3-O-rhamnoside exhibited a high affinity for Band 3 protein, presenting a binding energy of -8.39 kcal/mol compared to the standard ligand 4KU with -7.11 kcal/mol. The nine amino acids found to be important in this interaction include THR728, SER465, VAL729, ARG730, ILE531, LYS851, ILE528, PHE532, and PHE792. All hydrogels incorporating 2.5% v/v of extract could significantly enhance RBC adhesion controls ($p < 0.05$).

Conclusion: The hydroalcoholic extract from Juglans regia L. may be of value as an antioxidant and hemostatic agent. Quercetin-3-O-rhamnoside is suggested to be extracted from walnut leaves and directly incorporated into the hydrogel, and its hemostatic and antioxidant properties can be investigated at both in vitro and in vivo levels.

Keywords: Molecular docking, quercetin-3-O-rhamnoside, red blood cells, chitosan, gelatin, Juglans regia L.

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Introduction

Uncontrolled bleeding accounts for 30-40% of mortality resulting from injuries (1). The greatest mortality rate is due to significant blood loss before patients arrive at medical facilities, which may lead to severe complications such as nerve necrosis and limb amputation. Therefore, developing novel methods and products for hemorrhage control is urgently needed; it remains a focal point in pre-hospital emergency care research (2).

In the synthesis of modern hemostatic materials, polysaccharides, silicon-based materials, biological products, and self-assembling nanostructured peptides are commonly used in the forms of sponges, hydrogels, nanofibers, and particles (1, 3). Among these, hydrogels are promising candidates owing to their high water absorption capability, biocompatibility, and three-dimensional porous structure, which resembles the extracellular matrix (4). Hydrogels support wound healing by improving hemostasis due to the enrichment of blood cells and clotting factors through exudate absorption. These properties make hydrogels highly effective in managing moist wounds and facilitating granulation and epithelialization processes (5).

Hydrogels can be prepared using natural polymers like cellulose, alginate, chitosan, gelatin, and dextran, or synthetic ones such as polyvinyl alcohol, polyvinylpyrrolidone, and polyethylene glycol (6). Gelatin is a bio-based polypeptide polymer derived from collagen and has an excellent hydrogel-forming capacity. However, due to the absence of the triple-helix structure, it exhibits lower structural organization compared to collagen (7). Gelatin materials can absorb blood many times greater than their weight, concentrate coagulation factors and platelets at the site of injury, and exert pressure on the wound upon swelling (8). Besides, gelatin can form a structural matrix for clot formation (9). Although it is biodegradable, biocompatible, has good water solubility, and a strong hydrogen bonding capacity, the main drawbacks to the medical use of gelatin are its low mechanical strength and high degradation rate. Therefore, it is often combined with other materials, such as chitosan, to enhance its properties (10).

Chitosan is a natural, positively charged polysaccharide widely used in biomedical applications due to its excellent biocompatibility, biodegradability, non-immunogenicity, and antimicrobial properties. Its hemostatic effects occur via three main mechanisms: (I) The glucosamine groups in chitosan are positively charged and, therefore, attract negatively charged RBCs. This leads to agglutination and enhances clotting independent of the classical coagulation cascade. (II) Chitosan also stimulates platelet adhesion and aggregation, probably by increasing Ca2+ ion mobility and upregulating the GPIIb/IIIa complex on platelet surfaces. (III) Electrostatic attractions between ionized chitosan and fibrinogen would probably alter the structure-function properties of fibrinogen. Moreover, the molecular weight and degree of chitosan deacetylation also determine its properties as a hemostatic agent (11).

Meanwhile, since hemolysis, or the rupture of blood cells, can further weaken the blood's potential for coagulation and thereby worsen bleeding, the incorporation of antioxidants into such hemostatic materials halts bleeding by preventing blood cell damage from oxidative stress (12, 13). In red blood cells, the rigidity of the membrane is primarily imparted by the impaired mobility of the proteins embedded within its phospholipid bilayer. Among these critical transmembrane proteins on RBCs, Band 3 is highly important and plays a critical role in gas exchange, ionic balance, osmotic stability, mechanical properties, and maintaining the biconcave shape of RBCs. A large number of morphological disorders and anemias in RBCs are caused by oxidative stress impacting this protein. Thus, the finding of new protective molecules that maintain Band 3 structure under conditions of oxidative stress is highly relevant in bleeding management (14).

Recent efforts have focused on quercetin, a well-known antioxidant present in plant-based flavonoids, including citrus fruits, buckwheat, and onions. Previous works reported that walnut leaves are highly rich in antioxidant flavonoids, especially quercetin (15). However, the impacts of *Juglans regia L*. leaves on the coagulation process and the influence of its quercetin-3-O-rhamnoside on Band 3 proteins remain undefined. Thus, this study aimed to prepare a plant-based hydrogel with gelatin and chitosan, along with Iranian walnut leaf extract, and evaluate its hemostatic potential on blood samples extracted from healthy individuals. In-silico molecular docking analysis was done between Band 3 protein and quercetin-3-O-rhamnoside before in-vitro studies to predict the possibility of forming any interactions. This study explored the potential of such natural compounds for the development of effective strategies for wound management that are safe, accessible, and sustainable by leveraging their beneficial properties..

Materials and Methods

Molecular Docking Analysis

The three-dimensional structure of Band 3 protein was downloaded from The RCSB Protein Data Bank (PDB ID: 4YZF). The three-dimensional structure of quercetin-3-O-rhamnoside was retrieved from the PubChem database using CID: 5353915. AutoDock4 was used for carrying out the molecular docking analysis. Analysis and visualization of the results of the docking studies were conducted using ChimeraX and Studio Visualizer software, respectively.

Plant Leaves Collection and Hydroalcoholic Extract

Juglans regia leaves were collected in autumn, cleaned, and dried in the dark for 10 days. The plant materials were powdered by a mixer and kept in a dark condition. Then, 10 g of the powdered leaves were extracted with 350 mL of water to 70% ethanol solvent (1:1) for 8 hours using a Soxhlet extractor.

Assessment of Hemostatic Potential of Hydroalcoholic Extract through Prothrombin Time (PT) Test

This study was approved by the Ethical Committee of Tehran University of Medical Sciences, School of Allied Medical Sciences, with the following code: IR.TUMS.SPH.REC.1403.039. After the approval of the ethical committee and informed consent, 1 mL of blood was collected from healthy volunteers. Blood samples were drawn into citrated tubes and immediately centrifuged at 10,000 g for 5 minutes to separate the plasma containing coagulation factors from the blood cells. For hemostatic evaluation via PT measurement, which measures the extrinsic coagulation pathway, different concentrations of the extract in normal saline were made and tested: 0%, 2.5%, 5%, 10%, and 20% v/v. A negative control was made using normal saline to determine any effect that it may have on the coagulation factors; thus, a normal PT value was determined by testing untreated plasma. At the time of the assay, glass tubes were first pre-warmed in a water bath at 37°C for 5 minutes. Then, 50 µL of citrated plasma and 50 µL of the extract were added to the pre-warmed tubes and incubated for 5 minutes at 37°C. After that, PT reagent was added, and the time required for the first visible clot formation was measured using a digital chronometer with an accuracy of one second.

Preparation of Chitosan/Gelatin Hydrogel Films

A chitosan solution was prepared at the concentration of 1% w/v in 1% (v/v) acetic acid. This solution was stirred using a magnetic stirrer at 60°C for 5 hours to ensure better dissolution. Meanwhile, gelatin powder was dissolved in deionized water at 3% w/v and incubated at 2–6°C for 15 minutes. The gelatin solutions were then stirred using a magnetic stirrer at 55°C for 3 hours.

The prepared gelatin and chitosan solutions were then mixed in ratios of 1:1, 3:1, and 2:1 to yield 4 mL volumes for each ratio. These mixtures were incubated on a shaker at room temperature for 24 hours. The resulting solutions were poured into circular molds with a diameter of 3 mm and a volume of 2 mL using the casting method. The molds were then kept in the dark at room temperature for 3 days to develop solid hydrogel films. The stiffness and plasticity of the films were qualitatively evaluated by observation.

Incorporating the Hemostatic Extract into the Chitosan/Gelatin Hydrogel Films

Then, the optimized ratios of gelatin/chitosan polymers (3:1 and 2:1) were mixed, and hydroalcoholic extract was added to it to a final volume of 5000 µL at 2.5% v/v as the hemostatic dose. The resulting solution was then stirred at room temperature on a magnetic stirrer for 24 hours. The solution was cast into the pre-prepared molds and kept in the dark for 3 days.

Evaluation of the Ability of Red Blood Cell Adhesion to the Green Hydrogels

Using a spectrophotometric-based assessment, blood adhesiveness to the hydrogels was evaluated by calculating the optical density (OD) of free hemoglobin. For this purpose, anticoagulant-free whole blood (14 µL) was applied on the green hydrogels, followed by incubation at room temperature for 45 minutes. The hydrogels were subsequently transferred to 1000 µL of deionized water and shaken at 100 g for 30 seconds. The hydrogels were then rested at room temperature for 5 minutes. The free hemoglobin, as an indication of the amount of non-adhered RBCs, was measured spectrophotometrically at 540 nm. Sterilized gauze dressing was used as a commercial standard wound dressing for comparison. A positive control sample without hemostatic materials was also evaluated to establish the total free hemoglobin.

Statistical Analysis

The test was done using one-way ANOVA on SPSS software, while the visualization of data was performed on GraphPad Prism. A significance level of $p < 0.05$ was considered significant.

Results

Molecular Docking

Since Band 3 is a dimeric protein with six chains in each monomer, docking was performed exclusively on chains A, B, E, F, G, and H, while the other monomer was excluded (Figure 1). The protein's active site is located on chain A, where it may interact with the quercetin-3-O-rhamnoside molecule. The three-dimensional crystallographic structure of Band 3 and quercetin-3-O-rhamnoside was subjected to molecular docking, and the results were compared with the standard ligand of Band 3, i.e., 4KU, as a control. The binding affinity of quercetin to the Band 3 target molecule was analyzed over 200 runs (the cluster of runs is shown in Figure 2A), with the optimal interaction observed in run 46. This run exhibited a binding energy of -8.39 kcal/mol,

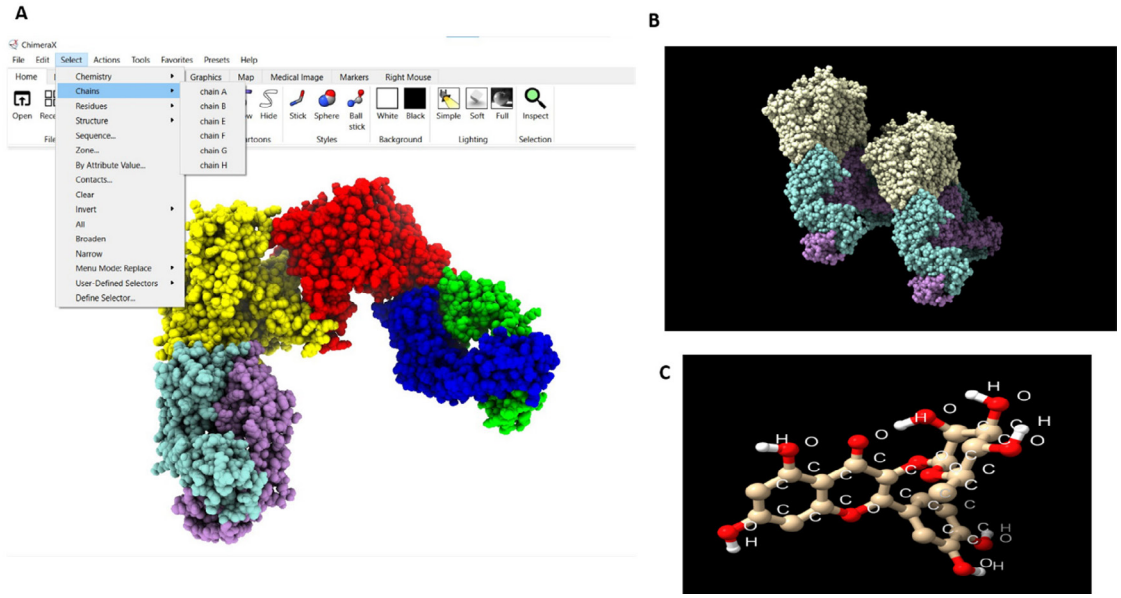

Figure 1: (A) Three-dimensional structure of Band 3 monomer, containing A, B, E, F, G, and H chains; (B) The dimeric structure of Band 3 protein; and (C) three-dimensional structure of Quercetin-3-O-rhamnoside.

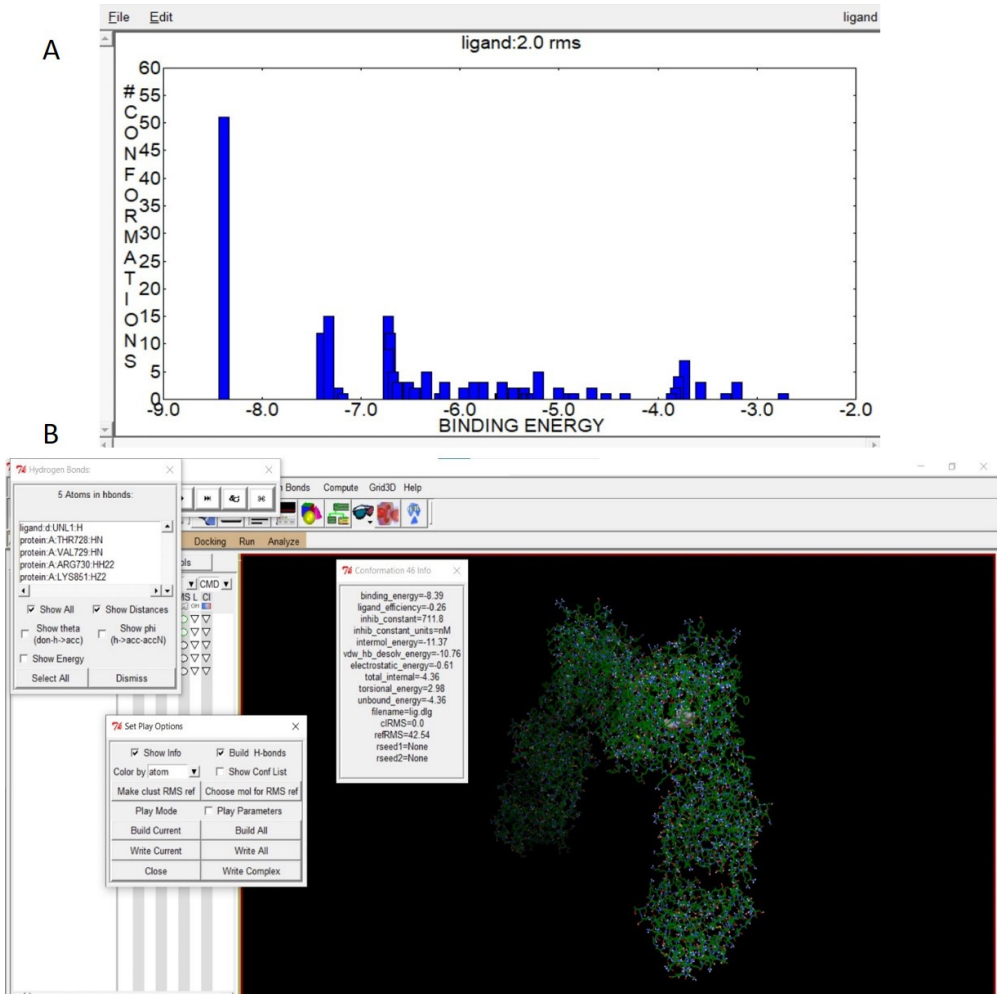

Figure 2: (A) Clustering diagram of docking runs performed to identify the optimal run with the most negative binding energy that indicated a more stable structure. The most stable structure was obtained in cycle 46 with a binding energy of -8.39 kcal/mol. (B) The results from the optimal docking at run = 46. The Band 3 protein molecule is shown in green, while Quercetin-3-O-rhamnoside is depicted in gray within chain A.

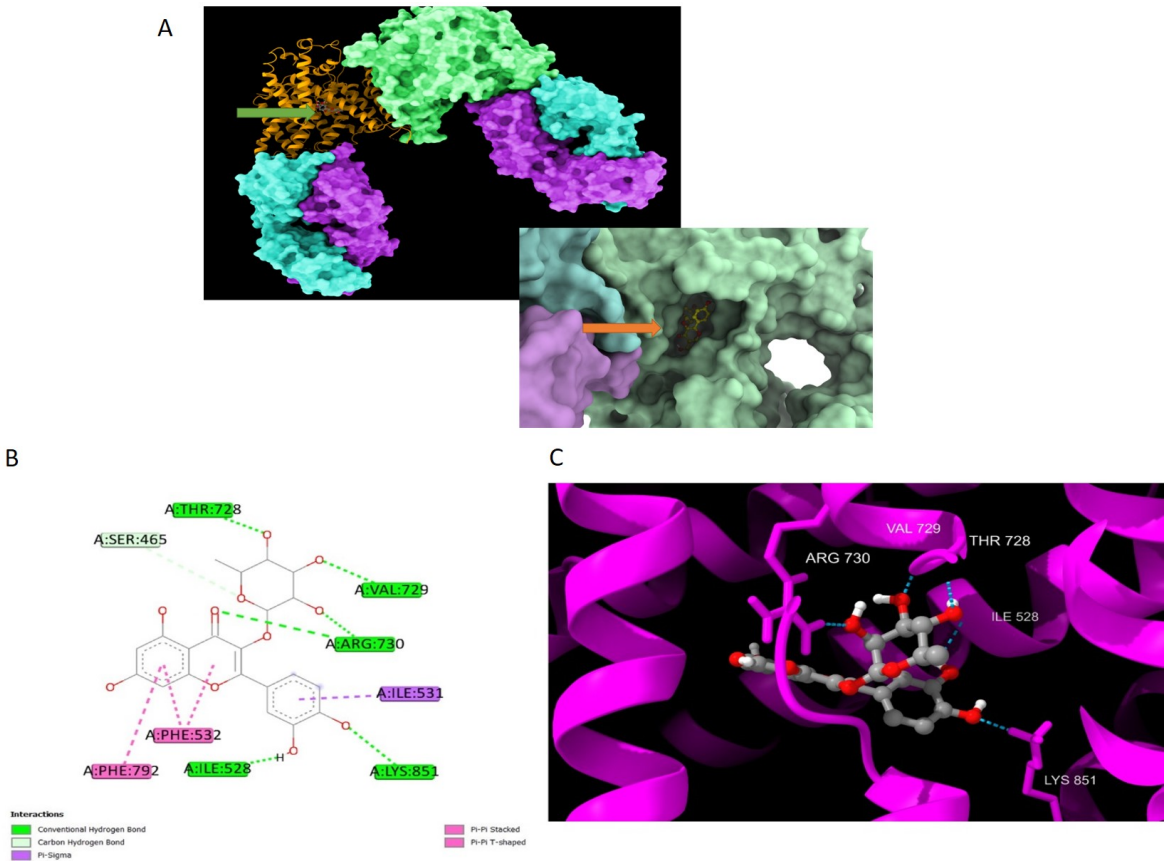

Figure 3: (A) It shows the optimized docking results in run=46 between chain A of the Band 3 protein and Quercetin-3-O-rhamnoside (indicated by the arrow) in Zoom in and Zoom out views. (B) It introduces the types of bonds between chain A of the Band 3 protein and Quercetin-3-O-rhamnoside. (C) it shows a three-dimensional representation of the hydrogen bonds.

which was notably more favorable than the standard 4KU ligand with a binding energy of -4.11 kcal/mol. The molecular docking results of run 46 are illustrated in Figure 2B. These findings strongly suggest that quercetin-3-O-rhamnoside could serve as a potential ligand for protecting against ROS-induced damage to the red blood cell membrane. Moreover, Figure 3A provides a three-dimensional representation of the optimized interaction in run 46 between Band 3 and quercetin-3-O-rhamnoside molecules. The potential bonds are depicted in Figure 3B. Among these intermolecular interactions, five were conventional hydrogen bonds, one was a carbon-hydrogen bond, one was a Pi-Sigma bond, and two were Pi-Pi interactions. The three-dimensional structure of the hydrogen bonds is shown in Figure 3C.

Determining Hemostatic Concentrations of the Extract and Loading Them into Hydrogels

A hydrogel with optimal mechanical strength was obtained using ratios of 1% chitosan in 3% gelatin and 1% chitosan in 2% gelatin. Observations revealed that a 2.5% v/v concentration of the extract significantly exhibited hemostatic properties (PT: 11±1.82 sec for the extract vs. 16.5±0.7 for plasma alone and 18±0.6 for normal saline; $p < 0.05$). However, the concentrations of 5%, 10%, and 20% of the extract prolonged PT time (Table 1). Accordingly, the hemostatic dilution of the extract was used in hydrogel preparation (Figure 4).

Adhesion Ability of Hydrogels to RBCs

The results are presented in Table 2 and Figure 4. Accordingly, the 3:1 gelatin/chitosan ratio of hydrogels ($OD_{Free\text{-}hemoglobin}$: 0.485±0.057) showed significantly lower free hemoglobin than the positive control and sterile gauze samples ($OD_{Free\text{-}hemoglobin}$: 1.489±0.007 and 1.157±0.026, respectively; $p < 0.0001$). Moreover, there were no significant differences in the amount of free hemoglobin between the 3:1 gelatin/chitosan ratio of hydrogels and the 2:1 one ($OD_{Free\text{-}hemoglobin}$: 0.485±0.057 and 0.518±0.008, respectively; $p = 0.5594$).

Discussion

Natural extracts have been of great interest in the

Table 1: Measuring hemostatic concentrations of the hydroalcoholic extract (1:1 water: 70% ethanol) from *Juglans regia L.* leaves.

Samples	Prothrombin Time (Sec)	Effects
Plasma (50μl)	16.5±0.7	Normal
Normal Saline (50μl)	18±0.6	Normal
Extract (2.5%v/v)	11±1.82	Hemostatic
Extract (5%v/v)	20±1	Prolonged
Extract (10%v/v)	31±1	Prolonged
Extract (20%v/v)	44±1	Prolonged

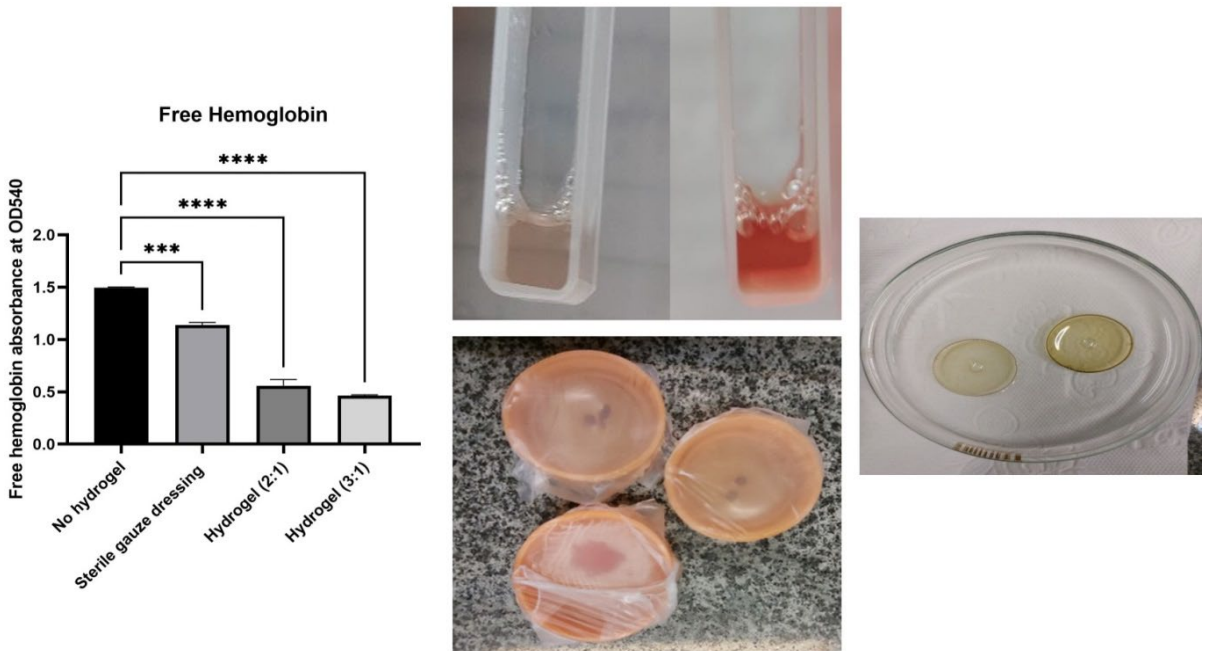

Figure 4: A film of green hydrogel synthesized from a combination of gelatin, chitosan, and the hydroalcoholic extract (1:1 water: 70% ethanol) from *Juglans regia L.* leaves at 2.5v/v%. Comparison of free hemoglobin among hydrogels, free-hydrogel positive controls, and sterile gauze showed that the synthesized hydrogels were able to absorb more blood compared to sterile gauze and the conditions without hydrogel. A statistical significance level of $p < 0.05$ was considered, and the data are presented as mean ± SD.

Table 2: Optical density (OD) of samples based on the amount of free hemoglobin.

Samples	OD	Adjusted P Value
Positive Controls	1.489±0.007	<0.0001
Steril Gauze	1.157±0.026	<0.0001
Hydrogels (G2:C1)	0.518±0.008	0.5594
Hydrogels (G3:C1)	0.485±0.057	Ref

synthesis of hemostatic materials in recent years because of their inherent bioactive properties (16, 17). Extensive studies have been conducted on plant-derived compounds, including extracts derived from leaves, fruits, and tree bark. Indeed, researchers aim to utilize the rich reservoirs of bioactive molecules present in natural sources to produce diverse hemostatic agents that are not only effective but also biocompatible and sustainable (16, 18, 19). In this context, the present study utilized walnut leaf extract, which contains bioactive compounds such as tannins and flavonoids known for their hemostatic and anti-inflammatory effects (20). The rationale of the present study was that these natural compounds could complement the intrinsic properties of chitosan and gelatin in terms of mucoadhesion, biocompatibility, and structural integrity (9, 21). This study well evidenced that the synergistic interactions between walnut leaf extract and the chitosan/gelatin matrix enhance the overall hemostatic efficacy of the hydrogel.

Docking results of the most abundant antioxidant, quercetin-3-O-rhamnoside, of walnut leaves are compared to previously reported studies which had docked another type of quercetin (PubChem CID 5280343, binding energy: -7.46 kcal/mol) or the standard ligand for Band 3, i.e., 4KU (binding energy: -7.11 kcal/mol) (22). The current study has also reflected the better binding energy of -8.39 kcal/mol, which showed that a band will be formed between the quercetin family and Band 3 protein. Previous studies showed the participation of three amino acids such as PHE532, ARG73, and

ILE528 in the interaction of quercetin (PubChem CID 5280343) with Band 3 (14), while this study identified nine amino acids in the interaction between quercetin-3-O-rhamnoside (PubChem CID: 5353915) and Band 3 including THR728, SER465, VAL729, ARG730, ILE531, LYS851, ILE528, PHE532, and PHE792. These findings strongly indicate that quercetin might act as a potential ligand in the prevention of ROS-induced RBC membrane damage.

In addition, this work did not use any chemical linker in the synthesis of the hydrogels. In other words, the synthesis of hemostatic hydrogels using natural polymers like chitosan and gelatin follows the principles of green chemistry and sustainable material development. This green chemistry approach, as demonstrated in this work, reduces the environmental footprint of conventional synthesis methods while ensuring the biocompatibility and safety of the resulting hydrogel (22). Since this hydrogel avoids the use of aggressive chemical agents, it maintains its potential biomedical applications for hemostasis and wound management.

In the study conducted by Habibpour et al. (2014), the effects of hydroalcoholic walnut leaf extract on blood parameters of male hypothyroid rats were investigated. According to their findings and other researchers, hydroalcoholic walnut leaf extract can protect red blood cells and hemoglobin against oxidative damage because of high antioxidant properties obtained from flavonoids and polyphenols (23). Another recent study, by Nasiri et al. (2022), used 75 male rats, all diabetic, randomly distributed among five groups, 15 in each: the untreated, Eucerin group, 2% and 5% Juglans regia L. ointment, and the reference drug phenytoin. The results of the study showed that topical application of Juglans regia L. leaf extract significantly enhanced the rate of wound healing in diabetic patients, particularly at the 5% concentration (24). In the present study, we have shown that a 2.5% v/v concentration of extract significantly exhibited hemostatic properties ($p < 0.05$), whereas 5%, 10%, and 20% concentrations prolonged PT time. Overall, the above studies support the present one in the fact that walnut leaf extract can be used effectively for hemostasis and wound healing.

Besides, research has documented that the chitosan/gelatin-based hemostatic polymer outperforms standard dressings by offering fast hemostasis, reduced blood loss, and speedy wound healing (25). Chitosan-based hemostatic dressings are used in many forms, such as bandages (e.g., Hemcon Chitosan Bandage) and sheets (e.g., Clo-Sur PAD). However, some dressings emit a strong acidic odor when acetic acid is used as a solvent during their preparation (26). In this study, although the chitosan film also had an acetic acid odor, its odor was reduced after being mixed with gelatin polymers and the extract. In addition, as an absorbent matrix, gelatin is able to lock in platelets and other coagulation factors within its structure to control bleeding. However, it readily dissolves in water, which could be a reason for its combination with other natural and synthetic polymers to enhance mechanical and chemical stability. At the same time, chitosan possesses good viscosity and layering properties and contains free amino acid groups that may be used for cross-linking. These two materials together form a natural and semi-continuous polymer structure mimicking a natural porous scaffold or dressing (27). In this study, the synthesized green hydrogels from gelatin and chitosan at the ratios of 3:1 and 2:1 could reduce free hemoglobin concentrations by 1.69% and 2.65%, respectively. This suggested their high ability to adhere to RBCs to reduce hemolysis—a desirable feature for biocompatible hemostatic materials. The results obtained in this study are similar to those from Sabino et al. (2020) for different hemostatic biomaterials (28). Due to the abundant supply of gelatin, chitosan, and walnut leaves in Iran, the results of this study propose a non-toxic, available, and sustainable hydrogel dressing.

Conclusion

In conclusion, by exploiting the hemostatic potentials of natural extracts in combination with biocompatible polymer matrices, our work contributes to the continuously developing realm of hemostatic material research. Our focus is on the need for efficient hemostatic agents that are biocompatible, sustainable, and therapeutically viable, thus enabling the second generation of wound care devices for a broad range of healthcare applications. The crystallographic three-dimensional structure of Band 3, PDB ID: 4YZF, and Quercetin-3-O-rhamnoside, PubChem CID: 5353915, were retrieved from the PDB and PubChem databases. From the molecular docking results, Quercetin-3-O-rhamnoside showed optimal binding affinity toward Band 3 protein with a binding energy of -8.39 kcal/mol, higher compared to the binding energy of the standard ligand 4KU (-7.11 kcal/mol); thus, this compound can be considered a potential ligand for protection against the damage of the RBC membrane induced by reactive oxygen species. In the best ratios of gelatin/chitosan polymers, 1:3 and 1:2, with 2.5% (v/v) hydroalcoholic extract of walnut leaves, the hemostatic effect was indicated on citrated plasma by PT test in laboratory experiments. This green hydrogel film showed significantly higher adhesion to red blood cells and a reduction in free hemoglobin concentration than controls without hydrogel and commercial sterile gauze ($p < 0.05$).

Future studies should be performed to optimize the formulation with the evaluation of its biocompatibility, loading capability, cytotoxicity, and long-term stability. Quercetin-3-O-rhamnoside can be extracted from walnut leaves and then directly incorporated into hydrogel. Its hemostatic and antioxidant properties may be studied both in vitro and in vivo.

Conflict of Interest

The authors have nothing to declare.

Ethical approval Statement

This study was approved by the Research Ethics committee of Tehran University of Medical Sciences under the identification code IR.TUMS.SPH.REC. 1403.039.

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