Research Article

In-vitro and *in-vivo* studies of Tamarind seed edible extract reveals anti-oxidative, anticoagulant, antiplatelet events

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Received: 9 March 2019 Revised: 10 May 2019 Accepted: 18 May 2019

Abstract

Objective: This study emphasizes antioxidant, anticoagulant and antiplatelet activities of Tamarind Seed Edible Extract (TSEE). **Materials and Methods**: Antioxidant activity was assessed by DPPH, Determination of oxidative stress marker, lipid peroxidation, Protein carbonyl content, and antiplatelet activity was tested using plasma recalcification time, bleeding time, APTT and PT Assays. Antiplatelet activity was studied by platelet aggregation studies using agonists ADP, Epinephrine and Collagen. **Results**: The TSEE showed similar protein banding pattern from the range of molecular mass 100kDa-14kDa under both reduced and Non-reduced conditions. It was signifying that the presence of monomeric protein in the extract. The TSEE showed proteolytic activity as it hydrolyzed casein and also the major role in the interference of plasma coagulation cascade and it induces clotting time. The TSEE extract was investigated for its role on platelet function, Surprisingly, TSEE inhibited the agonist ADP, Epinephrine and collagen-induced platelet aggregation in a dose-dependent manner and oxidative stress induced stress marker alteration in washed platelets is one among the many causes for the progression and development of thrombotic disorder like Alzheimer disease, arthritis, and diabetes millets and many chronic inflammatory disorders. TSEE were majorly used to determine the protective properties against the oxidative stress induced stress markers such as ROS, intracellular Ca2+ level, PCC, LPO, and thiols status. **Conclusion**: The TSEE shows the beneficial agent to protect the platelets from AAPH induced oxidative stress condition as well as agonist-induced platelet aggregation and its associated complication. Hence it considering the said facts that TSEE may be a better contender in the treatment of coagulation and haematological and cardiovascular disorders.

Keywords: Tamarind seed edible extract, antiplatelet, anticoagulant, antioxidant

Introduction

The antioxidant, anticoagulant and antiplatelet properties of herbs and plant seeds are of particular interest in the understanding of the impact of oxidative stress modification of drugs induced blood components for development of atherosclerosis and many other degenerative ailments. As many metabolic and stress-related disorder is closely associated with the much-complicated disorder and the use of plant seeds as a source of antioxidant activities (Sandesh et al., 2014). Oxidative stress has been occupied in aging many complicated diseases which including cancer, cardiovascular disorder especially atherosclerosis, diabetes and chronic inflammatory effect (Halliwell,1994). Tamarind seed (*Tamarindus indica*) is a monotypic taxon, having only a single species belongs the genus Tamarindus subfamily Caesalpinioideae belongs to the family of Fabaceae grown in cooler regions of the world. In addition, it also helps to lower the blood cholesterol level. In fruit, the pulp constitutes 30–50 %, the shell and fiber account for 11–30 % and the seed about 25–40 %.Tamarind seeds are flat and oval with a keen tip and stores several varieties of phytoconstituents that includes Flavonoids, diterpenes, and steroids, Phenolic compounds, cardiac glycosides, maleic acid, tartaric acid, mucilage, pectin, arabinose, xylose, galactose, glucose, and

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DOI: https://doi.org/10.31024/ajpp.2019.5.6.5

uronic acid. Tamarind seeds inhibit snake venom enzymes which cause local tissue damage, inflammation, and hypotension (Ushanandini et al., 2006). Polysaccharide from tamarind seeds has an immunomodulatory effect (Sreelekha et al., 1993) and was shown to improve dry eye syndrome, to assist the release of drug in the human body and intraocular penetration of Rufloxacin (Rolando et al., 2007; Ghelardi et al., 2004). Water extract of tamarind seed has been shown to reduce blood sugar level in Streptozotocin-induced diabetic male rats (Maiti et al., 2004). Despite, immense nutritional and therapeutic potential of various phytoconstituents of tamarind seed edible extract the beneficial role of proteins/phytoconstituent which present in the extract was least studied. Tamarind seeds are known to have high inhibitory activity against human neutrophil elastase (Fook et al., 2005). At presently using of traditional medicine/drugs from plant origin has been gaining much importance .The plant-based drugs are continuously using as part of the primary medical emergencies to cure various ailments. Thus, the aim of this work was to evaluate the anticoagulant, antiplatelet and antioxidant potential Tamarind seed edible extract and the results are presented.

Materials and methods

Reagents

Collagen type-I, Adenosine diphosphate (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. Fat-free casein, Molecular weight markers were from Bangalore Genie Private Limited, India. APTT and PT Reagents were purchased from AGAPE diagnostic Pvt. Ernakulam, Kerala, India. AAPH (2,2′ azobis (2-amidinopropane) hydrochloride), Thiobarbituric acid (TBA). All other chemicals and reagents used were analytical grade. The fresh blood sample was collected from healthy human donors for the washed platelets. 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. All other chemicals used were of analytical grade.

Ethics statement

Human blood was collected from healthy adult volunteers with transcribed informed consent according to the procedures of Institutional Human Ethical Committee, University of Mysore, Mysore. All the experimentations were conducted in accordance with the ethical guidelines and were approved by the Institutional Human Ethical Committee (IHEC-UOM No. 47Res/2014–15), University of Mysore, Mysore. Conducting animal experiments were permitted by the Institutional Animal Ethical Committee (UOM/IAEC/02/2016), University of Mysore, Mysore. The animal handling were proceeded in accordance with the guidelines of the Committee for the Purpose of monitoring and Supervision of Experiments on Animals (CPCSEA).

Preparation of tamarind seed edible Extract (TSEE) and Protein estimation

Tamarind fruits were procured from the grocer, Tumkur. Separate the fruits and seeds. The outer coat of tamarind seed was removed, and collect edible part and it was homogenized using phosphate buffer saline (PH 7.4) and centrifuged at 5000g for 20 min at 4^0C. The supernatant was separated and the sample obtained was lyophilized after getting powder stored at -20°C until use. This extracted sample was used throughout the study and referred as tamarind seed edible extract.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and periodic acid-Schiff (PAS) staining

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 10% was carried out according to the method of Laemmle et al.,1970. The crude TSEE (100µg) prepared under reducing and non-reducing conditions was used for SDS-PAGE. The electrophoresis was carried out using Tris (25mM), glycine (192mM) and SDS (0.1%) for 2hr at room temperature. After electrophoresis, the SDS-PAGE gels were stained with 0.1% Coomassie brilliant blue R-250 for detection of the protein bands and de-stained with 40% ethanol in 10% acetic acid and water (40: 10: 50v/v). Molecular weight standards from 200kDa to 14.3kDa were used. Glycoprotein staining was carried out according to the method of Leach et al.1980. After electrophoresis; the gel was fixed in 7.5% acetic acid solution and stored at room temperature for 1hr. Then the gel was washed with 1% nitric and kept in 0.2% aqueous periodic acid solution and stored at 4°C for 45min. After that, the gel was placed in Schiff's reagent at 4°C for 24hr and was de-stained using 10% acetic acid to visualize a pink color band.

Proteolytic activity by the colorimetric method

Proteolytic activity was assayed as described by Satake et al. (1963). Fat-free casein (0.4 ml, 2% in 0.2M Tris-HCl buffer, pH 7.6) was incubated with 100 µg of crude TSEE in a total volume of 1 ml for 2 hr and 30 min at 37°C. Undigested casein was precipitated by adding 1.5 ml of 0.44M trichloroacetic acid (TCA) and leaves it to stand for 30 min. The reaction mixture was then centrifuged at 2000 g for 10 min. Sodium carbonate (2.5 ml, 0.4M) and Folin–Ciocalteu's reagent (1:2) were added sequentially to

1 ml of the supernatant, and the color developed was read at 660 nm. One unit of the enzyme activity was defined as the amount of the enzyme required to cause an increase in optical density of 0.01 at 660 nm. TSEE specific activity was expressed as units/min/mg of protein.

Anticoagulant activity

Plasma re-calcification time

Plasma recalcification time was carried out using platelet rich plasma according to the method of Quick et al., (1935). Briefly, the crude TSEE (0-75µg) was pre-incubated with 0.2ml of citrated human plasma in the presence of 10mM Tris HCl (20µl) buffer pH 7.4 for 1min at 37°C, 20µl of 0.25M $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded.

Activated Partial Thromboplastin time (APTT) and Prothrombin time (PT)

Interference of TSEE in coagulation cascade is briefly determined, 100µl of normal citrated human plasma and TSEE (0–75µg) were pre-incubated for 1min. For APTT, 100µl reagent (LIQUICELIN-E Phospholipids preparation derived from with ellagic acid), which was activated for 3min at 37°C was added. The clotting was initiated by adding 100µl of 0.02M CaCl2 and the clotting time was measured. For PT, was initiated by adding 200µl of PT reagent (UNIPLASTIN– rabbit brain Thromboplastin). the visible clot in seconds. The APTT normalized ratio (INR)for PT at each point from the values of control plasma the buffer for an identical period of time.

In-vivo Bleeding time

The mice tail bleeding time was assayed by the method of Denis et al. (1998). Briefly, TSEE (0-200µg) in 30µl of PBS was injected intravenously through the tail vein of a group of six mice. After 10 min, mice were anesthetized using diethyl ether and a sharp cut of 3 mm length at the tail tip of the mouse was made. Immediately, the tail was vertically immersed into PBS which is pre-warmed to 37^0C. Bleeding time was recorded from the time bleeding started till it completely stopped and it was followed for 10 min (600 sec).

Whole blood clot retraction studies

Whole blood clot lysis assay was performed as per the method described by Prasad et al. (2006). Briefly, freshly drawn healthy human blood was transferred to pre-weighed sterilized micro-centrifuge tubes (500µl/tube) and incubated for 45min at 37°C. Serum was removed after the formation of a whole blood clot and each tube was weighed again. TSEE (0-150µg) was added individually to all the tubes thereafter incubated for 90min at 37°C. The obtained fluid was removed after incubation period and calculated the clot lysis percentage.

Preparation of platelet-rich plasma and platelet-poor plasma

The method of Ardlie and Han et al. (1974). was used to prepare human platelet-rich plasma (PRP) and platelet-poor plasma (PPP).the concentration of PRP was adjusted to 3.1×10^8 platelets/ ml with PPP. The PRP maintained at 37°C was used within 2hr aggregation process were carried out using plastic wares or siliconized glassware.

Preparation of washed platelets

Human platelets were prepared as reported to obtain platelet suspension withminimum leukocyte contamination with slight modifications. Blood was drawn fromantecubital vein of healthy drug-free non-smokers human volunteers with informed consent as per the guidelines of Institutional Human Ethical Committee University of Tumkur, Tumkur. All methods were in accordance with the IHEC guidelines and the study was approved by IHEC. The drawn blood was immediately mixed with acid citrate dextrose (ACD) anticoagulant (85 mM sodium citrate, 78 mM citric acid and 111 mM D-glucose) in the ratio 6:1 (blood: ACD v/v). The anticoagulated whole blood was then centrifuged at 330g for 5 min to obtain platelet-rich plasma (PRP). The PRP was diluted 1:1 with PBS and centrifuged at 240g for 10 min. Subsequently, the obtained supernatant was centrifuged at 500g and the obtained platelet pellet obtained was washed twice with CGS buffer (120mM sodium chloride, 12.9mM trisodium citrate, 30mM D-glucose, pH 6.5) and suspended in Tyrode's albumin buffer (145mM NaCl, 5mM KCl, 10mM HEPES, 0.5mM Na2HPO4, 1mM MgCl2, 6 mM glucose, and 0.3 % bovine serum albumin, BSA) pH 7.45. The cell count was determined using a Neubauer chamber and adjusted to 5×108 cells/mL in the final suspension using Tyrode's albumin buffer.

Antioxidant activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging activity

The antioxidant activities of TSEE with Ascorbic acid as positive control were evaluated by using DPPH radical scavenging assay (Sundaram et al., 2014). Briefly, samples using at various concentrations (0–100µg) were taken in test tubes and the final volume was made up to 1 mL using methanol. Freshly prepared 1 mL of 1.2 mM DPPH in methanol solution was added to the tubes containing the samples. The added mixture was incubated for 20 min at room temperature in the dark. The absorbance of the resulting mixture was recorded at 517 nm against blank and control using UV/Vis spectrophotometer (BioMate 3S, Thermo Scientific, USA).

Determination of reducing the ability

Ferric reducing abilities of TSEE compared with Ascorbic

acid as a positive control were estimated according to the method of Paul et al. (2015). with minor modifications. Briefly, samples (0–100µg) were mixed with 2.5 mL of 200 mM sodium phosphate buffer, pH 6.6 containing 1% potassium ferricyanide and the mixture was incubated at 50 °C for 20 min. At the end of incubation, 2.5 mL of 5% TCA was added and centrifuged at 450×g for 10 min. Further, 2.5 mL of supernatant was taken and mixed with 0.5 mL of aqueous 0.1% ferric chloride. The absorbance was measured at 700 nm against blank using UV/Vis spectrophotometer.

Determination of oxidative stress marker

Determination of endogenously generated reactive oxygen species (ROS)

Endogenous production of ROS in platelets was determined with slight modifications using CMH2DCFDA, a ROS sensitive fluorescent probe (Paulet al. 2015). Washed platelets (5 × 106 cells) taken in polystyrene 96-well microtiter plates were treated with AAPH (500 µM). For inhibition studies, platelets were pre-incubated with different doses of TSEE (0-200) for 10 min at 37^0C prior to AAPH treatment and the final volume was made up to 200µL with HBS and incubated at 37^0C for 1 hr. After incubation, control (untreated) and treated platelets were then incubated with 10µM CMH2DCFDA for 30 min at 37^0C, washed and the resulting fluorescence was recorded using a Varioskan multimode plate reader (Thermo Scientific, USA) by exciting the samples at 488 nm and measuring the resulting fluorescence at 530 nm.

Estimation of intracellular calcium

Washed platelets were treated with AAPH (500µM) in presence or absence of TSEE (0-200µg) as mentioned in the above section and the final volume made up to 200µL with HBS containing $CaCl_2$ (1.0 mM)\ and incubated for 1 hr at 37^0C. After incubation, the control (untreated) and treated platelets were then incubated for 45 min at room temperature with 2µM Fura-2/ AM, a fluorescence Ca2+ indicator. The cells were subsequently washed twice with the modified HBS to remove the extracellular dye and finally the platelet pellet was suspended in HBS. The Fura-2/AM absorption was determined by exciting the cells at 340 and 380 nm and the resulting fluorescence was measured at 500 nm. Data were presented as absorption ratios (340/380 nm).

***In-vitro* platelet viability and platelet count**

The method of Kropotkin et al.,1971 was used to test the platelet viability by incubating the PRP with various concentrations of TSEE (0–200µg), 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 TSEE (control).

Lipid peroxidation

Lipid peroxidation was measured according to the method of Ohakawa et al. (1979). Approximately 0-2mg of protein from lysate of washed platelets treated with an agonist AAPH (500µM) and TSEE (0-200µg/mL) was taken in dry test tubes, 1.5ml of acetic acid (pH 3.5, 20% v/v), SDS (8% w/v, 0.2ml) and 1.5ml thiobarbituric acid (0.8% w/v) was added, the reaction mixture was boiled at 45-60°C for 45min and centrifuged at 2000rpm for 10min. The formed Adducts were extracted into 1-butanol (3ml) and the TBARS (Thiobarbituric Acid-Reactive Substance) formed was read photometrically at 532nm and quantified using TMP (1, 1, 3, 3-tetramethoxypropane) as the standard.

Protein carbonyl content

DNPH assay is mainly used to measure the protein carbonyl content, according to the method described by Tunez et al. (2004), 1mg of protein from a lysate of washed platelets treated with an agonist AAPH (500µM) and TSEE (0-200µg/mL) was taken and an equal volume of 10mM DNPH in 2N HCl was added, incubated for 1hr shaking alternatingly at room temperature. The corresponding blank was carried out by adding only 2N HCl to the sample. After incubation, the mixture was precipitated with 20% Trichloroacetate (TCA) and centrifuged at 5000rpm, for 15min. The precipitate was washed twice with acetone by centrifuging at 10000rpm, for 15min and finally dissolved in 1ml of Tris buffer (20mM pH 7.4 containing 0.14M NaCl, 2% SDS) and the supernatant was recorded at 360nm. The difference in absorbance is determined and expressed as µm of carbonyl groups/mg protein, using molar extinction coefficient of 22mM-1cm-1.

Determination of total thiols

To determine the total thiols ,the method of Ardlie et al. (1974) was used, the washed platelets sample (0.05mg protein) was added to 0.375 mL of Tris–HCl buffer (0.2 M, pH 8.2) containing di-thio-bis-nitrobenzoic acid (DTNB, 10 mM) and 1.975 mL of methanol. Subsequent incubation for 30 min at room temperature the tubes were centrifuged at 3,000g for 10 min. The absorbance of the supernatant was measured at 410 nm and expressed as nmol of DTNB oxidized/mg protein.

Platelet aggregation

The turbid metric method of Born et al. (1962) was followed using a Chronology dual channel whole blood/optical Lumi aggregation system (Model-700). Washed platelets ($2.5x\ 10^7$) with various concentrations of TSEE (0–75µg) in the 0.25ml reaction volume. The aggregation was initiated independently with of agonists, such as ADP, epinephrine, and followed for 6 min.

Direct haemolytic activity

Direct erythrocytes lysis activity was evaluated by using washed human erythrocytes. Briefly, washed and phosphate buffered saline (PBS) (1:9v/v) were mixed; 1ml incubated independently with a concentration of TSEE (0-150 µg) for 1hr at 37^{0}C was stopped by adding 9ml of ice-cold PBS and centrifuged at 1000g for 10min at 37^{0}C released in the supernatant was measured at 540nm. Activity was expressed as a percentage of hemolysis against 100% lysis of cells the addition of water that served as positive control and phosphate buffered saline served as negative control.

Edema inducing activity

According to the method of Vishwanath et al.,1987 was followed. Groups of five injected separately into with different doses (0-200µg) of TSEE in 20µl saline. The left foot pads received 20µl saline alone served as control. After 1hr mice were anesthetized by diethyl ether inhalation. Hind limbs were removed at the ankle joint and weighed. Weight increased was the edema ratio, of edematous leg × 100/weight of the normal leg. Minimum edema dose (MED) as the amount required to cause an edema ratio of 120%.

Hemorrhagic activity

Hemorrhagic activity was assayed as described by Kondo et al.,1960.A different concentration of TSEE (0-200µg) was injected (intradermal) independently into the groups of five mice in 30µl saline. The group receiving saline alone serves as negative control and group receiving venom (2MHD) as a positive control. After 3hr, mice were anesthetized by diethyl ether inhalation. The dorsal patch of skin surface removed and observed against saline-injected control mice of hemorrhagic spot inner surface of was measured. The minimum hemorrhagic dose (MHD) as the amount of the protein-producing 10mm of hemorrhage in diameter.

Statistical analysis

The result data were presented as mean ± SD. Statistical analyses were performed by Student's T-test. A between the groups were considered if $P < 0.01$.

Results

Protein banding pattern, PAS staining and proteolytic activity of TSEE

For the identification protein banding pattern of TSEE in SDS-electrophoresis and PAS staining were carried out. Figure 1 signifies the protein banding pattern of TSEE in SDS-PAGE and PAS staining. The TSEE showed similar protein banding pattern from the molecular mass ranging from 100 kDa-14 kDa under both reduced and non-reduced condition (Figure 1A). Further, TSEE was analysed for if there any carbohydrate moiety content in the proteins; interestingly, it taken up PAS staining at the low-molecular-weight region around 29 kDa to 18 kDa that was compared with the positive control fibrinogen (Figure 1B).While the TSEE was positive to PAS staining and the fibrinogen that served as positive control took up the stain. The seed extract hydrolyzed casein and the specific activity was found to be 0.480 units/mg/min suggesting the proteolytic activity.

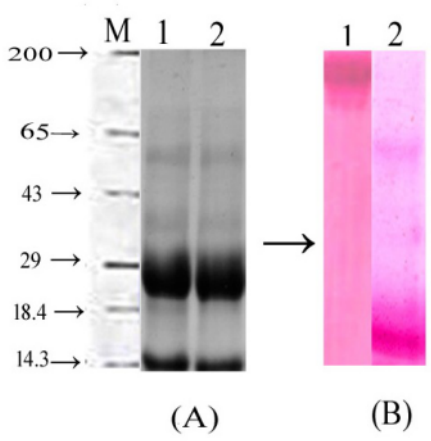

Figure 1. (A) SDS-PAGE 10% (B) Glycoprotein staining. (A) TSEE as shown in SDS-PAGE (10%): TSEE (100µg) under non-reduced (a1) and reduced conditions (a2), (B) PAS staining of TSEE: positive control fibrinogen (b1) and TSEE (b2). M molecular weight marker to bottom: myosin-H-chain (200), BSA (66.4), ovalbumin (44.3), carbonic anhydrase (29), lactalbumin (18.4) and lysozyme (14.3) BSA: bovine serum albumin, TSEE: tamarind seed edible extract.

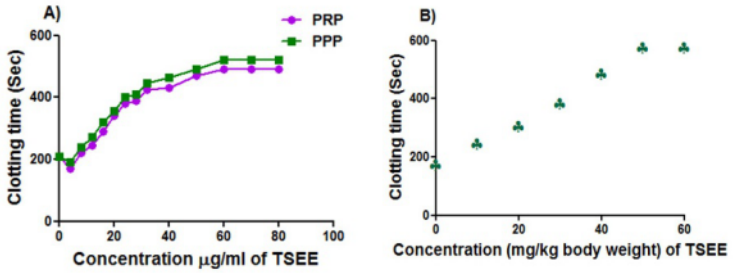

Figure 2. Effect of TSEE on plasma Recalcification time: (A) TSEE (0–75 µg) was pre-incubated with 0.2 ml of citrated human plasma in the presence of 20µl10 Mm Tris–HCl buffer pH 7.4 for 1 min at 37°C. 20µl of 0.25 M $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded. Effect of TSEE on the bleeding time: Tail bleeding time was measured 10 min after intravenous administration of PBS or various doses of TSEE. Each point represents the mean±SD of three independent experiments, P<0.01. Bleeding time longer than 10 min was expressed as>10 min.

Anticoagulant activity of tamarind seeds edible extracts

In order to identify the anticoagulant potential of TSEE; plasma Recalcification time and in-vivo bleeding time were evaluated. TSEE revealed the strong anticoagulant effect by interfering in plasma coagulation cascade. TSEE increases the Recalcification time of citrated human plasma from the control 210s and reached the maximum of 520s at the concentrations of 75µg and persisted intact at the increased dose up to 25µg. Figure 2A, represents the dose-dependent anticoagulant activity of TSEE. Additionally, the in-vivo anticoagulant activity of TSEE was determined by mouse tail bleeding assay. TSEE was Injected to mice intravenously,it significantly prolonged the bleeding time in a dose-dependent manner and the recorded bleeding time was more than 600 s ($P<0.01$) at the concentration of 200mg against the PBS-treated control of 180 s (Figure 2B). Furthermore, the major role of TSEE has evaluated the participation of on intrinsic/extrinsic pathway of the blood coagulation cascade was confirmed by measuring APTT and PT. Interestingly, TSEE significantly prolonged the plasma clotting time of only APTT (intrinsic pathway), but

Table 1. Dose dependent effect of TSEEE on clotting time of normal human plasma

TSEEE(µg)	PT Clotting time in sec	PT (INR values)	APTT clotting time in sec	APTT ratio
0	11.0±0.05	0.93±0.01	38.3±0.04	1.45±0.02
10	11.2±0.02	0.96±0.08	40.9±0.05	1.45±0.02
20	11.4±0.04	1.01±0.02	44.9±0.06	1.49±0.02
30	11.6±0.03	1.05±0.01	58.7±0.05	2.13±0.02
40	11.8±0.01	1.09±0.06	82.4±0.05	2.92±0.02
50	11.2±0.08	1.13±0.10	107.2±0.02	3.90±0.02
60	11.5±0.10	1.15±0.10	137.2±0.02	4.92±0.02
70	11.2±0.01	1.11±0.10	168.1±0.02	5.86±0.02
80	11.0±0.15	1.01±0.10	198.2±0.02	6.86±0.02
90	11.2±0.01	1.18±0.10	233.2±0.02	7.75±0.02
100	11.8±0.03	1.01±0.10	293.2±0.02	10.2±0.02

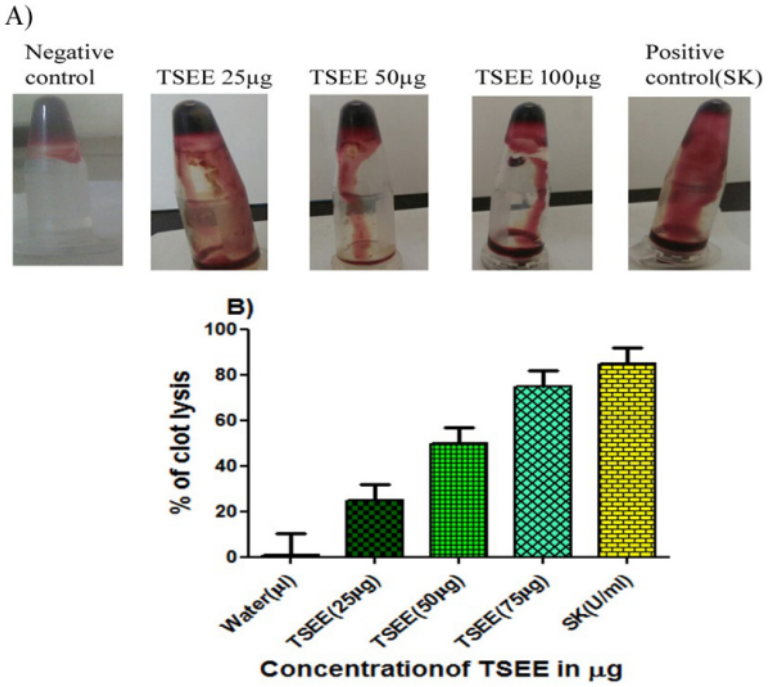

Figure 3. Whole blood clot lysis. (a) Whole blood clot lysis image (b) Percentage of whole blood clot lysis. (a) Freshly drawn healthy human blood was transferred to pre-weighed sterilized micro centrifuge tubes (500µL/tube) and incubated for 45min at 37°C. Serum was removed after the formation of whole blood clot then each tube was weighed again. TSEE (0-200µg) was added individually to all the tubes thereafter incubated for 90min at 37°C and calculated the clot lysis percentage. **(b)** Percentage of whole blood clot lysis.

it did not alter the clotting time of PT (extrinsic pathway), it shows the role of TSEE in participation in the intrinsic pathway of the blood coagulation cascade (Table 1).

Effect of TSEE on whole blood clot retraction

Subsequently, study the effect of TSEE whole blood clot retraction studies. Preincubation of whole blood with the increasing the concentration of TSEE it leads to a progressive inhibition in the extent of retraction (Figure 3). Interestingly TSEE dissolved the whole blood clots. When whole blood clot was incubated with TSEE (0-100µg) against positive control streptokinase (60 U/ml) it dissolves 80% of the whole blood clot.

Antioxidant potential of TSEE

TSEE induce the cellular antioxidant system by serving as a precursor for oxidative stress marker and also it proves the efficient free radical scavenging activity. Consequently, TSEE is used as an essential antioxidant it possesses the radical quenching property to fill in the place of TSEE. Thus TSEE were used to evaluate their free radical quenching property (Figure 4A). Initially, the scavenging properties of the TSEE were determined by DPPH free radical scavenging assay and there was a concentration-dependently scavenging free radical. In additionally reducing the ability of TSEE was evaluated by ferric reducing antioxidant power assay (Figure 4B). TSEE eventually shows the concentration-dependent ferric reducing ability. At the concentration of 200µg, there was a significant rise in the antioxidant potential of the TSEE and it compared with the ascorbic acid. Evaluated results suggest that TSEE shows the influenced amplified anti-oxidant potential activity.

Effect of TSEE on the endogenous generation of ROS and increase in intracellular calcium

Oxidative stress plays a pivotal role in the intrinsic mode of platelets oxidation, oxidative stress mediator such as ROS and intracellular calcium level were estimated. The platelets

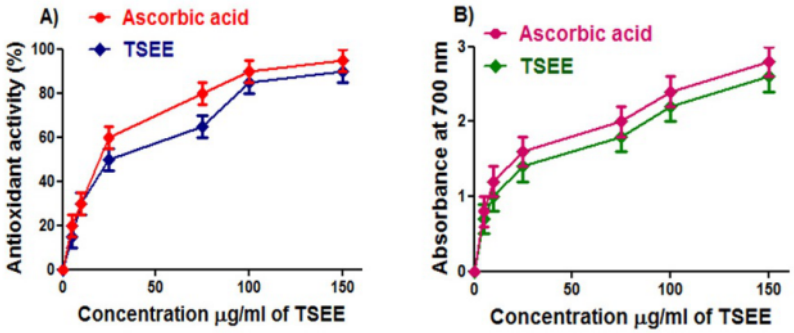

Figure 4. Free radical scavenging efficacy of TSEE in terms of a DPPH radical scavenging assay and B) Ferric ion reduction potential. Ascorbic acid was used as a standard. Values are presented as mean ± SEM (n = 5). *p < 0.01; *significant compared to TSEE concentration

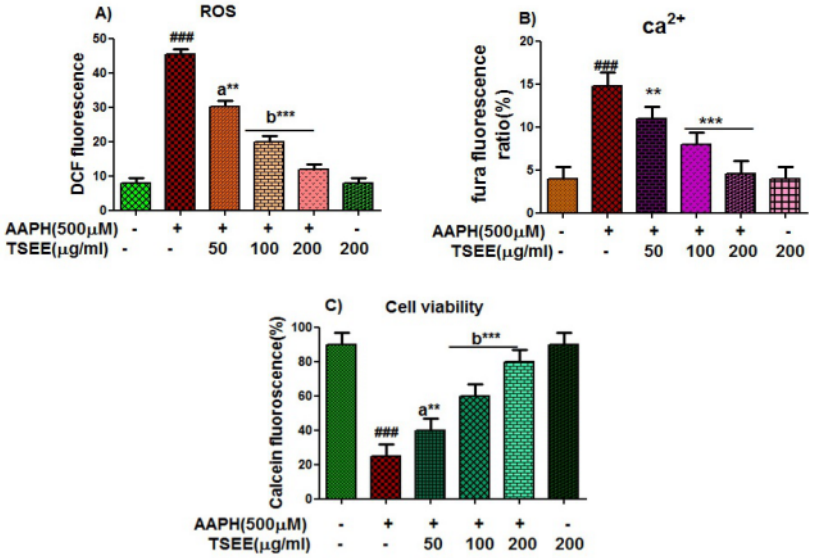

Figure 5. Effect of tamarind seed edible extract (A) Endogenous ROS generation and (B) Raise in intracellular calcium by a water-soluble free radical inducer AAPH. Values are presented as mean ± SEM (n = 3), expressed as percentage increase in DCF fluorescence and percentage increase in Fura-2/AM fluorescence, relative to control. ###p<0.001; significant compared to control. ***p<0.001; **p <0.04, *p<0.01; significant compared to AAPH.

treated with AAPH (500µM) it evoked significant ROS and intracellular calcium level. Therefore, TSEE were used for the evaluation of endogenous generation of ROS and Ca2+ level respectively compared with the control. AAPH induced ROS generation was eventually inhibited by TSEE. However, significant elevation of ROS production was seen at the concentration of 200µg (Figure 5A). Variation of intracellular calcium level, it's another kind of oxidative stress marker it enhances towards platelets apoptosis. Deployment of intracellular Ca2+ from a store of calcium in platelets enhances the dysfunction of platelets and release of cytochrome c from mitochondria into the cytosol. AAPH induced sustained level of intracellular Ca2+ level inhibition was evaluated by TSEE. AAPH induced intracellular Ca2+ level was dose-dependently inhibited by TSEE at the concentration of 100µg.there was a significant amelioration of intracellular Ca2+ level in platelets compared with control (Figure 5B). TSEE shows the more potential at the higher dose level. Finally appeared result clearly shows TSEE are able to inhibit the platelets ROS and intracellular Ca2+ content at the concentration of 100µg and thereby it was used for the evaluation of antioxidant potential in stressed platelets. Evaluating the AAPH treated platelets viability, AAPH treated cells shows a apoptotic activity. Interestingly, TSEE significantly inhibit the AAPH induced platelets apoptosis and restored the cell viability (Figure 5C). Treatment of platelets with (0-200µg) concentration of TSEE significantly inhibited the AAPH induced platelets apoptosis.

Modulatory effect of TSEE on AAPH-induced intracellular lipid peroxidation, Protein oxidation, and thiols status

Oxidation of lipids and protein is a key event that predicts the marked oxidative stress. Lipid peroxidation and protein carbonylation is a key marker to indicate the oxidative stress in cells. The current study evaluates the extent of lipid peroxidation was measured in terms of malondialdehyde (MDA) levels and a remarkable increase was spectacled in AAPH induced group. Interestingly, TSEE pre-treated groups completely elevate the lipid peroxidation level was observed. Whereas it compared with TSEE alone group, TSEE alone didn't show any significant alteration (Figure 6A). The currently appeared experimental result exposed the remarkable elevation of protein carbonylation in the AAPH group whereas it compared with an untreated group. Although in pre-incubation group TSEE reorganize the altered PCC by reducing the level to normality; while in the TSEE-alone didn't alter the level of protein carbonylation (Figure 6B). In order to evaluate the total thiols content in AAPH treated groups, there is a significant increase in thiols content, its reflecting image of oxidative stress in cells. In the TSEE pre-treated groups show significant inhibition in thiols content. While in the TSEE alone group there is no alteration occurred in the level of total thiols (Figure 6C).

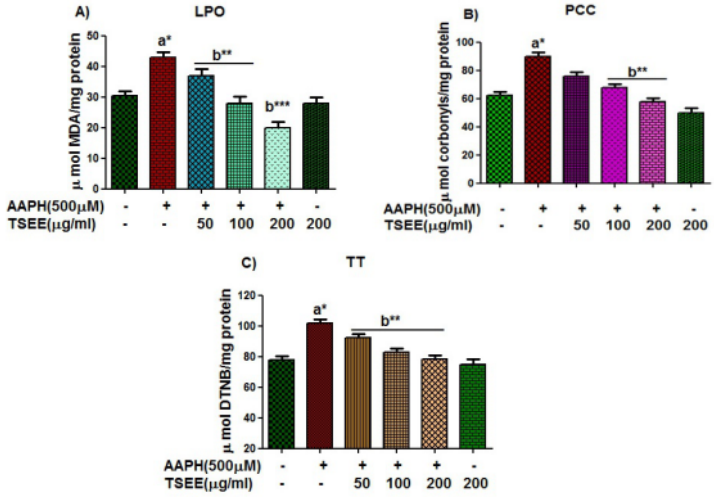

Figure 6. Effect of TSEE on (A) lipid peroxidation and (B) protein carbonyl content. (C) Total thiols estimation. AAPH was used as an agonist in induce lipid peroxidation and protein carbonyl content for 1h. For inhibition studies, platelets were pre-incubated with different doses of TSEE for 10 min at 37°C prior to p-TBC treatment. Values are presented as mean ± SEM (n = 3). [n = 3, p<0.05 (*), p<0.01 (**), p<0.001 (***/###); #: significant compared to untreated platelets, *: significant compared top-TBC treated platelets.]

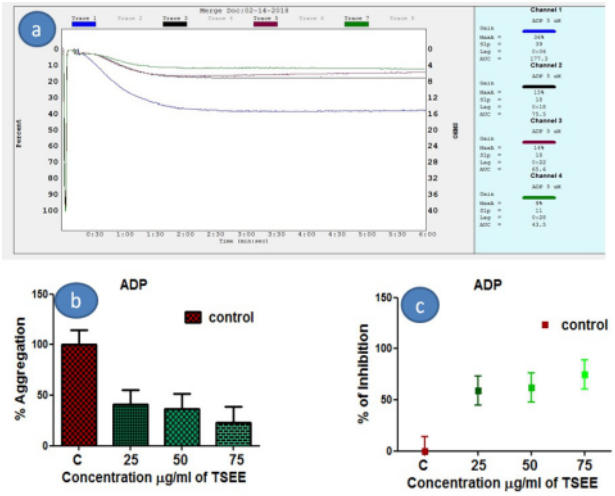

Figure 7. Platelet aggregation was initiated by adding ADP as an agonist. (A)Traces of platelet aggregation: Trace 1 (ADP 10 µM); Trace 2 (ADP 10 µM+25 µg of TSEE); Trace 3 (ADP 10 µM+50µg of TSEE); Trace 4 (ADP 10 µM+75µg of TSEE). The values represents of three independent experiments. (B) Dose-dependent platelet aggregation %. (C) Dose-dependent platelet aggregation inhibition %

Effect of TSEE on platelets function

Platelet hyperactivity is a major Couse to leads a thrombotic disorder and its associated complication. Therefore, in order to evaluate the antiplatelet property of the TSEE on platelet function, platelet aggregation induced by a physiological agonist such as ADP, Epinephrine, collagen and known antiplatelet drug N-acetyl cysteine (NAC) used as a positive control (Figure 7-10) TSEE inhibit the agonist-induced platelets aggregation at concentration-dependent level. Agonist-induced platelets aggregation was inhibited about 75%, 86%, and 96% respectively at the concentration of 75µg. And it's compared with the known antiplatelet agent N-acetylcysteine (NAC) inhibits the 96% at the concentration 20µM and it compared with the known agonist and it's inhibited by the TSEE (Figure 11). Among the all agonists observed TSEE inhibited in the order of ADP > epinephrine > collagen-induced aggregation.

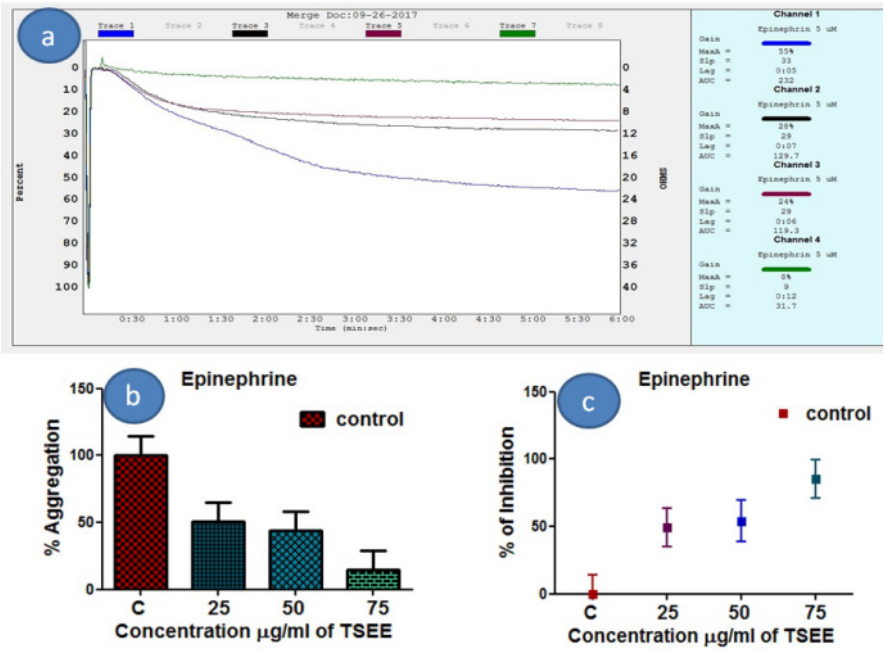

Figure 8. Platelet aggregation was initiated by adding Epinephrine as an agonist. Trace 1 (Epinephrine 5µM); Trace 2 (Epinephrine 5µM+25 µg of TSEE); Trace 3 (Epinephrine 5µM+50µg of TSEE); Trace 4 (Epinephrine 5µM+75 µg of TSEE). The values represent ±SD of three independent experiments. (B) Dose-dependent platelet aggregation %. (C) Dose-dependent platelet aggregation inhibition %

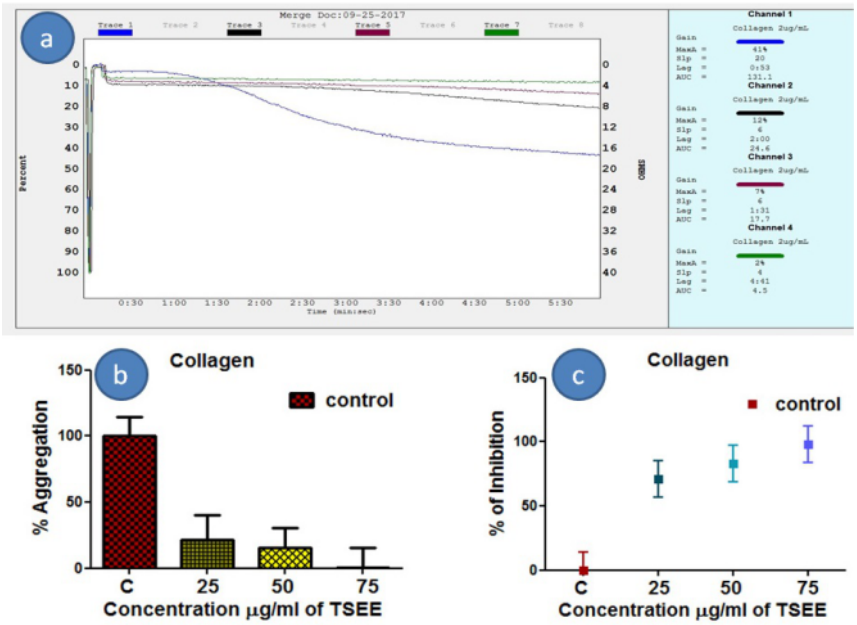

Figure 9. Platelet aggregation was initiated by adding collagen as an agonist. (A)Trace 1 (Collagen 2 µg/ml); Trace 2(Collagen 2 µg/ml+25 µg of TSEE); Trace 3 (Collagen 2 µg/ml+50 µg of TSEE); Trace 4 (Collagen 2 µg/ml+75 µg of TSEE). The values represent±SD of three independent experiments. (B) Dose-dependent platelet aggregation %. (C) Dose-dependent platelet aggregation inhibition %

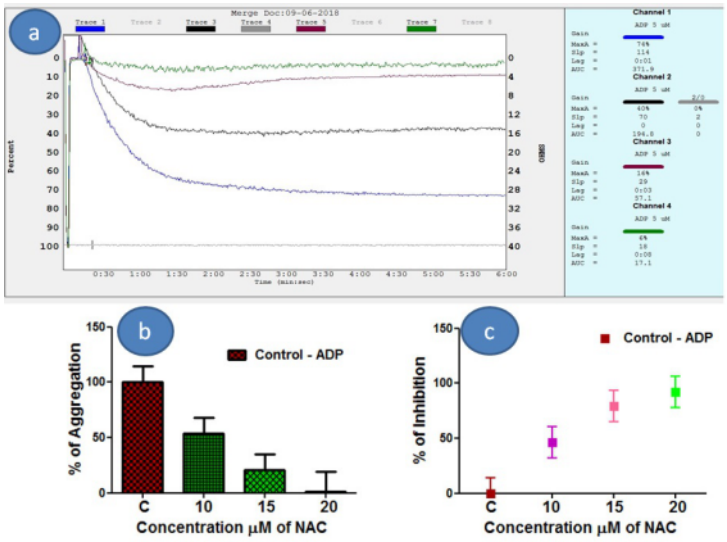

Figure 10. Platelet aggregation was initiated by adding ADP as an agonist. (A)Traces of platelet aggregation: Trace 1 (ADP 10 µM); Trace 2 (ADP 10 µM+10 µM of NAC); Trace 3 (ADP 10 µM+15µM of NAC); Trace 4 (ADP 10 µM+20 µM of NAC). The values represents of three independent experiments. (B) Dose-dependent platelet aggregation %. (C) Dose-dependent platelet aggregation inhibition.

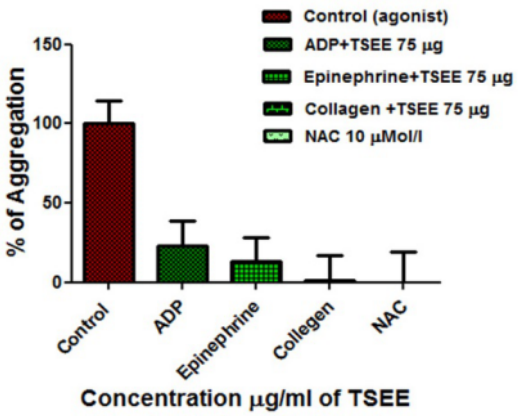

Figure 11. Comparative study of platelets aggregation with the N-acetyl cysteine (NAC) with the compare with the % of Platelet aggregation inhibition by using a different agonist like ADP, epinephrine and collagen as an agonist. (A): bar graph 1 (ADP 10 mM) (B): bar graph 2 (ADP 10 µM+75 µg TSEE); (A): bar graph 3 (Epinephrine 5 µM+75 µg TSEE); bar graph 4 (Collagen 2 µg+75 µg TSEE); bar graph 5 (ADP 10µM+20 µM TSEE); the values represents of three independent experiments.

Determination of in-vivo Non-toxic properties of TSEE

Erythrocyte is the main blood component in a human physiological system. It's mainly responsible for the carry oxygen and nutrient to various parts of the body. TSEE did not hydrolyze the red blood cells; and cause a hemorrhagic effect on mice and paw experimental mice up the concentration of 0-200µg, while positive control haemorrhage and edema in experimental mice, nontoxic property (Figure 12A and 12B).

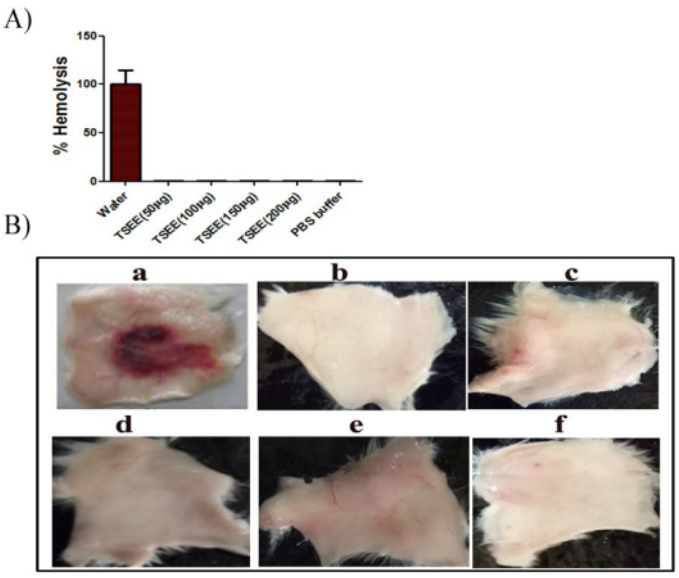

Figure 12. (A) Direct hemolytic activity of TSEE on human erythrocytes in dose dependent. (B)Dose-dependent haemorrhagic activity of Tamarind seed edible extract. (a) Saline, (b) positive control 2 MDH venom, (c) 50µg, (d) 100µg and (e) 200µg of TSEE independently into mice in an of 50µl intradermal. TSEE; Tamarind seed edible extract.

Discussion

In recent time therapeutic usage of tradition plants containing bioactive components like protease, flavonoids, and polyphenols have been gaining a much importance due to their therapeutic potential (Sandesh et al., 2014; Halliwell, 1994). Despite this anticancer, antiulcer, antihypertensive, antioxidant, anti-diabetic antifungal and antimicrobial activities, tamarind seed edible extract was studied. The aim of these study describes the bioactive components present in the tamarind seed buffer extract explores the anticoagulant, Antiplatelet, and antioxidant potential. TSEE protein banding pattern in look like similar under reducing and non-reducing condition, suggesting the presence of monomeric proteins. TSEE was positive to PAS staining only at the lower middle molecular weight region, suggesting the presence of glycoprotein. Proteolytic activity was also confirmed by colorimetric estimation. Interestingly, TSEE significantly induced the clotting time of citrated human plasma re-calcification time both in in-vitro and in-vivo mice tailing bleeding time revealed its strong anticoagulant effect. In order to identify the pathway of the blood coagulation cascade, Activated partial thromboplastin time (APTT) and prothrombin time (PT) assays were carried out. TSEE prolonged the clotting time of APTT but not PT suggesting its involvement in intrinsic pathway of blood coagulation cascade. Several potential anticoagulants in plants and animals were identified in a fermented food product, Japanese natto and Korean chungkook and soy sauce snake venoms,

earthworm secretions, dung beetles, food grade, microorganisms, marine creatures, herbal medicines (Drake et al., 1989). Because ourresults are reliable with the anticoagulant of ticks, earthworms, caterpillar, venoms of the snake, spider, and honey bees, and relatively nontoxic than those, it may serve as a better alternative medicine for thrombotic disorders as well as a cardiovascular complication.

AAPH induced Oxidative stress studies usually tells the determination of ROS generation, increased calcium level and also the determination of oxidative stress marker which includes the protein oxidation, lipid peroxidation and total thiol status (Drake et al., 1989; Kitchen et al., 2014). Some of the pathological condition linked with the oxidative stress that includes diabetes, malaria, chronic renal failure, uremic syndrome, anemia and thalassemia. In this study, our efforts are to identify the potency of TSEE (Veiga et al., 2000; Denson et al., 2002). Platelets activation which mainly leads to platelets aggregation that mainly induces the cardiovascular and thrombotic disorder, which are mainly considered as associated complication of diabetes (Veiga et al., 2000; Rachidi et al., 2013). In addition that, ROS play a major role to leads a several neurogenerative disease likes Alzheimer and Parkinson's and Huntington's disease several reports tells that tamarind seed high potency in depleting the oxidative stress (Rachidi et al., 2013).

Hemostasis is a coordinated physiological process, due to the effect of injury in an endothelial membrane, environmental factors, genetic factors, hyper coagulopathy, and abnormal blood flow and yet unidentified molecular pathway impairs the hemostasis that leads a thrombotic disorder (Rachidi et al., 2013; Poon et al., 2000). The rate of mortality and morbidity level has been increased due to the myocardial infarction, unstable angina, deep vein thrombosis, arterial thrombosis, atrial fibrillation, pulmonary embolism and cerebral stroke (Zare et al., 2013; Manoj et al). Although, *Tamarindus indica* reported to contain an innumerable medicinal application, and the antithrombotic and blood component protection were not explored. While, Kaeko Hayashi et al. (1994) showed the trypsin inhibitor-II from *Tamarindus indica* was prolonged the prothrombin time of human plasma (Poon et al., 2000). Meanwhile presently available anticoagulant and antiplatelet therapy have lots of limitation to offer safety against thrombotic disorders. Thus these limitations pave the way to the impetus for the identification of target anticoagulant and Antiplatelet agents from the natural source for the better handling of thrombotic disorder.

Thus, the current study explores the beneficial role of TSEE seed proteins and phytoconstituents in oxidative stress and thrombotic disorders. Interestingly, TSEE was nontoxic to experimental mice as it did not cause haemolysis, haemorrhage and edema.

Conclusion

In conclusion, this study for the first time reports the anticoagulant, antiplatelet and anti-oxidant activities of TSEE. The significant effect of tamarind seed edible extract shows the anticoagulation, antiplatelet and antioxidant activity of tamarind edible extract due to the presence of several bioactive components of the sample. Further identification and biochemical characterization of anticoagulant, antioxidant activity from the TSEE may present a valuable candidate in the treatment of thrombotic disorders.

Acknowledgments

The authors thank the Rajeev Gandhi national fellowship (RGNF), Government of India, New Delhi and UGC New Delhi for financial assistance.

Declaration of conflict of interest

The authors declared no potential conflict of interest with respect to the authorship and publication.

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Abbreviations

TSEE : Tamarind Seed Edible Extract

PCC : Protein Carbonyl Content

LPO : Lipid Per oxidation

PRP : Platelet Rich Plasma

PPP : Platelet Poor Plasma