Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Supple 1, 2015

Original Article

ANTICOAGULANT AND ANTIPLATELET ACTIVITIES OF JACKFRUIT (*ARTOCARPUS HETEROPHYLLUS)* SEED EXTRACT

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Received: 08 Jul 2015 Revised and Accepted: 27 Sep 2015

ABSTRACT

Objective: The current study focuses on the anticoagulant and antiplatelet activities of aqueous seed extract of Jackfruit (AqSEJ).

Methods: Anticoagulant effect of AqSEJ was tested using plasma recalcification time, mouse tail bleeding time, Activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT). Antiplatelet activity was examined by platelet aggregation studies using agonists such as ADP, Collagen and Epinephrine.

Results: The AqSEJ enhanced the clotting time of citrated human plasma from control 200±10 s to 740±14 s. The anticoagulant activity of AqSEJ was further strengthened by *in-vivo* mouse tail bleeding assay. The *i. v.* injection of AqSEJ significantly prolonged the bleeding time in a dose dependent manner. The recorded bleeding time was>10 min ($P<0.01$) at the concentration of 30 µg against the PBS treated control of 1.48±0.10 min with the IC_{50} values 37.5 µg/ml and 47.5 µg/ml respectively. Interestingly, AqSEJ specifically prolonged the clot formation process of only APTT but not PT, revealing the anticoagulation triggered by the extract could be due to its interference in an intrinsic pathway of the blood coagulation cascade. Furthermore, AqSEJ inhibited the agonists such as ADP, epinephrine and collagen induced platelet aggregation of about 66.7%, 39.2% and 37.0% respectively at the concentration of 200 µg.

Conclusion: AqSEJ showed anticoagulant and antiplatelet activities. Hence, it may serve as a better alternative for thrombotic disorders.

Keywords: Jackfruit Seeds, Moraceae, Anticoagulant activity, Ant platelet activity.

INTRODUCTION

Thrombotic disorders such as arterial thrombosis, atrial fibrillation, myocardial infarction/heart attack, unstable angina, deep vein thrombosis, pulmonary embolism and cerebral stroke increases the risk of cardiovascular/cerebrovascular complications and represent a major health problem worldwide including India. There are about 10 million cases annually reported exceeding the number of deaths from cancer and other diseases. It is estimated that the annual mortality rate of 60,000 in the United States and more than 50,000 in India. It is to note that, anticoagulants those inhibits the clot formation by blocking the action of clotting factors/coagulation factors and ant platelet agents those blocks the formation of blood clot by preventing the clumping of platelets are extensively being used in the treatment of thrombotic disorders [1, 2]. Thus, anticoagulant and ant platelet therapy is the effective therapy for the prevention and treatment of thrombotic disorders. While, the currently established anticoagulants such as, warfarin, dabigatran, unfractionated heparin (UFH), enoxaparin, fondaparinux, bivalirudin (thrombin inhibitors), low molecular weight heparin and antiplatelet agents such as, aspirin, thienopyridines, dipyridamole, dlopidogrel, dpoprostenol, abciximab, eptifibatide and tirofiban (glycoprotein IIb/IIIa inhibitors) are having numerous limitations with several side effects, including lack of reversibility, a sheer dose response, food and multiple drug-drug interactions, narrow therapeutic index, internal bleeding, birth defects and miscarriage [3-6]. Therefore, identifying the novel anticoagulant and ant platelet agents from the natural sources with least side effects is the challenging task to the researchers. In addition, the other hypothesis is that targeting coagulation cascade upstream from the common pathway provides a reduction in thrombin generation slow down the formation of fibrin clot or thrombosis. Investigating the target specific (factor Xa and thrombin) anticoagulant and ant platelet agents from the natural sources with least side effects helps in the better management of thrombotic disorders. Jackfruit seeds are richest sources of try sin, chymotrypsin, elastase inhibitors and lectin family glycoprotein's (Jacalin) [7-9]. Jackfruit seeds were found to exhibit anticancer, antihypertensive, antiulcer, antioxidant, antifungal, and antimicrobial properties [10-12]. In our previous study, characterization of is forms of proteases (serine and cysteine) and their ability to hydrolyze fibrinogen and fibrin clot was reported from the aqueous Jackfruit seed extract (AqSEJ) [13]. However, in the current study, the anticoagulant and antiplatelet activity of AqSEJ was investigated further and the results are presented.

MATERIALS AND METHODS

Materials

Collagen type-I, ADP, epinephrine were purchased from Sigma Chemicals Company, St. Louis, USA. UNIPLASTIN, LIQUICELIN-E and FIBROQUANT were purchased from Tulip Diagnostics Pvt. Ltd., Goa, India. All other chemicals and reagents used were analytical grade. Fresh blood sample was collected from healthy human donors. Swiss Wister albino mice weighing 20–25 g from the central animal house facility, Department of Studies in Zoology, University of Mysore, Mysore, India. Animal care and handling complied with the National Regulation for Animal Research.

Jackfruit seeds

Artocarpusheterophyllus Lam (Jackfruit) seeds were obtained in the month of April and May from Pittenahalli village, Tumkur district and identified by Dr. P. Sharanappa, University of Mysore. A voucher specimen (PS/52/18 FEB 2012)has been preserved at the herbarium of Bioscience Department, University of Mysore, Hassan PG campus, Hassan for future reference.

Preparation of jackfruit seed extract

Brown coat was removed from the Jackfruit seeds thoroughly chopped and homogenized using double distilled water and centrifuged at 2000 *g* for 20 min at 4 °C. The supernatant was collected and proteins were precipitated using 30% of ammonium sulphate. The precipitated protein sample was again centrifuged at 3,500 *g* for 20 min, pellet was dissolved and dialyzed overnight. The

protein sample obtained was stored at-20 °C until use. This extracted protein sample was used throughout the study and referred as aqueous seed extract of Jackfruit (AqSEJ).

Anticoagulant activity

Plasma recalcification time

The plasma recalcification time was determined as described earlier [14]. Briefly, the AqSEJ (0-50 µg) was pre-incubated with 0.2 ml of citrated human plasma in the presence of 10 mMTrisHCl (20 µl) buffer pH 7.4 for 1 min at 37 °C. Then 0.25 M $CaCl_2$ (20 µl) was added to the pre-incubated mixture and clotting time was recorded.

Bleeding time

The bleeding time was assayed as described previously [15]. Briefly, AqSEJ(0-50 µg) in 30 µl of PBS was injected intravenously through the tail vein of a group of five mice. After 10 min, mice were anaesthetized using diethyl ether and a sharp cut of 3 mm length at the tail tip of a mouse was made. Immediately, the tail was vertically immersed into PBS which is pre-warmed to 37 °C. Bleeding time was recorded from the time bleeding started till it completely stopped and it was followed for 10 min.

Activated partial thromboplastin time (APTT) and prothrombin time (PT)

Briefly, 100 µl of normal citrated human plasma and AqSEJ (0-50 µg) were pre-incubated for 1 min. APTT and PT were carried out according to the manufacturer protocol using the coagulation analyzer (Labitec, Germany). For APTT, 100 µl of LIQUICELIN-E phospholipids preparation derived from Rabbit brain with ellagic acid was added. The clotting was initiated by adding 100 µl of 0.02 M $CaCl_2$and the clotting time was measured. For PT, the clotting was initiated by adding 200 µl of PT reagent (UNIPLASTIN–rabbit brain thromboplastin). The time taken for the visible clot was recorded in seconds. The APTT ratio and the international normalized ratio (INR) for PT at each point were calculated from the values of control plasma incubated with the buffer for an identical period of time.

Preparation of platelet-rich plasma and platelet-poor plasma

The method of Ardlie and Han [16] was employed for the preparation of human platelet-rich plasma (PRP) and platelet-poor plasma (PPP). The platelet concentration of PRP was adjusted to 3.1×10^8 platelets/ml with PPP. The PRP maintained at 37°C was used within2 h for the aggregation process. All the above preparations were carried out using plastic wares or siliconizedglass wares.

Platelet aggregation

The turbid metric method of Born [17] was followed using a Chronology dual channel whole blood/optical lumi aggregation system (Model-700). Aliquots of PRP were pre-incubated with various concentrations of AqSEJ(0–200 µg) in 0.25 ml reaction volume. The aggregation was initiated independently by the addition of agonists, such as collagen, ADP and epinephrine and followed for 6 min.

In-vitro platelet viability and platelet count

The method of Kropotkin et al. [18] was used to test the platelet viability by incubating the PRP with various concentrations of AqSEJ(0–500 µg), and the platelet morphology was studied with a microscope using a Neubauer chamber. The data expressed as percentage viability, considering 100% viability in the absence of AqSEJ (control).

Determination of endogenously generated reactive oxygen species (ROS)

The endogenous Reactive Oxygen Species (ROS) in totalis determined according to the method of Li *et al.* [19]. In particular, H_2O_2 is determined according to the method of Barja [20]. PRP containing 1×10^5 platelets were diluted with 4-(2-hydroxyethyl)-1-piperazineethanesulfonicacid-buffered saline was incubated with different concentrations of AqSEJ (0–500 µg) at37°Cfor 15 min in a microtiter plate. Then, 100 µmol/lhomovanillic acid was added to the mixture and incubated for 30 min at room temperature. The fluorescence was recorded using a fluorescence plate reader by exciting the samples at 312 nm and the resulting fluorescence was measured at 420 nm.

Statistical analysis

The data are presented as mean±SEM of at least five animals in each group. Difference among the data were determined by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). Data were considered different at $P<0.01$.

RESULTS AND DISCUSSION

In our previous study, fibrin (ogen) olytic and non-toxic properties of AqSEJ was reported [13]. In the current study, anticoagulant and ant platelet proprieties of AqSEJ was carried out and the results are presented. AqSEJ showed strong anticoagulant effect in both in-vitro and in-vivo experiments. AqSEJ prolonged the clotting time of citrated human plasma from control 200±10 s to 740±14 s at the concentration of 20 µg (fig. 1). Furthermore, anticoagulant activity of AqSEJ was confirmed in-vivo by tail bleeding assay. The i. v. injection of AqSEJ significantly prolonged the bleeding time in a dose dependent manner. The recorded bleeding time was>10 min ($P<0.01$) at the concentration of 30 µg against the PBS treated control of 1.48±0.10 min with the IC50 values 37.5 µg/ml and 47.5 µg/ml respectively (fig. 2). Activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT) identify the efficacy/defect of the blood coagulation cascade that includes intrinsic, extrinsic and common pathways. Interestingly, AqSEJ specifically prolonged the clot formation process of only APTT but not PT revealing the anticoagulation triggered by the extract could be due to its interference in an intrinsic pathway of the blood coagulation cascade (table 1).

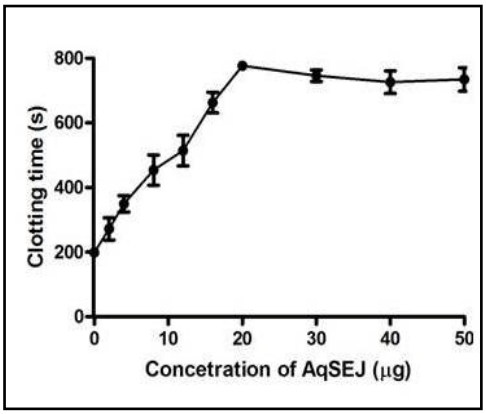

Fig. 1: Effect of AqSEJ on plasma recalcification time: (A) AqSEJ (2–50 µg) was pre-incubated with 0.2 ml of citrated human plasma in the presence of 20 µl10 Mm Tris–HCl buffer pH 7.4 for 1 min at 37 °C. 20 µl of 0.25 M $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded

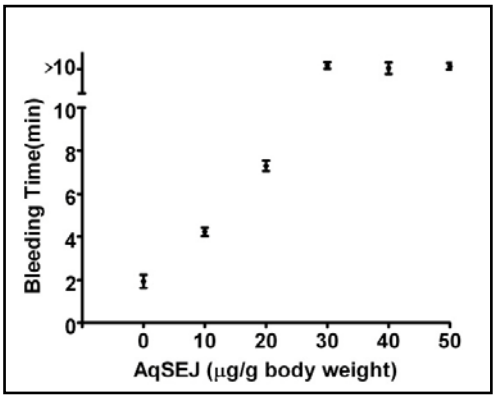

Fig. 2: Effect of AqSEJ on the bleeding time: Tail bleeding time was measured 10 min after intravenous administration of PBS or various doses of AqSEJ. Each point represents the mean±SD of three independent experiments, $P<0.01$. Bleeding time longer than 10 min was expressed as>10 min

Table 1: Dose dependent effect of Jack fruit seed extract on clotting time of normal human plasma

JFSE in µg	PT clotting time in sec	PT (INR values)	APTT clotting time in sec	APTT ratio
0	16.09±0.02	1.05±0.02	46.05±0.05	1.00±0.02
2	17.10±0.03	1.15±0.02	56.30±0.02	1.22±0.01
4	20.40±0.02	1.31±0.01	63.20±0.07	1.37±0.04
8	20.10±0.01	1.31±0.01	78.41±0.03	1.71±0.05
12	22.06±0.02	1.44±0.01	91.20±0.01	1.98±0.02
16	23.12±0.01	1.50±0.01	96.08±0.03	2.08±0.01
20	21.05±0.03	1.37±0.02	100.10±0.05	2.15±0.03
30	23.25±0.02	1.50±0.02	123.52±0.01	2.67±0.02
40	21.09±0.04	1.37±0.02	132.10±0.04	2.87±0.02
50	22.04±0.02	1.44±0.02	148.25±0.01	3.22±0.01

Homeostasis is a physiological process that drives immediate response to the external injury. It operates through complex system of two pathways, the contact activation pathway (intrinsic pathway) and the tissue factor pathway (extrinsic pathway). These pathways may proceed independently or together but culminate in the common pathway that is conversion of prothrombin (factor II) into thrombin (factor IIa). Thrombin hydrolyzes fibrinogen and converts it into fibrin clot that helps in the arrest of bleeding. Further the fibrin clot will be hydrolyzed by fibrinolytic enzyme the plasmin that facilitates the easy flow of blood in the arteries and helps in the wound healing. Thus homeostasis is a highly regulated path way and it has a strong balance between natural procoagulant, anticoagulants and fibrinolytic factors. While, Impairment of homeostasis leads to the hyperactivation of the coagulation factors of the said pathways is the major culprit for the thrombotic disorders. Anticoagulants from the natural sources are the preferable weapons for curing thrombotic disorders. Several anticoagulant agents from natural sources with their mechanism of action were reported from the various research groups. For instance, the anticoagulant from snake venom (Ancrod), fungi aspergillusoryzae (Brinase), coumarin derivative from sweet clover (acenocoumarol), hirudin derivative from siliva of leech (Bialirudin) are currently being used to treat thrombotic disorders (21-24).

Platelet activation by several physiological agonists namely, collagen, ADP, thrombin, epinephrine and platelet activating factor play a crucial role in the primary hemostasis. However, like coagulation factors hyper activation of the platelets due to genetic/environmental factors contribute equally for the thrombotic disorders. Eptifibatide, derivative from rattle snake venom that inhibits glycoprotein IIb/IIIa receptor on platelets is currently using in the treatment of coagulation disorders (21). In order to study the interference of AqSEJ on platelet function, platelet aggregation was analyzed using agonists such as ADP, epinephrine and collagen using platelet-rich plasma. AqSEJ inhibited the agonists such as ADP, epinephrine and collagen induced platelet aggregation of about 66.7%, 39.2% and 37.0% respectively at the concentration of 200 µg(fig. 3,4,5). Among agonists examined AqSEJ inhibited in the order of ADP>epinephrine>collagen induced aggregation. The respective IC50 values are given in the table 2.

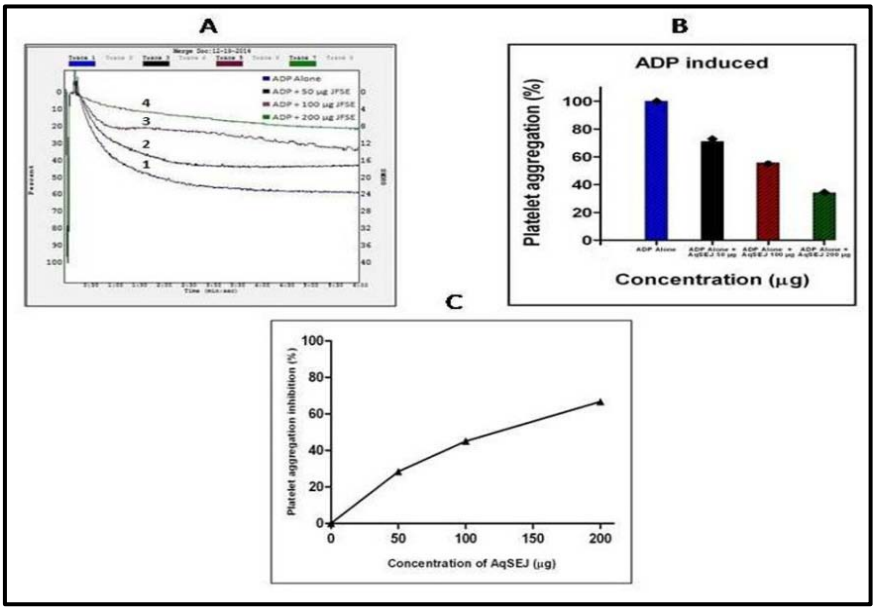

Fig. 3: Platelet aggregation was initiated by adding ADP as an agonist. (A)Traces of platelet aggregation: Trace 1 (ADP 10 µM); Trace 2 (ADP 10 µM+50 µg of AqSEJ); Trace 3 (ADP 10 µM+100 µg of AqSEJ); Trace 4 (ADP 10 µM+200 µg of AqSEJ). The values represent±SD of three independent experiments. (B) Dose dependent platelet aggregation %. (C) Dose dependent platelet aggregation inhibition %

Table 2: IC50 values of agonists collagen, thrombin, ADP, and epinephrine-induced platelet aggregation

S. No.	Name of the agonists	IC50values of AqSEJ (PRP) concentration (mg/ml)
01	ADP	0.488
02	Collagen	1.080
03	Epinephrine	1.020

ADP, adenosine diphosphate; PRP, platelet-rich plasma

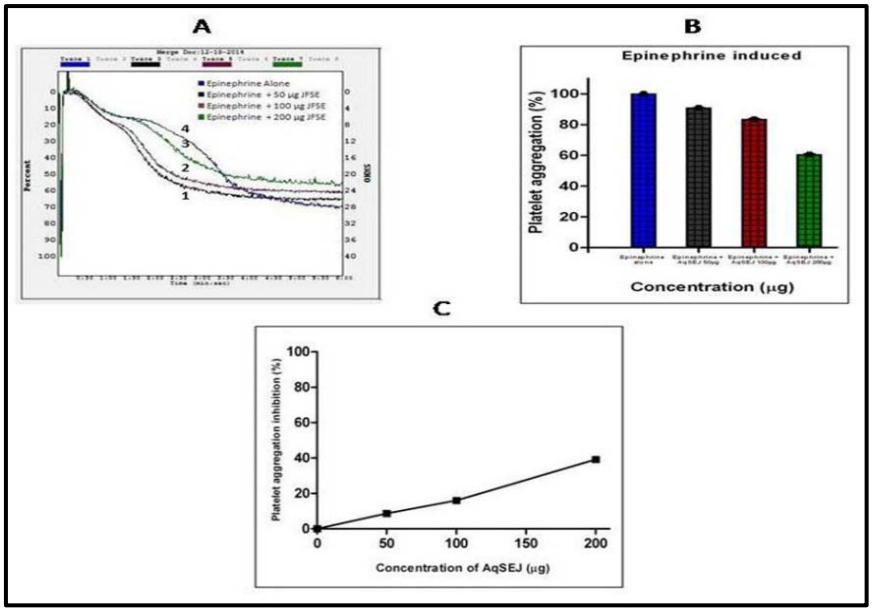

Fig. 4: Platelet aggregation was initiated by adding Epinephrine as an agonist. Trace 1 (Epinephrine 5µM); Trace 2 (Epinephrine 5µM+50 µg of AqSEJ); Trace 3 (Epinephrine 5µM+100 µg of AqSEJ); Trace 4 (Epinephrine 5µM+200 µg of AqSEJ). The values represent±SD of three independent experiments. (B) Dose dependent platelet aggregation %. (C) Dose dependent platelet aggregation inhibition %

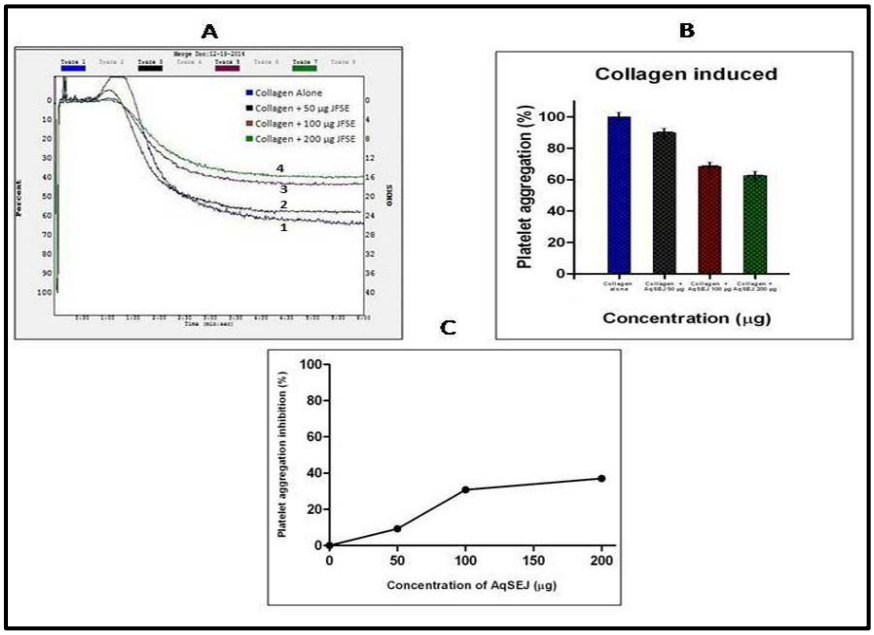

Fig. 5: Platelet aggregation was initiated by adding collagen as an agonist. (A)Trace 1 (Collagen 2 µg/ml); Trace 2(Collagen 2 µg/ml+50 µg of AqSEJ); Trace 3 (Collagen 2 µg/ml+100 µg of AqSEJ); Trace 4 (Collagen 2 µg/ml+200 µg of AqSEJ). The values represent±SD of three independent experiments. (B) Dose dependent platelet aggregation %. (C) Dose dependent platelet aggregation inhibition %

Although, the observed ant platelet activity of AqSEJ could be due to the participation of proteolysis enzyme/try sin inhibitors, it is too preliminary to say its exact mechanism of action. Ethanolic extracts of Acheranthusaspera (leaves), Tridaxprocumbens(whole plant), aqueous extract of Abutilon indicum(leaves), Acheranthusaspera (whole plant), Soshiho-tang, Cannabis and dicoumarin from Viola yedoensis Makino, Phenanthrenes and flavonoids from Calanthearisanensis (leaves), Artocarpuscommunis (root cortex) found to exhibit ant platelet activity [25-31].

Furthermore, AqSEJ was tested for its probable role on the generation of Reactive Oxygen Species (ROS), in particular superoxide radical and hydrogen peroxide. Interestingly, AqSEJ did not alter the level of ROS in both PRP and washed platelets up to the tested dose of 500 µg. It is known fact that the process of platelet aggregation constantly escorted through preliminary shape change and generation of Reactive oxygen species (ROS). Nonetheless, AqSEJ did not generate ROS, while inhibited ADP, epinephrine and collagen induced platelet aggregation the observed event could be due to receptor mediated but not through the change in the platelet shape. It's indeed very interesting to sightsee whether AqSEJ blocking agonists or hydrolyzing the platelet surface receptors.

CONCLUSION

In conclusion, this study for the first time attempted to sight seethe anticoagulant and ant platelet properties of AqSEJ. Hence, further identification and biochemical characterization of active molecule

from the AqSEJ, may present a promising alternative in the treatment of thrombotic disorders.

ACKNOWLEDGMENT

The authors thank the Vision Group on Science and Technology, Government of Karnataka, Bangalore and UGC New Delhi for financial assistance.

CONFLICT OF INTERESTS

No competing financial interest exists

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