Journal of Experimental Pharmacology

Dovepress
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ORIGINAL RESEARCH

Cytotoxicity and Coagulation Effects of Methanolic Extracts of *Punica granatum, Quercus infectoria, and Achillea millefolium*: an in vitro Study

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Objective: Considering the historical use of three plant species *Punica granatum, Quercus infectoria*, and *Achillea millefolium* in traditional medicine for hemostatic purposes, along with the variation observed in some experimental research, the aim of this study was to comparatively evaluate the effect of methanol extracts derived from these three medicinal plants on blood clotting time in vitro.

Methods: Methanolic (70%) extracts of *P. granatum* flower, *Q. infectoria* galls and *A. millefolium* aerial parts were prepared by maceration method. The total tannin and phenolic content were determined using a colorimetric method. The cytotoxic activity of extracts on NIH/3T3 cell lines was evaluated by the colorimetric MTT assay. Non-toxic concentrations were tested in vitro on blood coagulation profiles including prothrombin time (PT) and activated partial thromboplastin time (aPTT) of healthy human volunteers.

Results: *Q. infectoria* had the highest phenolic content at 287.18 mg gal/g, followed by *P. granatum* and *A. millefolium. P. granatum* exhibited the highest tannin content (119.21 mg catechin/g DW), followed by *Q. infectoria* (109.30 mg catechin/g DW) and *A. millefolium* (54.35mg catechin/g DW). The highest total flavonoid content was found in *P. granatum, A. millefolium*, and *Q. infectoria* with 98, 85.5, 5.22 mg Rutin/g DW, respectively. Non-toxic concentrations were selected to perform coagulation experiments. Based on the plant-derived coagulation assays, pomegranate extract notably reduced the PT to 11.57 ± 0.37 s compared with the control value of 14.47 ± 0.41 s. Conversely, gall oak extract markedly increased the coagulation time to 17.07 ± 1.07 s. Similarly, pomegranate significantly decreased aPTT to 33.83 ± 0.79 s relative to the control (36.30 ± 2.04 s), whereas gall oak considerably prolonged it to 50.00 ± 2.04 s.

Conclusion: Methanolic extracts of *P. granatum* show promise as procoagulants, while *Q. infectoria* acts as an anticoagulant, and *A. millefolium* remains neutral in this context.

Keywords: *Punica granatum*, *Quercus infectoria*, *Achillea millefolium*, coagulation profile, cytotoxicity, in vitro assay

Introduction

Despite advances in medicine, hemorrhagic shock is still the leading cause of death in civilian trauma, childbirth, and the battlefield.[1] Although the initiation of hemostasis and control of bleeding is an automatic process that helps maintain the hemodynamic status of the body, if bleeding continues, the person will die. Considering that humans have about 5 liters of blood and damage to a large artery or vein can put patients' lives at risk, controlling bleeding is a very important issue. Topically applied hemostatic drugs have many clinical applications in achieving hemostasis. This process occurs in several sequential steps, including blood vessel constriction, activation of the coagulation cascade, and blood clotting.[2] The hemostatic potential of medicinal plants and their components has long been known and can play a key role in preventing death from bleeding. In this study, three medicinal plants were selected based on their reported activity in

https://doi.org/10.2147/JEP.S580084
Received: 11 November 2025
Accepted: 4 February 2026
Published: 17 February 2026

Persian Traditional Medicine (PMT). *Achillea millefolium* commonly known as yarrow is a plant in the family Asteraceae used in the treatment of disorders related to the digestive system, including digestive problems, indigestion, bloating, abdominal pain, and diarrhea.[3] Several studies have shown its effectiveness in reducing internal organ bleeding in rat and human.[4,5]

Pomegranate (*Punica granatum* L.) is a medicinal plant from the family Lythraceae, whose original origin is Iran, Afghanistan, the Caucasus, and northern India. This plant has long been used in traditional Iranian medicine to prevent liver bleeding and heal wounds.[6] In the latest study, the positive effects of pomegranate on bleeding control have been cited.[7,8] In PTM, pomegranate flowers (Golnar) are specifically renowned for their hemostatic and astringent properties. Classical texts, such as those by Avicenna (The Canon of Medicine), recommend its use for managing bleeding, wounds, and oral gum disorders. The flowers are particularly rich in hydrolyzable tannins (especially punicalagins and ellagic acid derivatives), which are potent protein-precipitating agents. These tannins are known to denature blood proteins and promote platelet aggregation, providing a mechanistic basis for their hemostatic action. While the fruit peel also contains tannins, the flower is the traditional and most cited part for blood staunching.

Quercus infectoria gall is a natural product derived from the *Q. infectoria* Olivier oak tree and has a long history in traditional medicine, particularly in the Middle East.[9] Galls are rich in tannins, especially gallo-tannins and gallic acid, and phenolic acids, and therefore have astringent, anti-inflammatory, and hemostatic properties.[10] They are not the tree's standard vegetative part but are pathological growths induced by insect larvae, which PTM explicitly identifies and uses for their exceptional astringency, and several studies have reported a positive effect on wound healing, reduced inflammation, and increased cellular infiltration. These three plants contain high levels of polyphenolic compounds, with tannins being particularly abundant. Research indicates that tannins, as plant-derived compounds, can influence platelet function, blood coagulation, the fibrinolytic system, and endothelial activity. They also exhibit anti-thrombotic properties. While many studies highlight the beneficial impact of tannins on hemostasis, some drawbacks have been noted, including their limited bioavailability and the potent effects of their metabolites.[11]

Considering the historical application of these three botanical species within Persian medicine for hemostatic purposes, along with the diversity observed in some experimental research, the objective of this study was to evaluate the comparative impact of methanolic extracts derived from these three medicinal plants on in vitro coagulation time. Acknowledging that methanolic extracts facilitate the extraction of a broader spectrum of bioactive constituents (including flavonoids and terpenoids) compared to their aqueous counterparts, which are integral to pharmacological mechanisms such as platelet aggregation and hemostasis,[12] methanolic extracts of the aforementioned three medicinal plants were employed in this research. While the hemostatic potential of these botanicals is supported by tradition, translating traditional use into evidence-based application requires rigorous safety evaluation. A fundamental prerequisite for any substance intended to modulate physiological processes in vivo is the establishment of a non-toxic concentration range. Therefore, prior to assessing bioactivity, it is critical to screen for cytotoxicity to identify concentrations that are biologically active yet safe for human cells, thereby ensuring that any observed coagulant effects are not confounded by general cellular toxicity.

Materials and Methods

Plant Material Collection and Preparation of Plant Extract

Galls of *Q. infectoria* were gathered from Kermanshah in July 2023. A voucher specimen of *Q. infectoria* was deposited in the agriculture Faculty, Herbarium Center of Razi (968RUH). Leaves of *A. millefolium* were provided from the market. Authentication of the plant was done at Kerman University of Medical Sciences. A voucher specimen of *A. millefolium* was deposited at the Faculty of Pharmacy (KF 1234). A voucher specimen of *P. granatum* was authenticated by a professional herbalist and was deposited in the herbarium of the Faculty of Pharmacy, Kerman University of Medical Sciences (KF1634-1).

About 500 g of *P. granatum* flowers, *Q. infectoria* galls and *A. millefolium* aerial parts were extracted separately. The extracts were concentrated under vacuum in a rotary evaporator (Heidolph WB 2000, Germany), dried completely in an oven at 40°C, and stored at −20°C until testing.

Total Phenolic Content (TPC), Total Flavonoids (TF) and Total Tannin Content (TTC) of Plant Extracts

TPC was measured using the Folin-Ciocalteu method, mixing 1 mL of plant extract or gallic acid with 1 mL of Folin-Ciocalteu reagent and sodium carbonate. Absorbance was recorded at 700 nm by a UV-Visible spectrophotometer (Lambda 25, PerkinElmer, USA), and data were expressed as mg gallic acid equivalent/g dried extract. A calibration curve was made with different gallic acid concentrations.[13]

The total flavonoid content (TFC) was determined using the aluminum chloride colorimetric method. Briefly, 1 mL of the appropriately diluted methanolic extract was mixed with 1 mL of a 2% aluminum chloride ($AlCl_3$) methanolic solution and 3 mL of a 5% sodium acetate (CH_3COONa) solution. A reagent blank was prepared simultaneously by replacing the extract with 1 mL of 70% methanol. The reaction mixtures were vortexed and then incubated in the dark for 2.5 hours at 25 ± 1°C. After incubation, the absorbance was measured at 445 nm against the reagent blank using a PerkinElmer Lambda 25 spectrophotometer. Quantification was performed using a standard calibration curve of rutin (31.28–500 μg/mL) prepared in 70% methanol and subjected to the same assay procedure. All measurements were performed in triplicate (n = 3) for each independent extract. The total flavonoid content was expressed as milligrams of rutin equivalents per gram of dry extract.[14]

For measuring TTC, 0.5 mL of the extract was added to a test tube that was subsequently covered with aluminum foil, followed by 3 mL of 4% vanillin-methanol solution along with 1.5 mL of HCl. The mixture was incubated for 15 minutes in the dark at 20°C. Then, the absorbance of the solution was measured using a spectrophotometer (Lambda 25, Perkin Elmer, USA) at 500 nm. Calibration was conducted using a catechin solution at a concentration of 30 mg/L. The outcomes were articulated as milligrams of catechin equivalents per gram of dry extract.[15]

Cytotoxicity Assay

The cytotoxic activity of *P. granatum, Q. infectoria*, and *A. millefolium* on NIH/3T3 cell lines were evaluated by the colorimetric MTT assay. Cells were maintained at 37°C in a humidified incubator with 5% CO_2 using DMEM supplemented with penicillin, streptomycin, and 10% FBS. To assess the cytotoxicity of the methanolic extract at various concentrations, the MTT assay was employed. For this, 8,000 cells were plated into each well of 96-well plates and exposed to the different concentrations of the extracts for 48 hours. Afterwards, MTT solution was added, and after a 3-hour incubation, formazan crystals were dissolved using DMSO. Absorbance was then read at 490 nm, and cell viability was determined. Each experiment was performed in triplicate.[16,17]

Plasma Preparation

Blood samples were collected from four healthy adult volunteers who provided written consent. Participants were surveyed regarding their lack of medication use in the past week. The blood was mixed with trisodium citrate, centrifuged at 2500 g for 10–15 min, and the plasma was refrigerated for later use.

Prothrombin Time (PT)

For PT determination, 200 μL of pre-warmed PT reagent was pipetted into a test tube or cuvette and kept at 37°C. Then, 190 μL of patient plasma or 10 μL of normal saline (for control) and 4%, 0.001% and 2% of *P. granatum, Q. infectoria* and *A. millefolium*, respectively, were added to the reagent. The timer was started and the solution was gently mixed. The time required for clot formation was recorded, which is prothrombin time (PT).[18]

Activated Partial Thromboplastin Time (aPTT)

To determine aPTT, 200 μL of aPTT reagent was Pipetted into a test tube. About 190 μL of patient plasma or 10 μL of normal saline (for control) and 4%, 0.001% and 2% of *P. granatum, Q. infectoria* and *A. millefolium*, respectively, were added to the tube. The mixture was incubated at 37°C for 3 minutes. About 100μL of pre-warmed $CaCl_2$ solution was added and the timer was turned on while the mixture gently mixed. The time required for clot formation was recorded, which is aPTT.[18]

Statistical Analysis

Data were statistically analyzed by independent *t*-test using GraphPad Prism 10.0. P-values less than 0.05 ($P < 0.05$) were considered statistically significant. The results were expressed as Mean ± SD.

Results

Plant Extract

The extract yields of *Q. infectoria, A. millefolium* and *P. granatum* were 25.2%, 12.2% and 35.8% (dry extract weight/dry matter), respectively.

Total Phenolic Content (TPC) and Total Tannin Content (TTC) of Plant Extracts

TPC of the examined plants was expressed as mg gallic acid equivalents per gram dry extract. Among the analyzed samples, *Q. infectoria* showed the highest TPC (287.18 ± 4.12 mg gallic acid equivalent/g dried extract), followed by *P. granatum* (60.51 ± 2.26 mg gallic acid equivalent/g dried extract) and *A. millefolium* (7.59 ± 0.62 mg gallic acid equivalent/g dried extract).

The measured TTC values were significantly different between the three plant species. *P. granatum* exhibited the highest tannin content (119.21 mg catechin/g dried extract), followed by *Q. infectoria* (109.30 mg catechin/g dried extract) and *A. millefolium* (54.35 mg catechin/g dried extract).

The highest total flavonoid content was found in *P. granatum, A. millefolium*, and *Q. infectoria* with 98, 85.5, 5.22 mg Rutin/g dried extract, respectively.

Cytotoxicity Assay

The results of cytotoxicity showed that IC_{50} for pomegranate, yarrow and gall oak were 18.6%, 3.3% and 13.3%, respectively. Concentrations of 4%, 0.001% and 2% of P. *granatum, Q. infectoria* and *A. millefolium* that which did not show significant differences with the control and were therefore non-toxic, were selected for coagulation experiments. Figure 1a–c shows the cytotoxicity results of *Punica granatum, Quercus infectoria* and *Achillea millefolium* at different concentrations, respectively.

In vitro Prothrombin Time (PT)

According to the plant-based coagulation tests, pomegranate significantly shortened prothrombin time (PT) to 11.57 ± 0.37 compared to the control group's 14.47 ± 0.41. In contrast, gall oak considerably prolonged coagulation time, reaching 17.07 ± 1.07 (Figure 2).

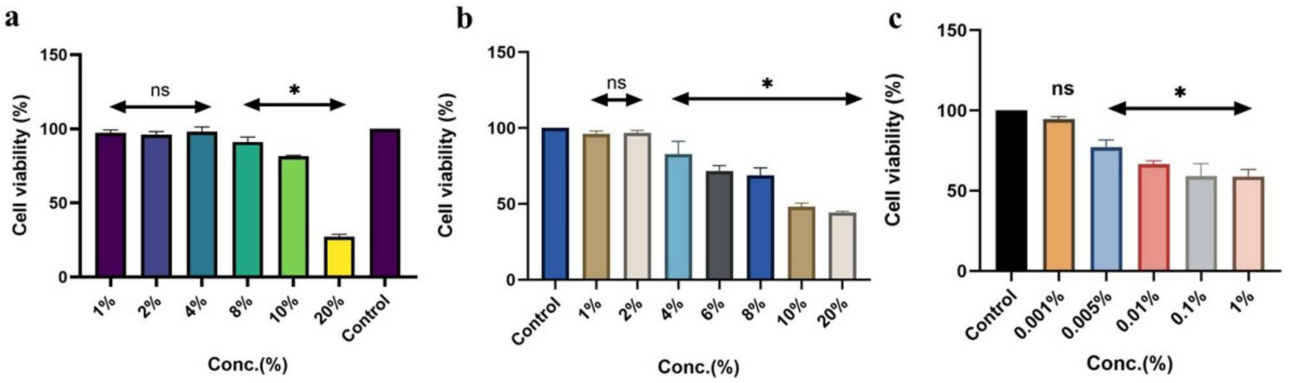

Figure 1 The cytotoxicity results of *Punica granatum* (**a**), *Quercus infectoria* (**b**) and *Achillea millefolium* (**c**) at different concentrations. Data are presented as Mean ± SEM (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$), while "ns" indicates no significant difference, in cytotoxicity between the control group and the tested plant extract concentrations.

 https://doi.org/10.2147/JEP.S580084

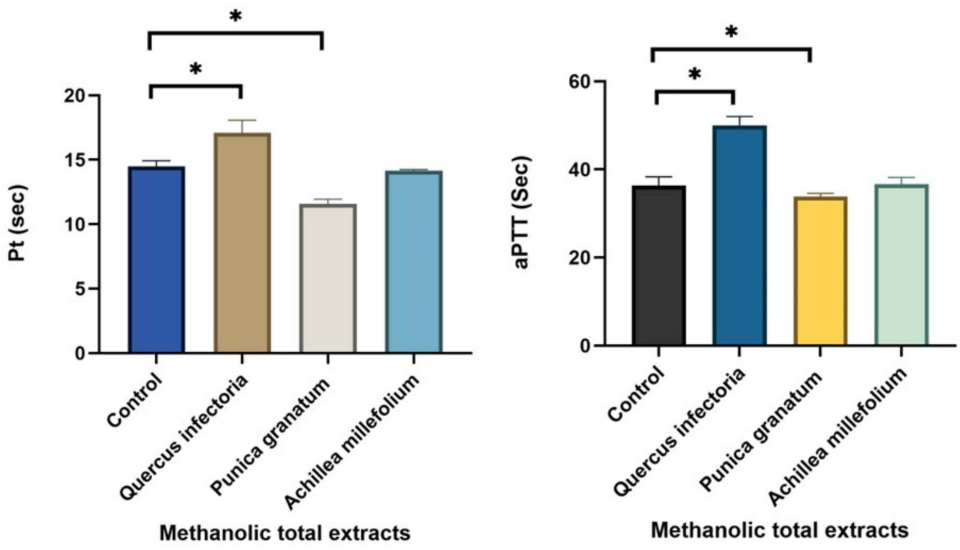

Figure 2 In vitro Prothrombin time and activated partial thromboplastin time of normal human plasma treated with *Punica granatum, Quercus infectoria* and *Achillea millefolium* at nontoxic concentration. Results are Mean ± SEM (n = 4). The asterisk (*) indicates a statistically significant difference ($p < 0.05$).

In vitro Activated Partial Thromboplastin Time (aPTT)

Figure 2 shows that pomegranate significantly reduced activated partial thromboplastin time (aPTT) to 33.83 ± 0.79 compared to the control value of 36.30 ± 2.04. In contrast, gall oak significantly extended thromboplastin time, reaching 50.00 ± 2.04.

Discussion

The current investigation assessed the in vitro influences of methanolic extracts derived from *P. granatum* (pomegranate flower), *Q. infectoria* (gall oak galls), and *A. millefolium* (yarrow aerial parts) on human blood coagulation metrics, specifically time (PT) and (aPTT). The extracts were analyzed for TPC, TF, and TTC, and then the nontoxic concentration of the extracts was determined based on cytotoxicity assessments. The results demonstrate differential modulation of coagulation by these extracts, with *P. granatum* exhibiting procoagulant activity, *Q. infectoria* showing anticoagulant effects, and *A. millefolium* displaying no significant impact on PT or aPTT at the tested concentration. TPC analysis showed that *Q. infectoria* had the highest level, followed by *P. granatum* and *A. millefolium*. These findings are consistent with previous reports on the rich polyphenolic composition of these plants.

For instance, *Q. infectoria* galls are known for their high tannin content, primarily gallotannins and gallic acid, which contribute to their astringent and hemostatic properties in traditional medicine.[19] Similarly, *P. granatum* peels and flowers contain hydrolysable tannins and flavonoids, with TPC values in various extracts range from 50–200 mg/g depending on extraction methods.[20] *A. millefolium*, while lower in TPC, includes phenolic acids and flavonoids such as apigenin and luteolin, consistent with studies reporting TPC around 5–15 mg/g in aerial parts.[21] The variation in phenolic and tannin contents may influence their biological activities, as these compounds can interact with proteins in the coagulation cascade, potentially modulating fibrin formation or platelet aggregation.[18]

In the coagulation assays, *P. granatum* extract significantly shortened PT and aPTT, indicating procoagulant effects likely through enhancement of the extrinsic and intrinsic pathways. This is consistent with prior in vitro and in vivo studies where *P. granatum* extracts reduced clotting times and inhibited platelet aggregation via polyphenolic interactions with fibrinogen and thrombin.[8] In a study by Gashtasbi et al (2014) in women who had heavy menstrual bleeding, they reported that there was no significant difference between the two groups that received pomegranate flower extract or tranexamic acid after the intervention and bleeding decreased and hemoglobin levels increased in both groups.[8] Pomegranate flower extract has been shown to have positive effects on the early stages of experimentally induced bleeding disorder in rats.[7] In contrast, *Q. infectoria* extract prolonged PT and aPTT, exhibiting anticoagulant activity.

This aligns with reports of methanolic plant extracts increasing PT and aPTT by interfering with factor activation, as seen in studies on various species where tannins inhibit thrombin or factor Xa.[18] However, this contrasts with traditional uses of *Q. infectoria* for hemostasis, where topical applications promote wound healing without systemic anticoagulant effects, as noted in rat models.[22] The results of the study by Iminjan et al (2014) are in line with the results of this study and have reported that *Q. infectoria* galls did not have an effect on systemic coagulation parameters such as PT and PTT and it seems that it does not have a significant systemic effect on blood coagulation.[23]

Yarrow extract at 2% concentration showed no significant changes in PT or aPTT, which may indicate neutral effects on systemic coagulation in vitro, despite traditional claims of its hemostatic properties. This is supported by studies showing local hemostatic efficacy in rat liver hemorrhage models without affecting plasma parameters, which is likely due to local vasoconstriction induced by flavonoids rather than direct coagulation factor modulation.[4] However, some contradictory results have also been reported. For instance, a systematic review study in Iran reported that yarrow significantly reduced menstrual pain and bleeding in women,[4,5] Pitrika et al showed that the aerial part of the yarrow plant has been significantly shown to reduce to 36% liver bleeding in Wistar rats.[24] Jankel et al showed that consuming large amounts of *A. millefolium* can reduce the rate of blood clotting. Taking *A. millefolium* with medicines that slow down blood clotting may increase the chance of bruising and bleeding, drugs such as Aspirin, Clopidogrel, Diclofenac, Ibuprofen, Naproxen, Dalteparin, Enoxaparin.[25]

The fact that pomegranate flowers, due to having high tannins and flavonoids compounds, showed a greater blood-thinning effect indicates these compounds might play a role in this. Consistent with a potential mechanism for our findings, Lafdil et al documented that *P. granatum* extract provided 80% antithrombotic protection in mice. Their mechanistic studies revealed dose-dependent inhibition of platelet aggregation in vitro, alongside prolonged bleeding and coagulation times in vitro and ex vivo in rats. HPLC analysis correlated these effects with a high concentration of polyphenols and flavonoids, suggesting these constituents mediate the observed antiplatelet and anticoagulant actions, which were not associated with toxicity.[26] *A. millefolium*'s lower phenolic profile might explain its lack of effect, highlighting the dose- and compound-specific nature of plant-based modulators. These findings corroborate traditional Iranian medicine uses for bleeding control but reveal inconsistencies, such as *Q. infectoria*'s anticoagulant profile, which may limit its systemic application.[5] The potent anticoagulant activity of *Q. infectoria* is likely due to its unique polyphenolic composition, which may interfere with critical coagulation factors like calcium, rather than promoting protein precipitation. The stark contrast with pomegranate, despite both being tannin-rich, highlights that the type and mechanism of tannins are crucial.

Limitations include the in vitro nature of the study, which may not reflect in vivo pharmacokinetics or interactions with whole blood components like platelets. The use of plasma from only four volunteers introduces variability, and the single-species focus (from southeast Iran) limits generalizability due to genetic and environmental differences in plant chemotypes. Future research should incorporate in vivo models, platelet aggregation assays, and molecular docking studies to elucidate mechanisms, alongside clinical trials for conditions like menorrhagia or postoperative bleeding.[27]

In conclusion, methanolic extracts of *P. granatum* show promise as procoagulants, while *Q. infectoria* acts as an anticoagulant, and *A. millefolium* remains neutral in this context. These findings indicate that there is a need to further investigate these plants for hemostatic treatments and indicate the need to identify the main constituents and subsequently standardize the extracts. The strong procoagulant activity of *P. granatum* aligns perfectly with its exceptionally high tannin content, a class of compounds known to precipitate fibrinogen and promote clotting. Conversely, the potent anticoagulant effect of *Q. infectoria*, despite its high phenolic content, suggests a different mechanism, potentially through the chelation of essential calcium ions or inhibition of key coagulation enzymes by its specific tannin profile.

Limitations: We acknowledge that the inclusion of a positive control, such as warfarinized plasma for the PT assay or heparinized plasma for the aPTT assay, would have strengthened the experimental validation. Their omission in the current study is a limitation.

Blood from four healthy donors was used for this initial in vitro screening study. This sample size was chosen to allow for a paired, proof-of-concept assessment of extract activity while acknowledging the limitation in statistical power for detecting small effects.

Ethics Approval and Informed Consent

The research received ethical approval from the Ethics Committee of Kerman University of Medical Sciences with approval number IR.KMU.REC.1402.232. All participants were provided with written informed consent forms outlining the study details and investigator contact information. Their agreement to take part in the study was confirmed through signed consent forms. The authors declare that this study complies with the Declaration of Helsinki.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This work was supported by Kerman University of Medical Sciences, [grant numbers 402000472].

Disclosure

The authors declare that they have no competing interests in this work.

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