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Inhibition of thrombin, an unexplored function of retinoic acid☆

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ABSTRACT

Retinoic acid, a derivative of vitamin A, is known to possess *in vivo* anti-inflammatory, anti-platelet and fibrinolytic activities. We have investigated the *in vitro* thrombin and platelet aggregation inhibitory activities of vitamin A (retinol) and its derivatives, retinoic acid and retinaldehyde. The thrombin enzymatic assay was performed fluorimetrically to assess the inhibition of thrombin (Sigma and plasma). Retinoic acid, retinaldehyde and retinol exhibited potent inhibition of thrombin, with IC_{50} values of 67μg/ml, 74μg/ml and 152μg/ml, respectively for the inhibition of thrombin (Sigma); and 49μg/ml, 74μg/ml and 178μg/ml, respectively for the inhibition of thrombin (plasma). Amongst vitamin A and its derivatives, retinoic acid showed the highest inhibition of both the forms of thrombin. Vitamin A and its derivatives also displayed remarkable inhibition of platelet aggregation. This is the first report of vitamin A and its derivatives showing inhibition of thrombin and platelet aggregation *in vitro*.

1. Introduction

Vitamin A is crucial for vision in animals [1,2]. Vitamin A (retinol) and its derivatives namely; retinoic acid and retinaldehyde have different biological activities. Deficiency of vitamin A (avitaminosis A) severely impairs the vision, manifesting as nyctalopia (night blindness) and xerophthalmia (a broad spectrum of ocular diseases) [3]. Xerophthalmia is characterized by different eye signs (graded by World Health Organization) such as Conjunctival xerosis, Bitot's spots, Corneal xerosis, Corneal ulceration, keratomalacia, Corneal scarring, Xerophthlamic fundus and retinopathy [3–7].

Liver is the primary storage site of vitamin A where retinol is stored as retinyl esters within lipid droplets [8,9]. Retinyl ester supplementation has a marked effect on the regeneration of liver in rats subjected to partial hepatectomy [10]. Retinyl esters are also present in the pigment epithelium (PE) of retina in the eye and are therefore involved in the absorption of scattered light, formation of blood-retinal barrier, facilitation of optic nerve signal transduction, renewal of all-*trans*-retinaldehyde, phagocytosis of outer segment membranes, secretion of signalling molecules and facilitation of immunity to the eye [11].

Retinaldehyde, which is formed either from β-carotene (mediated by the enzyme β-carotene dioxygenase) [12] or retinol {through the enzyme retinol dehydrogenase (RDH) [13]}, is present in the rod cells of the retina and participates in the visual cycle responsible for the light/dark vision [14].

Retinoic acid is formed from retinaldehyde, which is mediated by the enzyme retinaldehyde dehydrogenase (RALDH) [15]. Although, retinoic acid has no direct role in the visual function, it determines the number of photoreceptor cells present on the retina [16] and also plays a pivotal role in the mucous secretion by lacrimal glands in the eye [3–7]. It has decisive roles in glycoprotein metabolism, epidermal cell differentiation, innate immunity, embryonic development [17] and hematopoiesis [18]. Retinoic acid has decisive roles in anti-cancer therapy by reduction of multidrug resistance in cancer cells [19,20] and induction of differentiation in embryonal carcinoma cell lines [21].

Abbreviations: ADP, adenosine diphosphate; AFU, arbitrary fluorescence units; AMC, 7-amino, 4-methyl coumarin; PE, pigment epithelium; PRP, platelet rich plasma; RBP, retinol binding protein

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Retinoic acid also plays a role in the differentiation of leucocytes such as natural killer (NK) cells [22] and T-cells [23], thus providing innate immunity against infections. Vitamin A also influences the secretion of the human growth hormone [24]. Retinoic acid anhydride promotes growth in vitamin A-deficient animals [25,26]. Retinoyl β-glucuronide, a natural bioactive form of retinoic acid, functions as a detoxification molecule to treat skin disorders [27].

Thrombosis is the condition resulting due to obstruction of flow of blood in blood vessels leading to aberrant coagulation. Thrombotic disorders can be classified into arterial and venous thrombotic disorders. Arterial thrombosis leads to coronary heart disease and ischemic stroke [28], while venous thrombotic disorders manifest either as deep-vein thrombosis or pulmonary embolism [29]. Therefore, anti-thrombotic drugs are necessary to combat these disorders by prevention of thrombosis using thrombin inhibitors and anti-platelet drugs.

The process of atherosclerosis is a complex phenomenon which involves a cross talk between various processes such as inflammation, platelet activation and blood coagulation. There are reports which have shown retinoic acid to possess anti-platelet and anti-inflammatory [30] properties. Retinoic acid is also known to enhance the *in vivo* fibrinolytic activity of tissue plasminogen activator (t-PA) [31]. Retinoic acid has been shown reduce atheroma [32], increase vasodilation [33,34], reduce endothelial smooth muscle cell proliferation [35] and decrease foam cell formation [36] during the process of atherosclerosis. Retinoic acid in the diet of rabbits have shown decreased intimal thickening and thereby inhibited restenosis after balloon angioplasty [37,38]. Thus, retinoic acid is a vital molecule on account of its function to inhibit blood coagulation as well as prevention of atherosclerosis.

Our study compares anti-platelet activity of retinoic acid with retinol and retinaldehyde for the first time *in vitro*. The novel finding of the present study is the demonstration of thrombin inhibitory activity of vitamin A (retinol) and its derivatives - retinoic acid and retinaldehyde.

2. Materials and methods

2.1. Materials

Tris HCl, sodium chloride, calcium chloride, trisodium citrate and milliQ water were purchased from Merck-millipore (India). Bovine thrombin, argatroban monohydrate, retinoic acid, retinol, retinaldehyde, heparin (sodium salt), ticlopidine, dimethyl sulfoxide (DMSO) and adenosine diphosphate (ADP) were obtained from Sigma-Aldrich (Bangalore, India). Thrombin substrate III, fluorogenic (benzoyl phenylalanylvalylarginyl 7-amino, 4-methyl coumarin) was obtained Calbiochem (USA). 1.7 ml microcentrifuge tubes (eppendorf tubes) were purchased from Genaxy. Greiner chimney flat black ELISA plates (96-well) were obtained from Thermo Scientific (USA).

2.2. Animals

Male wistar rats, procured from the Central Animal Facility (CAF), Indian Institute of Science, were housed in a pathogen-free environment under standard laboratory conditions. Experiments were performed as per the guidelines of the Institutional Animal Ethics Committee (IAEC).

2.3. Thrombin inhibition assay

This assay uses a fluorogenic synthetic substrate of thrombin, a tripeptide linked to an inactive fluorophore (AMC: 7-amino, 4-methyl coumarin), called thrombin substrate III [39]. Thrombin cleaves the substrate to release the active fluorophore - AMC, which is detected fluorimetrically with excitation at 370 nm and emission at 450 nm. This fluorescence is recorded as arbitrary fluorescence units (AFU). Thrombin substrate III (Calbiochem) was dissolved in 100% dimethyl sulfoxide (DMSO) at a concentration of 20 mM. From this, a working stock of 2 mM was prepared using milliQ water. Thrombin (Sigma) was dissolved in 0.9% NaCl to make a concentration of 100U/ml. The test compounds were dissolved in 100% DMSO and the final concentration of DMSO having the test compound was 2% in the assay mix. Firstly, 160 μM of thrombin substrate III was added to the 96-well greiner chimney flat black ELISA plate, followed by 5 μl of the test compound (Concentration of the test compounds in the assay ranging from 50μg/ml to 200μg/ml). For the control, 5 μl of DMSO was used. Then, 200 μl of Tris buffer [0.24% Tris HCl, 0.88% NaCl, 0.5% bovine serum albumin (BSA) and 0.02% $CaCl_2$, pH 7.5] was added followed by the addition of 0.6U/ml thrombin. The 96 well ELISA plate was subjected to gentle shaking to uniformly mix the assay components and then incubated at 37 °C for 90min in an incubator. The fluorescence was then measured using a TECAN 96 well ELISA plate reader. The percentage inhibition of thrombin was calculated as the percentage decrease in fluorescence (AFU) by the test compound as compared to the control (DMSO). The mean values of percentage inhibition of thrombin are given in the figures. In this assay, the experiments were repeated consistently at each concentration of each of vitamin A (retinol) and its derivatives namely, retinoic acid and retinaldehyde. The positive control used for inhibition of thrombin (Sigma) was argatroban monohydrate.

Blood was collected from male wistar rats by heart puncture method in presence of 3.8% trisodium citrate (pH 6.5), an anticoagulant and centrifuged at 5000rpm to obtain plasma. For thrombin inhibition assay of plasma thrombin, the assay conditions used were similar to that given above for thrombin (Sigma), wherein 200 μl of plasma (isolated from rat blood) was added instead of Tris buffer and then a small concentration of thrombin (Sigma) (0.3U/ml) was added to act as an agonist to promote generation of thrombin from prothrombin present in plasma *in situ* [40]. The positive control used for inhibition of thrombin (plasma) was heparin.

2.4. Platelet aggregation assay

Blood was collected from male wistar rats by heart puncture method in presence of trisodium citrate buffer (pH 6.5), an anticoagulant and centrifuged at 1000rpm. The supernatant having platelet rich plasma (PRP) was collected. 450 μl of PRP was aliquoted into a cuvette and incubated at 37 °C. 5 μl of the test compound dissolved in 100% DMSO (concentration of the test compounds in the assay ranging from 40μg/ml to 120μg/ml) was added to PRP and incubated for 10 min at 37 °C. Platelet aggregation was induced by the addition of 50 μM adenosine diphosphate (ADP), an agonist. Platelet aggregation was measured by the decrease in absorbance of PRP as a function of time recorded in a platelet aggregometer (computerized dual channel Chronolog Aggregometer, Chrono-Log Corporation, Havertown, PA). A steep decrease in absorbance was observed when platelet aggregation occurred. This decrease is less when the test compound inhibits platelet aggregation [41]. The mean values of percentage inhibition of platelet aggregation (with reference to control – DMSO) are presented. In this assay, the experiments were repeated consistently at each concentration of each of vitamin A (retinol) and its derivatives. The positive control used for inhibition of platelet aggregation was ticlopidine.

3. Results

3.1. Thrombin inhibitory activity of vitamin a and its derivatives

Vitamin A (retinol) and its derivatives namely, retinoic acid and retinaldehyde were tested for their potential to inhibit thrombin. In this assay, retinoic acid, retinaldehyde and retinol at a concentration of 200μg/ml showed 92%, 87% and 59% inhibition of thrombin (Sigma), respectively (Fig. 1A). The IC_{50} values of retinoic acid, retinaldehyde and retinol for the inhibition of thrombin (Sigma) were 67μg/ml, 74μg/ml and 152μg/ml, respectively (Table 1), which were calculated based

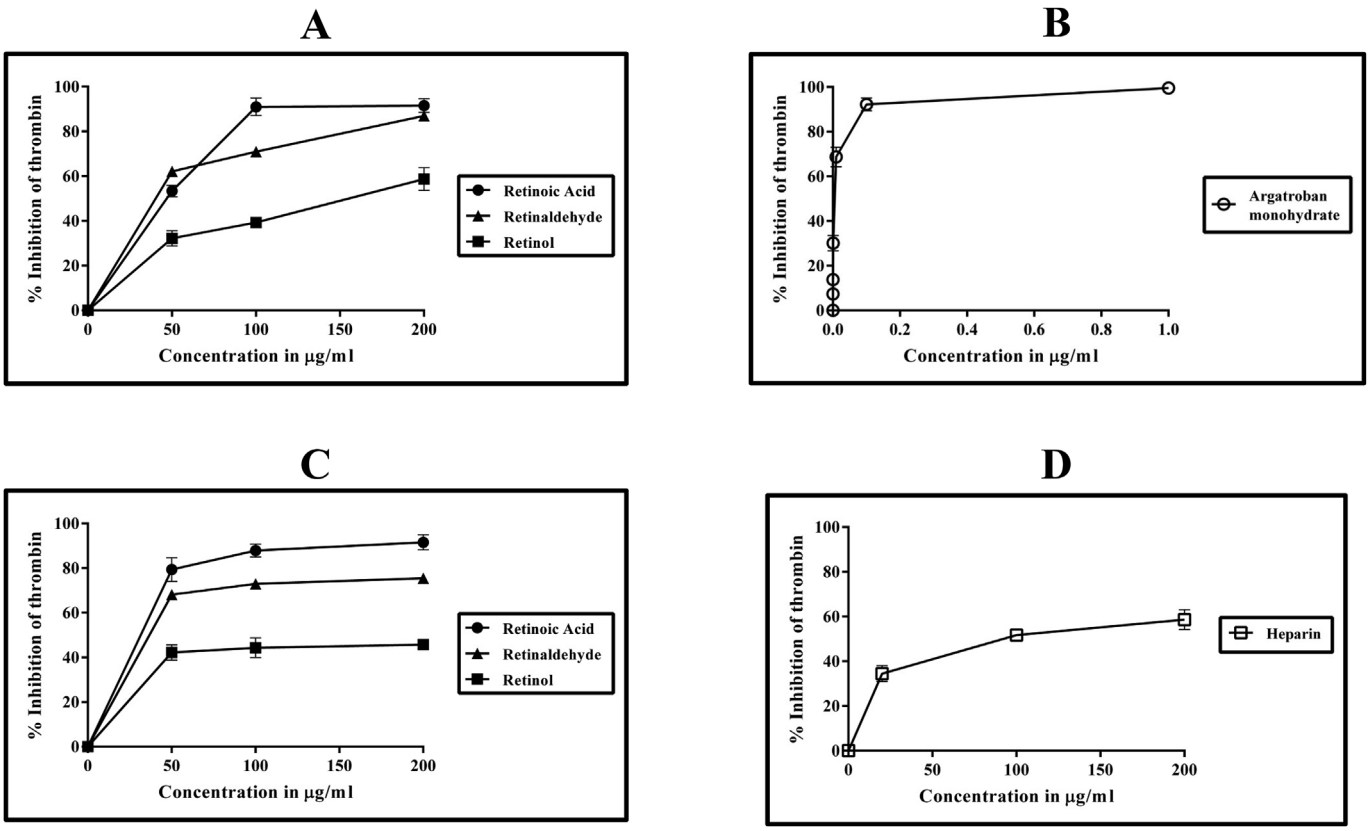

Fig. 1. Thrombin inhibitory activity of vitamin A and its derivatives.
Inhibition of activity of thrombin (Sigma) by (**A**) retinoic acid (●), retinaldehyde (▲), retinol (■) and (**B**) the positive control argatroban monohydrate (⊖). Inhibition of activity of thrombin (plasma) by (**C**) retinoic acid (●), retinaldehyde (▲) retinol (■) and (**D**) the positive control heparin (⊟).

Table 1
IC_{50} values for the inhibition of thrombin and inhibition of platelet aggregation by the vitamin A, its derivatives and the respective positive controls.

Vitamin A forms	IC_{50} values for Inhibition of thrombin (µg/ml)		IC_{50} values for Inhibition of platelet aggregation (µg/ml)
	Sigma thrombin	Plasma thrombin	
Retinoic acid	67	49	49
Retinaldehyde	74	74	33
Retinol	152	178	40
Positive control	0.037 (Argatroban monohydrate)	137 (Heparin)	55 (Ticlopidine)

on the different concentrations of these molecules added into the assay medium. The positive control used for inhibition of thrombin (Sigma) was argatroban monohydrate which exhibited 94% inhibition of thrombin (Sigma) at a concentration of 0.1 µg/ml (Fig. 1B), with IC_{50} value of 0.037µg/ml (Table 1).

These molecules were also tested for their potential to inhibit thrombin present in rat plasma (natural source). In this assay, 200µg/ml of retinoic acid, retinaldehyde and retinol showed 92%, 76%, and 46% inhibition of thrombin (plasma), respectively (Fig. 1C). The IC_{50} values of retinoic acid, retinaldehyde and retinol for the inhibition of thrombin (plasma) were 49µg/ml, 74µg/ml and 178µg/ml, respectively (Table 1), which were calculated based on the different concentrations of these molecules added into the assay medium. The positive control used for the assay, heparin exhibited 59% inhibition of plasma thrombin at a concentration of 200 µg/ml (Fig. 1D), with IC_{50} value of 137 µg/ml (Table 1).

It is evident from Fig. 1A,C that with reference to inhibition of thrombin (both Sigma and plasma), retinoic acid is more potent when compared to retinaldehyde and retinol. Both retinoic acid and retinaldehyde, being more polar, are more inhibitory of thrombin as compared to retinol. The acid (retinoic acid) and aldehyde (retinaldehyde) derivatives of vitamin A are relatively more soluble in the aqueous buffer and can bind to the thrombin protein. The formation of a Schiff's base of retinaldehyde as the basis of inhibition of thrombin is unlikely and their inhibitory mechanism due to their polarity is more likely in this process. The albumin added stabilizes the minute quantity of thrombin enzyme used in the assay. The molecules of interest which need to be tested for their inhibitory properties on thrombin can thus be speculated to first bind to the bulk albumin and then get exchanged with thrombin protein during the assay. This hypothesis also helps us to understand the reason for inhibition of thrombin by the retinoid molecules.

With thrombin (Sigma), vitamin A (retinol) and its derivatives showed a concentration-dependent increase in inhibition, but with thrombin (plasma), only retinoic acid did so. Thus, vitamin A (retinol) and its derivatives showed formidable inhibition of thrombin.

3.2. Platelet aggregation inhibitory activity of vitamin a and its derivatives

In this experiment, ADP was used as an agonist to induce platelet aggregation. Vitamin A (retinol) showed 98% inhibition of platelet aggregation, while the derivatives of vitamin A retinoic acid and retinaldehyde exhibited 95% inhibition of platelet aggregation at a concentration of 120µg/ml (Fig. 2A–C). The IC_{50} values of retinoic acid, retinaldehyde and retinol for the inhibition of platelet aggregation were 49µg/ml, 33µg/ml and 40µg/ml respectively (Table 1). The positive control ticlopidine also showed 99% inhibition of platelet aggregation at a concentration of 120µg/ml (Fig. 2D). The IC_{50} value of ticlopidine

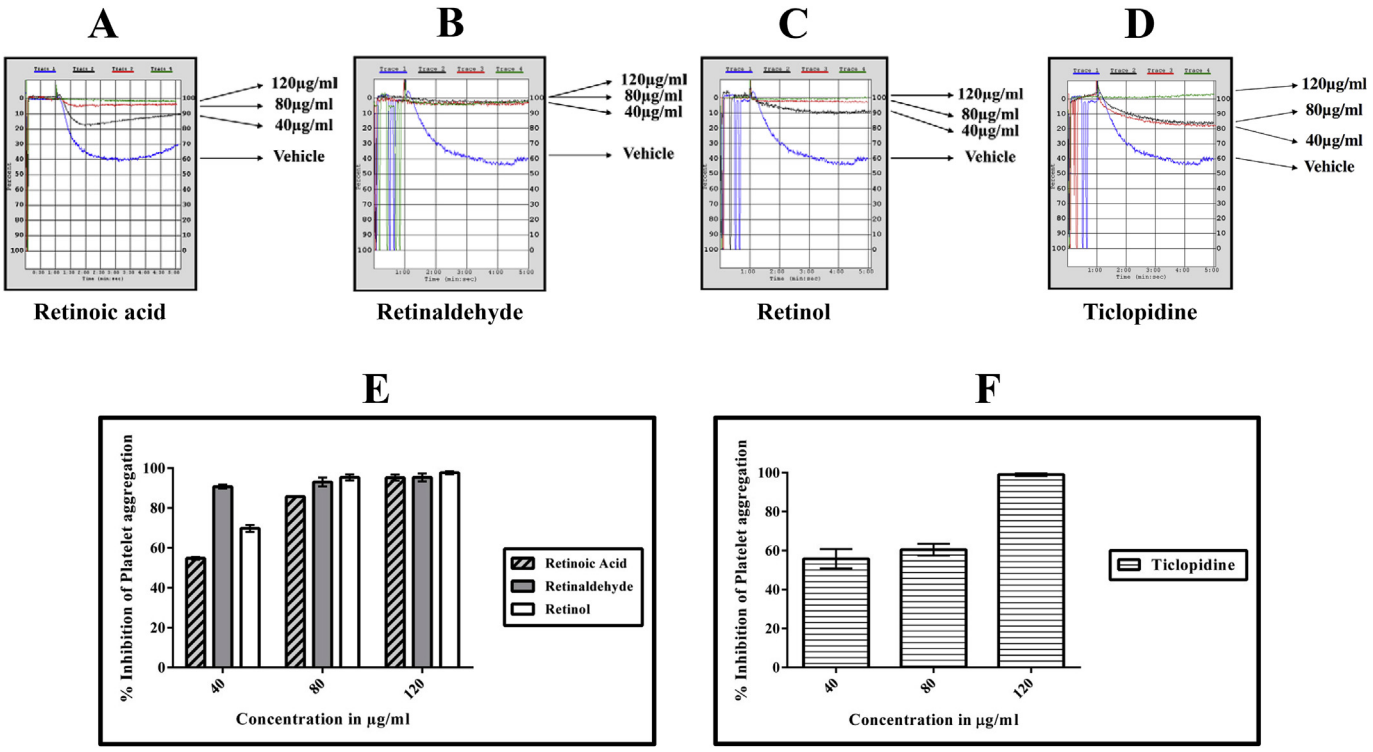

Fig. 2. Platelet aggregation inhibitory activity of vitamin A and its derivatives.
Inhibition of platelet aggregation by (**A**) retinoic acid, (**B**) retinaldehyde, (**C**) retinol and (**D**) the positive control ticlopidine. (**D**) Graphical representation of inhibition of platelet aggregation by (**E**) retinoic acid (▨), retinaldehyde (▬), retinol (▭) and (**F**) the positive control ticlopidine (▭).

for inhibition of platelet aggregation was found to be 55 µg/ml (Table 1). Among the three molecules tested, retinaldehyde showed the highest inhibition of platelet aggregation.

4. Discussion

In the present study, we have compared vitamin A (retinol) and its derivatives namely, retinoic acid and retinaldehyde in the inhibition of thrombin and platelet aggregation. It is evident (from the results section) that vitamin A and its derivatives have prominent anti-thrombotic properties. Although there have been reports which have mentioned the *in vivo* inhibition of platelet aggregation and the enhancement of fibrinolytic activity by retinoic acid, the work presented in this manuscript describes for the first time the inhibition of thrombin (both Sigma and plasma) and *in vitro* platelet aggregation by vitamin A (retinol) and its derivatives – retinoic acid and retinaldehyde.

Retinoic acid, retinaldehyde and retinol have a structural relationship as all three molecules are retinoids. Retinaldehyde has an aldehyde functional group, while retinoic acid has a carboxylic acid functional group and vitamin A (retinol) has an alcohol as its functional group. Retinaldehyde and retinoic acid have more polarity due to their functional groups when compared to retinol. The structural activity relationship between vitamin A (retinol) and its derivatives retinaldehyde and retinoic acid is indicated by the ratio of their IC_{50} values (µg/ml) for inhibition of thrombin (Sigma) being 152:74:67, inhibition of thrombin (plasma) being 178:74:49 and inhibition of platelet aggregation being 40:33:49, respectively. The ratio of IC_{50} (µg/ml) values for inhibition of thrombin (both forms) implies that with increase in polarity there is a decrease in the IC_{50} values, thereby indicating that with increase in polarity of the derivatives of vitamin A, there is increase in the inhibitory action of thrombin. But, in the case of inhibition of platelet aggregation, the ratio of IC_{50} values indicates retinaldehyde to show the highest inhibition, amongst vitamin A and its derivatives. Retinoids are hydrophobic molecules and these are introduced into the assay medium (aqueous) through dimethyl sulfoxide (DMSO) as a vehicle. It is possible that all the retinoid molecules in the assay medium might not be available to bind to thrombin because of their decreased water solubility. Hence, the effective concentration of the retinoid molecules in the assay medium may be lower than the mentioned concentration. This might have an impact on the IC_{50} values for thrombin inhibitory activity.

Inhibition of thrombin and platelet aggregation is an important aspect while considering a therapeutic solution to inhibit blood clotting as a preventive measure in case of medical conditions such as cardiovascular disease, stroke and deep vein thrombosis. Anti-platelet drugs such as aspirin, ticlopidine and clopidogrel are administered in case of arterial thrombotic conditions such as cardiovascular disease and stroke; while anti-thrombin drugs such as heparin, argatroban and hirudin are administered in case of both arterial and venous thrombotic conditions. In the case of venous thrombotic conditions, anti-thrombin drugs are very crucial to prevent clotting and therefore, in the pursuit of novel anti-thrombin drugs, the work presented opens a window on the inhibition of thrombin by naturally-occurring vitamin A. The future pursuit in this direction is to perform experiments to examine the *in vivo* effects of vitamin A and its derivatives in inhibition of thrombosis, wherein direct thrombin inhibition by vitamin A and its derivatives can be expected to occur *in vivo*. If vitamin A and its derivatives show promising results towards anti-thrombosis, then, these molecules will be one of the most potent prospective drugs. The work presented in this manuscript also opens up opportunities for researchers to perform experiments on the inhibition of thrombin and platelet aggregation by retinoid molecules from natural sources such as trisporic acid [42], isotretinoin (13-cis-retinoic acid), alitretinoin (9-cis-retinoic acid), etretinate, adapalene etc. [43].

The present study opens a window to understand blood coagulation in animals by highlighting the thrombin inhibitory activity of retinoic acid and this further helps us to decipher some key aspects of the process of atherosclerosis.

Conflicts of interests

The authors hereby declare that there are no conflicts of interest.

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