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Effect of mangiferin isolated from *Mangifera indica* leaves on *in vitro* blood coagulation and cell migration activities

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ABSTRACT

Background: Mangiferin, a natural compound has been reported to possess a variety of biological activities such as anticancer, anti-diabetic, antimicrobial, antioxidant, cardioprotective activities, etc. Screening on blood coagulation activity effects of mangiferin might be helpful for further activities investigation.

Objective: This study aimed to isolate mangiferin from mango leaves and evaluate its blood coagulation and anticancer activities against human lung cancer cell lines (A549 cells).

Materials and methods: Mangiferin was extracted from mango leaves and characterized by IR, NMR, and mass spectroscopic techniques. It was determined *in vitro* activities as follows: blood clotting time, platelet aggregation, activated partial thromboplastin time (aPTT), prothrombin time (PT), clot lysis, fibrinolysis, and cell migration assay.

Results: It was found that mangiferin had a significantly slower effect on inducing blood clots than the control group, with the coagulation value of 13.09±2.97 minutes and decreasing platelet aggregation at an inhibition percentage value of 14.1±1.2. There was significant (p<0.05) prolongation of PT and aPTT activities tested with the mangiferin at the value of 12.4±1.2 and 29.9±3.1 seconds, respectively. However, mangiferin was unable to cause fibrin clot dissolution on fibrinolysis test. Mangiferin also showed anticancer activity against A549 cells by inhibition of cell migration assay.

Conclusion: Mangiferin showed antiplatelet aggregation activity and prolongation of prothrombin time (PT) and activated partial thromboplastin time (aPTT) assay without fibrinolysis activity. In addition, mangiferin showed anticancer activity against A549 cells by inhibiting cell migration.

Introduction

Blood coagulation, also known as blood clotting, is a necessary process that helps block a bleeding when a blood vessel is injured. Nevertheless, blood clot formation in the vessel without any apparent injury can cause several complications in the human body. The undesired blood clot interferes with the free flow of blood, resulting in several life-threatening diseases. Meanwhile, thrombolytic activity is the process of breaking down the blood clot, which helps improving the blood flow.[1] In fact, platelet dysfunction and coagulation defects are becoming more associated with the onset and progression of several cardiovascular diseases, including atherosclerosis, thrombosis, peripheral artery disease, myocardial infarction, and ischemic stroke.

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Several anticoagulant agents used in clinical practices to prevent or reduce coagulation of blood consist of heparins, vitamin K-antagonists, coumarin derivatives, and factor Xa inhibitors. Thrombolytic agents used for dissolving blood clots are streptokinase, urokinase, recombinant tissue plasminogen activators, etc.[2] Some drugs have limitations of use and unwanted effects; novel potential drugs still need to be discovered and developed.[3] Natural products continue to be the focus of drug discovery research, and several drugs have been derived from plants. Nowadays, active constituents and crude extracts from plants have been widely screened for various biological activities.[4] Mangiferin, the major constituent obtained at significant level from leaves and barks of *Mangifera indica*, is a xanthone C-glucoside having a glucose substituent at position 2 of xanthone skeleton. Mangiferin has been reported to have numerous biological activities[5,6] such as anticancer,[7,8] antidiabetic,[9-13] antioxidant,[14] anti-inflammatory,[15] antiviral,[16] antibacterial,[17,18] cardiovascular protection[19] and hepatoprotective activities.[20] Due to its low toxicity, mangiferin has been widely used in nutritional supplement.[21] Furthermore, many mangiferin analogues have been synthesized and evaluated for their biological activities.[22] Interestingly, several studies have reported that mangiferin exerts markedly antineoplastic effects toward many types of cancer such as prostate cancer,[23] colon cancer,[24] leukemia,[25] and lung cancer.[26]

The present study aimed to investigate the potential effects of mangiferin on *in vitro* blood coagulation and anticancer activities.

Materials and methods

Reagent kits for prothrombin time (PT) and activated partial thromboplastin time (aPTT) (Siemens, Germany), 100 U/mL penicillin and 100 µg/mL of streptomycin (Invitrogen, USA), Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA), fetal bovine serum (FBS) (Invitrogen, USA) and other analytical grade chemicals were purchased from commercial vendors. Nuclear magnetic resonance (NMR) spectra were recorded using a 500-MHz Jeol NMR spectrometer. The infrared (IR) spectra were taken on a Perkinelmer Spectrum 100 FTIR spectrometer. Mass analysis was performed using a Joel JMS-S3000 mass spectrometer.

Extraction and purification of mangiferin

Several methods of mangiferin extraction have been reported.[27,28] The extraction and isolation of mangiferin herein was carried out following modified methods from Jutiviboonsuk *et al*.[29] and Shindea *et al*.[30] The leaves of 'Nam Dok Mai' mango cultivar collected from Samut Prakarn province were extracted with methanol (Merck, Germany) in a soxhlet apparatus for 48 hrs. The extract solvent was concentrated under reduced pressure to yield semisolid mass which was chromatographed over silica gel column eluted with dichloromethane (Merck, Germany): methanol (1:1) which gave amorphous mangiferin powder. Then, crystallization with 70% ethanol gave the needle-shaped crystals of mangiferin which were characterized by melting point, IR, NMR, and mass spectroscopy compared with mangiferin standard.

Blood sample preparation

Blood samples were collected from 60 healthy volunteers with no nonsteroidal anti-inflammatory drugs (NSAIDs). Normal sodium citrated plasma was prepared as follows. Nine milliliters of blood was drawn by venipuncture into centrifuge tubes containing 1 mL of 3.2% trisodium citrate solution. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by centrifugation at 1,000 rpm and 3,000 rpm for 15 min, respectively. This research was approved by Research Ethics Committee, Huachiew Chalermprakiet University.

Blood coagulation

Modified whole blood clotting time (modified WBCT) assay[31]

Clotting tubes containing 10 µL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany) (control tube) and 10 µL of 500 µg/mL mangiferin in DMSO (test tube) were incubated at 37 °C. Then, one mL of fresh blood from healthy volunteers was added into each incubated tube. After 5 min, the blood clot formation was determined at 30 sec interval. The time was recorded immediately after complete blood clot.

Platelet aggregation assay[32]

Briefly, 450 µL of platelet-rich plasma (PRP) ($2.5x10^8$ platelets/mL) was mixed with 4.5 µL of 50 mg/mL mangiferin to make the final concentration of 500 µg/mL, incubated at 37 °C for 3 min. After that, twenty microliters of 35 mM adenosine diphosphate (ADP) (Sigma-Aldrich, Germany) were added into 140 µL of the pre-incubated PRP. Platelet aggregation was measured as the increase in light transmission with a CS2500 (Siemens Sysmex®, Germany) analyzer at 660 nm using PPP as the baseline of 100% aggregation.

Activated partial thromboplastin time (aPTT) and prothrombin time (PT) [33] test

In short, 50 µL of normal sodium citrated plasma was incubated with mangiferin (at a final concentration of 500 µg/mL) for 1 min and 100 µL of tissue thromboplastin solution was added. The PT was recorded for the fibrin clot formation. For the aPTT test, 50 µL of normal sodium citrate plasma was incubated with mangiferin at a final concentration of 500 µg/mL for 1 min. Then, 50 µL of aPTT solution was added and incubated at 37 °C for 3 min. After that, 50 µL of 0.25 M calcium chloride ($CaCl_2$) (Sigma-Aldrich, Germany) was added and the time was recorded as a clot was formed. The obtained values were compared with the control unit (vehicle control).

Fibrinolysis activity assay

Clot lysis[34]

In brief, 500 µL of blood from healthy volunteers was drawn into a sterile microcentrifuge tube (the microcentrifuge tube had been weighed and recorded

previously), incubated at 37 °C for 45 min to form a complete clot. The serum was removed, leaving only the clot. The tube was weighed and the value obtained was calculated to find the clot weight (clot weight = weight of the tube in which the clot formed - weight of the tube). After that, mangiferin 100 µL was added to a final concentration of 500 µg/mL and incubated at 37 °C for 90 min and the liquid was aspirated and the weighed of the remaining clot was calculated. The results were displayed in the form of % lysis using phosphate buffer saline (PBS) (Invitrogen, USA) and streptokinase (Sigma-Aldrich, Germany) as negative and positive controls, respectively.

% Lysis = 100 - (clot weight after testing x 100)/initial clot weight)

Fibrinolytic activity

In brief, 100 µL of normal sodium citrated plasma (from 20 volunteers, pooled together) was mixed with 30 µL of 0.25 M $CaCl_2$, incubated at 37 °C for 3 hrs to form a soft fibrin clot. After that, washed the clot with PBS, then added 400 µL of 0.2 M Tris-HCl buffer pH 8.5 (Sigma-Aldrich, Germany) and 100 µL of mangiferin to make the final concentration of 500 µg/mL incubated at 37 °C for 2 hrs. Then, 750 µL of 0.44 M trichloroacetic acid was added at room temperature for 30 min and centrifuged at 3,300 rpm for 15 min. The supernatant was transferred to a new tube, followed by the addition of 1.25 mL of 0.4M sodium carbonate (Na_2CO_3) (Sigma-Aldrich, Germany) and 250 µL of 1:3 diluted Folin Ciocalteu's reagent (Sigma-Aldrich, Germany), leaved for 30 min and the absorbance was recorded at 660 nm. One unit of fibrinolytic activity, which is absorbance increased by 0.01 at 660 nm/hr at 37 °C was calculated.

Anticancer activity

Cell culture

Human lung cancer cell lines (A549 cells) were purchased from the American Type Culture Collection (ATCC). A549 cells were cultured in DMEM, supplemented with 10% FBS and 100 U/mL penicillin and 100 µg/mL of streptomycin. Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere.

***Cell cytotoxicity assay*[35,36]**

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed for cytotoxicity assessment. A549 ($5x10^3$ cells/mL) cells were seeded into 96-well culture plate. Following which, 100 µL fresh medium containing various final concentrations (1-1,000 µg/mL) of mangiferin were added and incubated at 37 °C for 72 hrs. The medium was then replaced with fresh medium containing MTT solution (Bio Basic, Canada) at the final concentration of 0.5 mg/mL. After formazan formation, 50 µL DMSO was added to dissolve the crystals. The samples were measured at 570 nm, along with the reference at 630 nm, using a microplate reader. The data was analyzed using GraphPad Prism v 6.00 software for Windows (GraphPad Software, Inc.). All experiments were performed independently in triplicate.

***Cell migration assay*[37,38,39]**

Effect of mangiferin on cell migration was performed by *in vitro* scratch assay. A549 cells were cultured ($1x10^5$ cell/well) in a 24-well plate. The wound was generated by scratching the cell layer using a 10 µL pipette tip when A549 cells reached approximately 90% confluency. Cells were washed 3 times with PBS and incubated with variour concentrations of mangiferin (0-1,000 µg/mL). The cell images were captured immediately (t=0 hr) under a microscope and then captured again after 24, 48, and 72 hrs of incubation. The area of wound was determined by Image J software. The cell migration was expressed as the percentage change in normalized measurement area to the original open area or wound closure:

$$\%\text{Wound closure} = [(A_{t=0} - A_{t=\Delta t}) / A_{t=0}] \times 100$$

Where $A_{t=0}$ is the area of the wound measured immediately after scratching and $A_{t=\Delta t}$ is the area of the wound measured at t hr after scratching.

Statistical analysis

Statistical analysis of the results was performed using PSPP software version 0.10.4.62724. Data were presented as the mean±SD. The comparison between the groups was carried out by independent samples *t*-test. The results were considered significant at a value of $p<0.05$.

Results

The melting point and molecular weight of isolated mangiferin were 269-271 °C and 422.3 g/mol, respectively. The FT-IR spectral data of isolated mangiferin showed remarkable OH stretch band at 3363 cm^{-1}, conjugated C=O stretch peak at 1648 cm^{-1} and C-O stretch peak at 1250 cm^{-1}. The chemical structure and the interpretation of NMR data of isolated mangiferin were shown in Figure 1 and Table 1, respectively. The ^{1}H-NMR signals at δ 6.35 (1H, H-4), 6.85 (1H, H-5), and 7.38 (1H, H-8) represented Ar-H. The down-field singlet proton at δ 13.80 indicated Ar-OH hydrogen bonding with the carbonyl moiety. The chemical shifts of the sugar moiety were observed at δ 3.15-4.60 ppm. The ^{13}C-NMR indicated 19 carbons atoms in the molecule. The signals of quaternary aromatic carbons were observed at δ 161.8 (C-1), 107.5 (C-2), 163.8 (C-3), 156.2 (C-4a), 101.3 (C-4b), 150.9 (C-6), 143.9 (C-7), 111.4 (C-8a), 154.5 (C-8b), and 179.0 (C=O), and methine carbons at δ 93.3 (C-4), 102.5 (C-5), and 107.8 (C-8). The signals of sugar carbons were observed at δ 61.5-81.6 ppm.

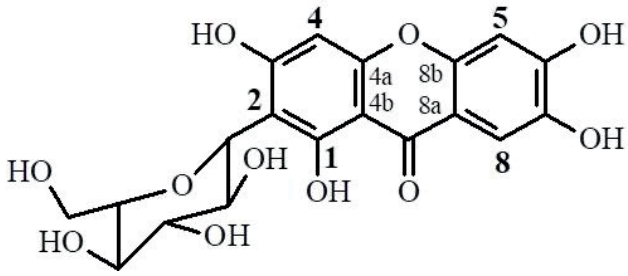

Figure 1 *Chemical structure of mangiferin.*

Table 1 Interpretation of ^{1}H-NMR (500MHz), ^{13}C (125MHz) data of mangiferin.

Position	^{1}H(δ,ppm)	^{13}C(δ,ppm)
1	13.8 (1-OH)	161.8
2		107.5
3		163.8
4	6.35, s	93.3
4a		156.2
4b		101.3
5	6.85, s	102.5
6		150.9
7		143.9
8	7.38, s	107.8
8a		111.4
8b		154.5
CO		179.0
Sugar	3.15-4.60	61.5-81.6

The effect of mangiferin on blood clotting using modified whole blood clotting time method revealed that mangiferin had a significantly slower effect on inducing blood clots than the control group (p<0.05). Mangiferin had a blood coagulation value of 13.09±2.97 minutes compared with the control set with a blood coagulation value of 12.06±2.34 minutes as shown in Table 2.

Table 2 Effect of mangiferin on blood clotting using modified whole blood clotting time method.

Sample (N=60)	Whole blood clot time (WBCT) (min)
Mangiferin (500 µg/mL)	13.09±2.97
Vehicle control	12.06±2.34

It was found that mangiferin decreased platelet aggregation in a statistically significant (p<0.05). Mangiferin had a platelet aggregation value of 65.3±11.1 compared with the control set which had a value of 76.0±6.6 min. The inhibition percentage of platelet aggregation activity of 500 µg/mL mangiferin was 14.1±1.2 as shown in Table 3.

Table 3 Effect of mangiferin on ADP-induced platelet aggregation.

Sample (N=20)	Platelet aggregation (%)	Inhibition (%)
Mangiferin (500 µg/mL)	65.3±11.1	14.1±1.2
Vehicle control	76.0±6.6	

Mangiferin affected the function of coagulation factors in fibrin clot formation in both the extrinsic and intrinsic pathways. Mangiferin at the concentration of 500 µg/mL prolonged the PT with a value of 12.4±1.2 sec slower than the control set with a value of 10.9±0.9 sec, in a statistical significance (p<0.05). Mangiferin showed significant (p<0.05) prolongation of aPTT compared with the control at the value of 29.9±3.1 sec as shown in Table 4.

Table 4 Anticoagulation activity of mangiferin.

Sample (N=60)	Coagulation test (s)	
	Prothrombin time (PT)	Activated partial thromboplastin time (aPTT)
Mangiferin (500 µg/mL)	12.4 ± 1.2	29.9 ± 3.1
Vehicle control	10.9 ± 0.9	27.4 ± 2.6

The study of mangiferin on clot lysis by blood clot lysis time method indicated that mangiferin at a concentration of 500 µg/mL caused blood clot lysis equal to 5.8 ±2.9% whereas the control group had clot lysis at a value of 6.9±1.0%, which was not significantly different at the 95 % confidence level (p>0.05). Studying the effect of mangiferin on the breaking down of fibrin clot by fibrinolytic activity assay revealed that mangiferin was unable to cause fibrin clot dissolution with a value of 0.5±0.4 compared with the control set with statistical significance 5.5±2.1 (p<0.05) as shown in Table 5.

Table 5 Fibrinolysis activity of mangiferin.

Sample (N=20)	Fibrinolysis	
	Blood clot lysis (%)	One unit of fibrinolytic activity
Mangiferin (500 µg/mL)	5.8±2.9	0.5±0.4
Vehicle control	6.9±1.0	5.5±2.1

To determine the effect of mangiferin on cell migration by *in vitro* scratch assay, A549 cells were cultured in the presence of various concentrations of mangiferin. The 90% confluent A549 cells were wounded using a pipette tip. After incubation, the wound closure was observed and imaged under an inverted microscope. Migration of A549 cell line treated with mangiferin (for 72 hrs) was inhibited with the percentage of wound closure reduced to 59.0, 41.1, and 26.8% at the concentration of 250, 500 and 1,000 µg/mL, respectively. Mangiferin at a concentration of 1,000 µg/mL significantly inhibited cell migration with the percentage of wound closure of 17.2, 23.1, and 26.8% after 24, 48 and 72 hrs, respectively (Figure 2) without cytotoxic effects as confirmed by MTT assay (Figure 3).

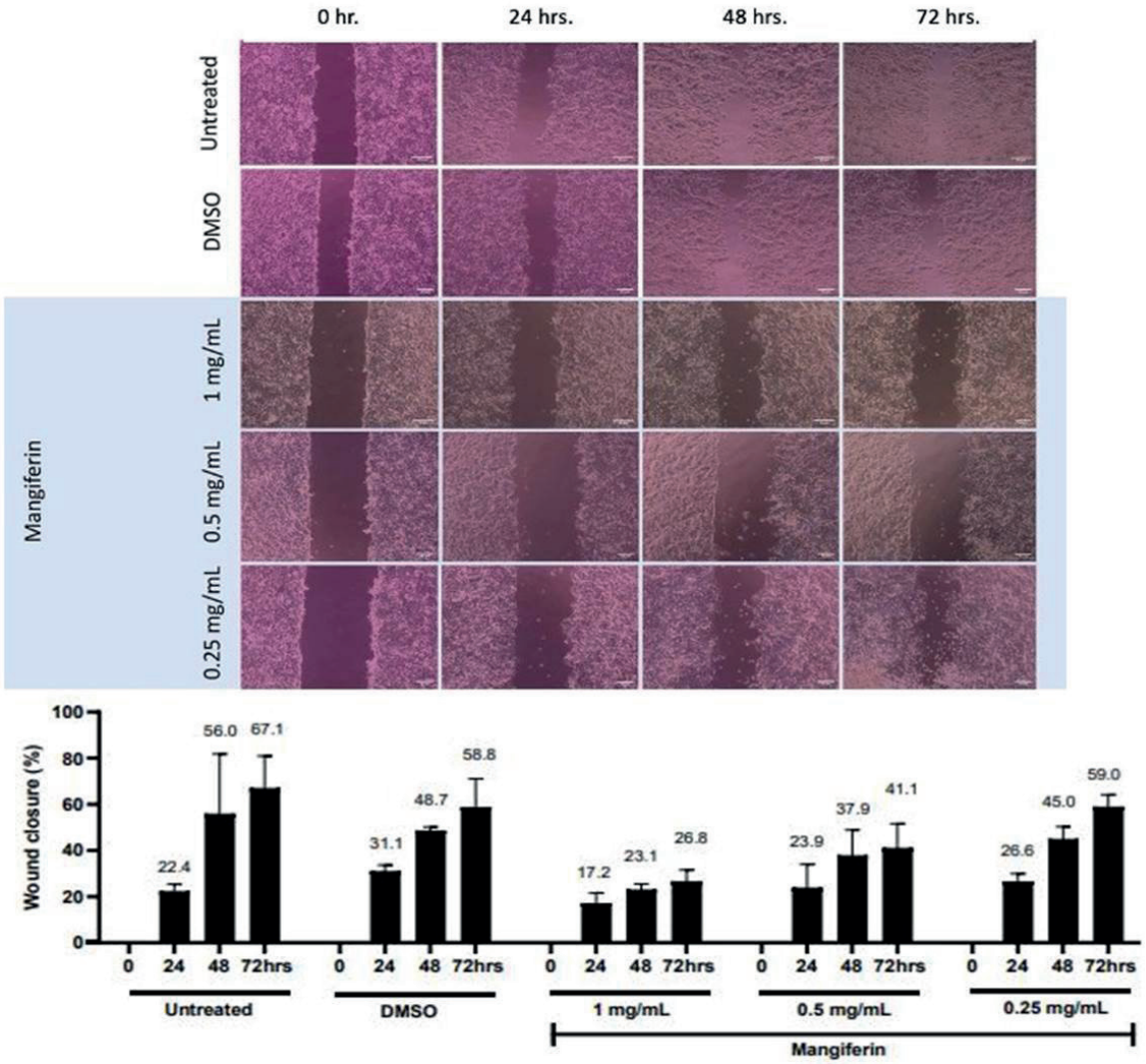

Figure 2 *Image of wound and cell migration to close the wound taken at time 0, 24, 48 and 72 hrs after wounding and the effect of mangiferin on cell migration in A549 cells. (plotted as the percentage of wound closure)*

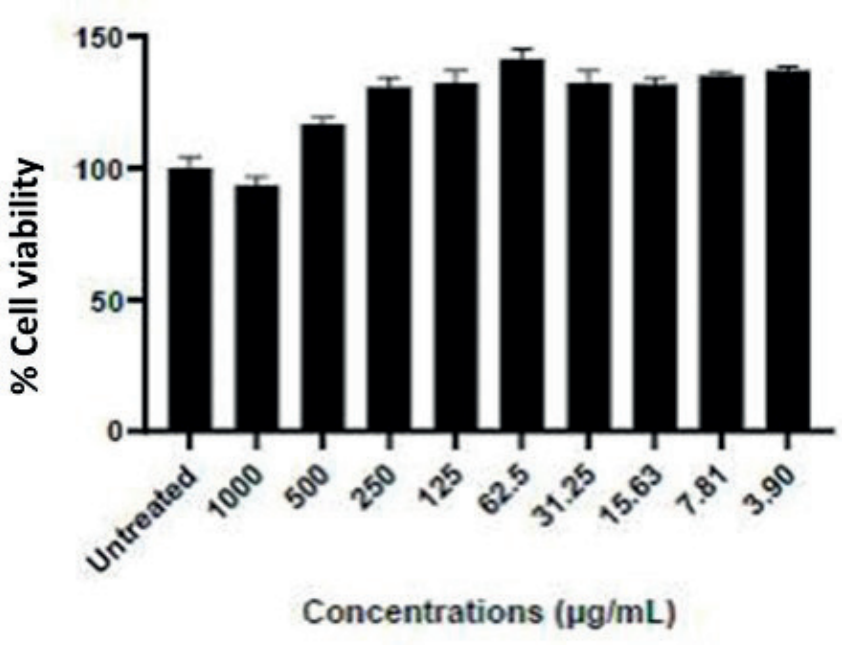

Figure 3 *Effect of mangiferin on the viability of A549 cells.*

Discussion

The extraction of mangiferin from mango leaves used a soxhlet extractor to remove fatty interference substances, then the isolation and purification of mangiferin were performed by column chromatography and crystallization with ethanol to yield 2.7% of mangiferin crystals. The obtained mangiferin characterized by IR, NMR and MS spectroscopic techniques was subjected to evaluate the biological activities. The present study investigated antiplatelet aggregation, anticoagulant, and anticancer activities of mangiferin. Our previous study on cell toxicity of mangiferin indicated that 500 µg/mL of mangiferin had no cytotoxicity on A549 cells (unpublished). Therefore, all the final concentration of mangiferin used in this test experiments were 500 µg/mL. The antiplatelet aggregation activity of mangiferin using conventional light transmission platelet aggregation assay was evaluated on human platelets whose aggregation was induced by using ADP as an agonist. The results showed that mangiferin at a concentration of 500 µg/mL inhibited platelet aggregation in a statistically significant with the inhibition percentage value of 14.1±1.2. This assay was consistent with the research by María Elena Alañón and colleagues who reported that mangiferin extracted from mango seeds had the effect of inhibiting platelet aggregation at a concentration of 1 mg/mL.[32] In addition, using the modified whole blood clotting time method which was one of the simplest methods to evaluate how well the primary blood clotting process was functioning, indicated that mangiferin delayed the time of a blood coagulation at a value of 13.09±2.97 min with statistical significance (p<0.05) when compared with the control set.

The anticoagulant activity of mangiferin was performed *in vitro* in human plasma by prothrombin time (PT) and activated partial thromboplastin time (aPTT) test. PT was mainly applied to measure the activity of coagulation factors of extrinsic and common coagulation pathways while aPTT was used to evaluate the function of coagulation factors of the intrinsic and common coagulation pathways. The prolongation of aPTT, suggested the inhibition of the intrinsic and/or the common pathway. On the other hand, PT prolongation gave information about the inhibition of the extrinsic and/or the common pathway. It was found that mangiferin had the effect of inhibiting the formation of fibrin clot being significantly slower than the control group (p<0.05) for both the PT and aPTT tests. Previous research reported that mangiferin heptasulfate (a polysulfated derivative of mangiferin) interfered blood coagulation by inhibiting the activity of coagulation factor Xa.[40] Consequently, it was considered that mangiferin might exert anticoagulant activity by interfering the function of coagulation factor Xa.

In order to determine the fibrinolysis action of mangiferin, the blood clot lysis and fibrolytic activity assay were also evaluated. The study showed that mangiferin had no fibrinolytic acitivity at the concentration of 500 µg/mL. Qurat U. Ain and colleagues reported that aqueous-methanolic extract of *Mangifera indica* leaves had a significant increment in clot lysis, PT and aPTT due to the presence of polyphenols, flavonoids, alkaloids in its extract.[41] Our studies revealed that isolated mangiferin from *Mangifera indica* leaves had an increment in both PT and aPTT without clot lysis activities. Nevertheless, mangiferin showing no clot lysis activity due to the inappropriate concentration used or entirely lacking fibrinolytic action should be further determined. Several studies reported that mangiferin had many biological activities, especially, anticancer activity against several types of cancers.[5,6]

The anti-lung cancer activity of mangiferin was attractive. was attractive. One *in vitro* experiment showed that mangiferin exerted anti-lung cancer by an influence on cell cycle arrest and induced apoptosis in A549 cells.[26] Another report indicated that mangiferin likely regulated proliferation and apoptosis in lung cancer LUAD cells by reducing the expression levels of miR-92a and miR-27b.[42] Cancer cell migration is one of the important steps in cancer spread and invasion. Inhibitions of A549 cells migration have been widely reported for evaluation of lung carcinogenesis and cancer cells growth.[43-45] In this study the inhibition of cell migration of A549 cells was investigated to determine the anticancer activity of mangiferin by using wound healing (or scratch) assay.

The present data showed that mangiferin significantly decreased the migratory ability of A549 cells in a dose-dependent manner. The cell migration inhibition effect of mangiferin did not result from cytotoxicity as indicated by cytotoxicity assay. It had been previously reported that mangiferin displayed anticancer effects on A549 cells through various mechanisms, such as induction of apoptosis and reduction of miR-92a and miR-27b expression.[26,42]

Our studies demonstrated that mangiferin was able to inhibit cell migration and invasion on A549 cells without cytotoxicity, however, the migration of cancer cells involved multiple mechanistic pathways, the precise molecular mechanism of cell migration inhibition should be further studied.

Conclusion

Mangiferin is a natural bioactive compound possessed a variety of biological activities. In this study, it was clearly showed that mangiferin possessed antiplatelet aggregration and anticoagulation activities without fibrinolysis effect. Nevertheless, the obvious mechanisms of blood coagulation inhibition and optimum concentration used for those activities must be further investigated. In addition, mangiferin showed anticancer activity against A549 cell line by inhibition of cell migration through wound healing assay.

Conflict of interest

No conflict of interest to declare.

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