INTERNATIONAL RESEARCH JOURNAL OF PHARMACY
www.irjponline.com
ISSN 2230 – 8407

Research Article

ANTIOXIDANT, ANTI-CANCER AND ANTICOAGULANT PROPERTIES OF ETHANOLIC EXTRACT OF *TAMARINDUS INDICA* SEED COAT

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Article Received on: 12/04/19 Approved for publication: 28/05/19

DOI: 10.7897/2230-8407.1006204

ABSTRACT

The existing piece of work validates the antioxidant, anticancer and anticoagulant properties of Ethanolic Extract of *Tamarindus Indica* Seed Coat (EETISC). The feasible secondary metabolite in the EETISC extract was analysed on the RP-HPLC column chromatography with PDA detector. In addition, the extract showed DPPH and superoxide radicals scavenging activities supporting its antioxidant activity. Curiously, EETISC extract showed anti-cancer activity as it exhibited potent pro-apoptotic activity in the EAT tumour model and the antiangiogenic properties in chorioallantoic membrane assay. Interestingly, EETISC showed anticoagulation property by inhibiting intrinsic pathway of blood coagulation cascade, suggesting its therapeutic potential in cardio and cerebrovascular complications. Moreover, EETISC extract did not cause edema and haemorrhage in the experimental mice study and did not hydrolyze RBC's suggesting its nontoxic property. In conclusion, EETISC is exhibited manifold therapeutic applications thus it may be a promising therapeutic agent for worries such as oxidative stress, cancer cells and thrombosis.

Key words: Tamarind Seed Coat Ethanol Extract, Antioxidant, Anticoagulant, Proapoptotic, Anti angiogenic effect

INTRODUCTION

The oxidative stress caused due to an imbalance between the free radicals generation, reactive metabolites and their elimination by the natural antioxidants leads to the damage of key biomolecules and cell function [1]. Moreover, the ROS such as, (O_2^-, H_2O_2, OH•) and organic peroxides (-O-O-) produced during endogenous metabolic reactions significantly damage the biochemical functions at the cellular and molecular level that in-turn induce somatic and neoplastic mutation [2- 4]. Thus, the initiation of cancer and its progression is coupled with the oxidative stress [5, 6]. About millions of people suffering from various types of cancer due to stress [7]. The mortality and morbidity rate is being increased in both developed and underdeveloped countries because of change in life style, a major contributor of stress [8, 9]. In addition to the said fact, stress may also have direct influence on the blood coagulation system. Investigation by various research groups suggested that the stress found to provoke the activation of sympathoadrenal medullary system that in-turn activates both the coagulation cascade and the fibrinolytic system end up in hypercoagulability or thrombosis [10, 11]. Thrombosis is a pathological phenomenon where the formation of unusual clot takes place in the arteries and veins leads to thrombotic disorders [12]. Thrombotic disorders increase the risk of cardio/cerebrovascular complication. The mortality and morbidity rate has been increased radically in the recent time [13]. Usage of medicinal plant extract and their isolated compounds in treating various ailments is an ancient practice [14]. The extract prepared from the various parts of the medicinal plants such as roots, stems, seeds, barks and leaves have been widely used [15]. *Tamarindus Indica* L belongs to the family *Fabaceae* and subfamily *Caesalpinioideae,* though native to Africa but it grown elsewhere the world [16]. Tamarindus indica generally called Tamarind is a pod like fruit having edible part and a seed. Edible part is being used in multi cuisines and traditional medicine around the world. While, the seed possess the kernel and seed coat is a key source for plethora of phytochemicals mainly polyphenols, alkaloids, flavonoids, triterpenes, polysaccharides and other unknown factors responsible for healing bustle for several human pathophysiological conditions [17-19]. The major active component characterized so far from tamarind seed extract includes Threo isocitric acid, galactosyl glycerol, procyanidin B2, areca tannin B1, catechin, rutin, and embelin [13,20]. Despite, its immense therapeutic applications the role of tamarind seed extract on thrombosis and cancer was least explored. Therefore, the current work focuses on the anticoagulant, antioxidant and anticancer effect of ethanolic extract of tamarind seed coat extract and the results are presented.

MATERIALS AND METHODS

Reagents

All other fundamental chemicals were of analytical grade. Ehrich Ascites Tumor cells (EAT cells): Dulbecco's modified Eagle's medium, foetal bovine serum (Sigma, USA) and antibiotics, 2, 2-diphenyl-1-picrylhydrazyl (DPPH). Nitro blue tetrazolium (NBT), APTT and PT Reagents were purchased from AGAPPE diagnostic Pvt. Ernakulum, Kerala, India. 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.

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, Mysuru. 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)

Plant material and preparation of tamarind seed coat extract

Tamarind seeds were purchased from local market Tumkur thoroughly washed them and dried at room temperature for 24h. Seed coats were separated mechanically, homogenized using pestle and mortar by adding 95% ethanol. The ethanol extract obtained was filtered using Whatman no-1 filter paper and allowed to evaporate the alcohol content. The obtained filtrate was completely dissolved in water and it was used for all the assays.

Analysis of Phytoconstituents in the EETISC extract using HPLC-PDA instrument

Phyto constituents present in the EETISC extract was analyzed using the HPLC-PDA instrument. About 20µl of EETISC sample was injected to a C18 column (150 mm × 4.60 mm, particle size 5µm). Mobile Phase water (Solvent A) and Acetonitrile (Solvent B) was applied. Flow rate was set to 1mL/min and the sample was scanned from various wave length ranges from 200-800nm.

Fourier Transform Infrared Spectrophotometer (FTIR)

Fourier Transform Infrared Spectrophotometer (FTIR) is perhaps the most commanding device for classifying the types of chemical bonds (functional groups) present in compounds. The wavelength of light absorbed is distinctive of the chemical bond as can be seen in the annotated spectrum. Through understanding the infrared absorption spectrum, the chemical bonds in a molecule can be determined. Dried powders of EETISC were used for FTIR analysis. 10mg of the dried extract powder was encapsulated in 100mg of KBr pellet, in order to prepare translucent sample discs. The powdered sample of each plant specimen was loaded in FTIR spectroscope (Agilent Technologies), with a Scan range from 1000 to 4000 cm 1 with a resolution of 4cm.

ANTICOAGULANT ACTIVITY

Plasma re-calcification time

The plasma re-calcification time was determined according to the method of Quick et al. (21). Briefly, EETISC (0-60µg) 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.

Mouse tail bleeding time

The bleeding time was assayed by the method of Denis et al. [22]. In brief, EETISC (0–50µg) in 30ml of phosphate buffered saline (PBS) was injected intravenously through the tail vein of a group of six mice. After 10min, the mice were anesthetized using diethyl ether and a sharp cut of 3mm length at the tail tip of mouse was made. Immediately, the tail was vertically immersed into PBS, which was prewarmed to 37^0C. The bleeding time was recorded from the time the bleeding started till it completely stopped and it was followed for 10min (600 s).

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

In Brief, 100µl of normal citrated human plasma an EETISC (0–40µg) were pre-incubated for 1min. For APTT, 100µl reagent (LIQUICELIN-E Phospholipids preparation derived from Rabbit brain with ellagic acid), which was activated for 3min at 37°C was added. The clotting was initiated by adding 100µl of 0.02M $CaCl_2$ and the clotting time was measured. For PT, the clotting was initiated by adding 200µl 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 was calculated from the values of control plasma incubated with the buffer for identical period of time.

ANTIOXIDANT ACTIVITY

DPPH (2, 2-diphenyl-1-picrlhydrazyl) Radical Scavenging Activity

Different concentration of EETISC (0-100µg) was mixed with 100µl of DPPH. The mixture was homogenised and incubated for 30min at room temperature in the dark. The absorbance was read at 517nm in a UV spectrophotometer. Ascorbic acid was used as the antioxidant standard. The experiments were performed in duplicates. The percentage of free-radical scavenging activity was expressed with the following formula:

% Antioxidant activity= Absorbance Control-Absorbance test sample / Absorbance Control X100

Superoxide dismutase (SOD)

The assay of SOD was based on the reduction of nitro blue tetrazolium (NBT) to water in-soluble blue formazone. In 0.5ml homogenate 1ml of 50mM sodium carbonate 0.4ml of 24mM NBT and 0.2ml of 0.1mM EDTA were added. The reaction was initiated by adding 0.4ml of 1mM hydroxylamine hydrochloride. Zero-time absorbance was taken at 560nm followed by recording the absorbance after 5min at 25°c. The control was simultaneously run without homogenate. Units of SOD were expressed as the amount of enzyme required to inhibit the reaction by 50%. The specific activity was expressed as units per mg protein.

ANTICANCER ACTIVITY

Preparation of cell culture media: **Cell culture and maintenance**

EAT cells were procured from the National Centre for Cell Science, Pune, India. The cells were grown in suspension culture in Dulbecco's modified Eagle's medium (Life Technologies, USA) containing 10% foetal bovine serum (Sigma, USA) and

antibiotics (100 U/ml penicillin and 100 Ag/ml streptomycin) in a humidified atmosphere of 5% CO2 at 37^0C. For all experiments, Dulbecco's modified Eagle's Medium containing 5% foetal bovine serum was used.

Primary culturing of Ehrich Ascites Tumor cells (EAT cells)

EAT cells were withdrawn aseptically from mice 5-8days following routine tumour inoculation. The cells were mixed with volume of sterile ammonium chloride to lyse any erythrocytes and centrifuged at 2000rpm for 3min in graduated conical centrifuge tubes. The supernatant fluid was discarded; the sediment cells were re-suspended in approximately 4 volumes of medium DMEM (Dulbecco's modified Eagle's medium) and centrifuged again at 800rpm for 3min. The packed cells were re-suspended in a small volume of medium DMEM, counted in haemocytometer, and aliquots containing approximately $6x10^6$cells distributed in to T25 flasks.

Giemsa staining

EAT cells grown in 12-well plates ($5x10^5$ cells/well) were treated with EETISC for 2h. After washing once with phosphate-buffered saline, the cells were smeared on slides and fixed with methanol-acetic acid (3:1) and stained with 1% Geimsa for 5min. Excess stain was washed off with distilled water for 5min.Then slides were air dried and photographed using Leica inverted fluorescent microscope (Leica DM 1000).

Ethidium Bromide/ Acridine Orange Staining

Nuclear staining was performed according to the method described by Srinivas et al. [23] Briefly, EAT cells, either treated or untreated with EETISC solution for 16h in vitro, were smeared on a clean glass slide and fixed with methanol and acetic acid (3:1) and air-dried. The cells were hydrated with PBS and stained with a mixture (1:1) of acridine orange/ethidium bromide (4µg/mL) and/or Giemsa solutions. They were immediately washed with PBS and viewed under a Leitz-Diaplan fluorescent microscope for acridine orange/ethidium bromide staining.

In vitro cytotoxicity of EETISC assessed by Trypan blue dye exclusion assay

Mouse mammary carcinoma, EAT cells growing in exponential phase, were seeded in 6 well culture plates at 3×10^4 cells per well in 2ml complete DMEM medium. After 16h, the media was replaced with complete medium containing 50µl of EETISC to the respective wells. The negative control cells were vehicle (0.1% DMSO) treated and incubated for 16h at 37°C with 5% CO2 in humidified incubator. The semi adherent cells were harvested by centrifugation at 2500 x g for 5min. The cell pellet was then re-suspended in 100µl of PBS; 10µl of the cell suspension was stained with an equal volume of 0.4% trypan blue in 980µl of PBS and incubated for 2min at 37°C. The total number of viable cells was estimated by counting using a Neubauer Chamber.

DNA isolation and gel electrophoresis

EAT cells (5×10^6 cells) were suspended in basal DMEM media and either untreated or treated with (10, 20 and 25µM) different concentration of EETISC for 2h at 37^0C.The reactions were terminated using ice-cold HBSS and supernatant was removed by centrifugation. Cells were lysed in a lysis buffer containing 50mMTris–HCl, pH 8.0 and 0.5% SDS, and incubated for 30min at 37◦C. The cell lysate was subjected to 8M potassium acetate precipitation and left for 1h at 4°C.The supernatant was subjected to phenol/chloroform/ isoamyl alcohol (25:24:1) mixture, followed by chloroform extraction. DNA was precipitated by adding 1:2 volumes of ice-cold ethanol. The precipitated DNA was digested with 20µg/ml RNase at 37^0C for 30min. The DNA was quantified and resolved on 1.5% agarose gel, viewed under UV-light and documented using UV-Bio Doc system.

ANTIANGIOGENIC ACTIVITY

Chorioallantoic membrane (CAM) assay

The Chick Chorioallantoic Membrane (CAM) assay was done as described by Gururaj et al. 24]. The fertilized eggs were incubated at 37°C in a humidified and sterile atmosphere for 10 days. On the 11^{th} day a window was opened on the eggshell. PBS, VEGF (vascular endothelial growth factor) and the various concentration of EETISC (0-100µg) was placed on sterile discs and the discs were inverted over the CAM and immediately window was sealed. The eggs were incubated for about two days. On the day 13^{th} the window was opened and analyzed for the change in micro vessel density in the area below the discs and it was photographed using the Nikon digital camera.

TOXICITY STUDIES

Direct hemolytic activity

Direct hemolytic activity was determined by using washed human erythrocytes. Briefly, packed human erythrocytes and phosphate buffered saline (PBS) (1:9v/v) were mixed; 1ml of this suspension was incubated independently with the various concentration of EETISC (0-200µg) for 1h at 37°C. The reaction was stopped by adding 9ml of ice cold PBS and centrifuged at 1000g for 10min at 37°C. The amount of haemoglobin released in the supernatant was measured at 540nm used by (Thermo Scientific BioMate-6 UV-Vis). Activity was expressed as percent of haemolysis against 100% lysis of cells due to addition of water that served as positive control and phosphate buffered saline served as negative control.

Edema-inducing activity

The procedure of Sannanaik et al. [25] was followed. Groups of five mice were injected separately into the right foot pads with different doses (0–200µg) of EETISC in 20ml saline. The left foot pads received 20ml saline alone served as controls. After 1h, the mice were anesthetized by diethyl ether inhalation. The hind limbs were removed at the ankle joint and weighed. Weight increased was calculated as the edema ratio, which equals the weight of edematous leg_100/weight of normal leg. Minimum Edema Dose (MED) was defined as the amount of protein required to cause an edema ratio of 120%.

Hemorrhagic activity

Hemorrhagic activity was assayed as designated by Kondo et al. [32]. A different concentration of EETISC (0–200µg) was injected (intradermal) independently into the groups of five mice in 30µl saline. The cluster receiving saline alone serves as a negative control and the cluster receiving venom [2 minimum hemorrhagic doses (MHD)] as a positive control. After 3h, the mice were anesthetized by diethyl ether inhalation. Dorsal patch of the skin surface was carefully removed and observed for hemorrhage against saline-injected control mice. The diameter of the hemorrhagic spot on the inner surface of the skin was measured. Minimum hemorrhagic doses (MHD) were defined as

the amount of the protein producing 10mm of hemorrhage in diameter.

Table 1: Dose dependent effect of EETISC on clotting time of normal human plasma

EETISC (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
4	11.2±0.02	0.96±0.08	39.9±0.05	1.45±0.02
8	11.4±0.04	1.01±0.02	40.9±0.06	1.49±0.02
12	11.6±0.03	1.05±0.01	58.7±0.05	2.13±0.02
16	11.8±0.01	1.09±0.06	80.4±0.05	2.92±0.02
20	11.2±0.06	1.13±0.10	107.2±0.02	3.90±0.02
24	11.5±0.10	1.15±0.10	135.2±0.02	4.92±0.02
28	11.2±0.01	1.11±0.10	161.1±0.02	5.86±0.02
32	11.0±0.15	1.01±0.10	188.2±0.02	6.86±0.02
36	11.2±0.01	1.18±0.10	213.2±0.02	7.75±0.02
40	11.8±0.03	1.01±0.10	283.2±0.02	9.2±0.02

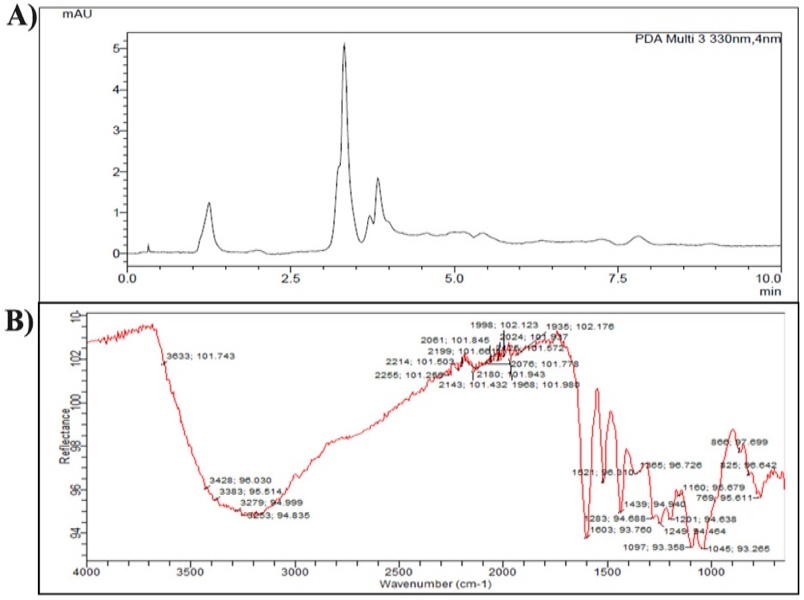

Fig.1 Represents RP-HPLC and FTIR profile of EETISC. **(A)** EETISC was subjected to RP-HPLC using C_{18} Column (5 mm, 0.21X25 cm) column that had been pre-equilibrated with Acetonitrile. The column was eluted using linear gradient from solvent A (HPLC water) and solvent B (Acetonitrile) for 60 min. The protein was eluted at a flow rate of 1 ml/min and monitored at 200-800 nm. **(B)** The FTIR (Fourier Transform Infrared) spectra of EETIS showcasing the probable functional groups of EETISC.

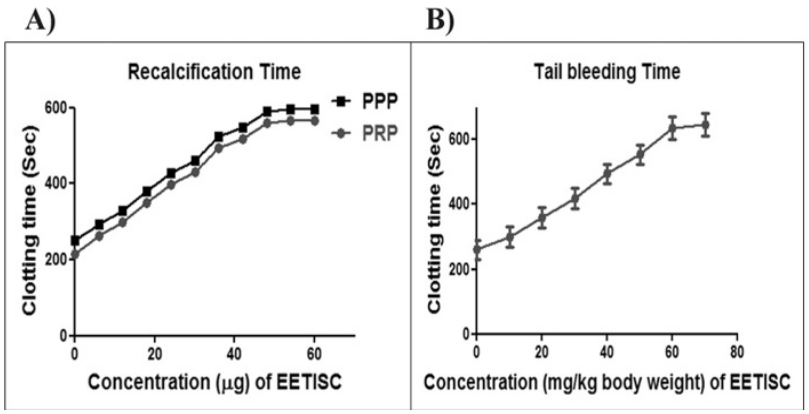

Fig.2 Represents the plasma recalcification time and Mouse tail bleeding assays. **(A)** EETISC (0–40µg) was pre-incubated with 0.2ml of citrated human plasma in the presence of 20µl 10Mmol/l Tris–HCl buffer (pH 7.4) for 1min at 37°C. Twenty microliter of 0.25mol/l $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded. Effect EETISC on the tail bleeding time. **(B)** Tail bleeding time was measured 10min after intravenous administration of PBS or various doses of EETISC. Each point represents the mean of SD of three independent experiments ($P<0.01$). Bleeding time longer than 800s was expressed as above 800s.

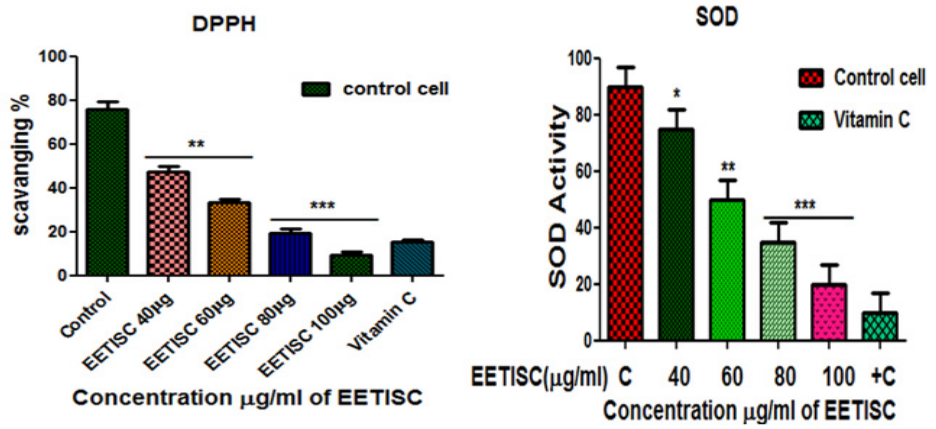

Fig.3 Represents the DPPH scavenging and inhibition of SOD by EETISC**. (A)** Dose dependent DPPH scavenging ability of EETISC. **(B)** a Superoxide radicals scavenging activity of EETISC**.**

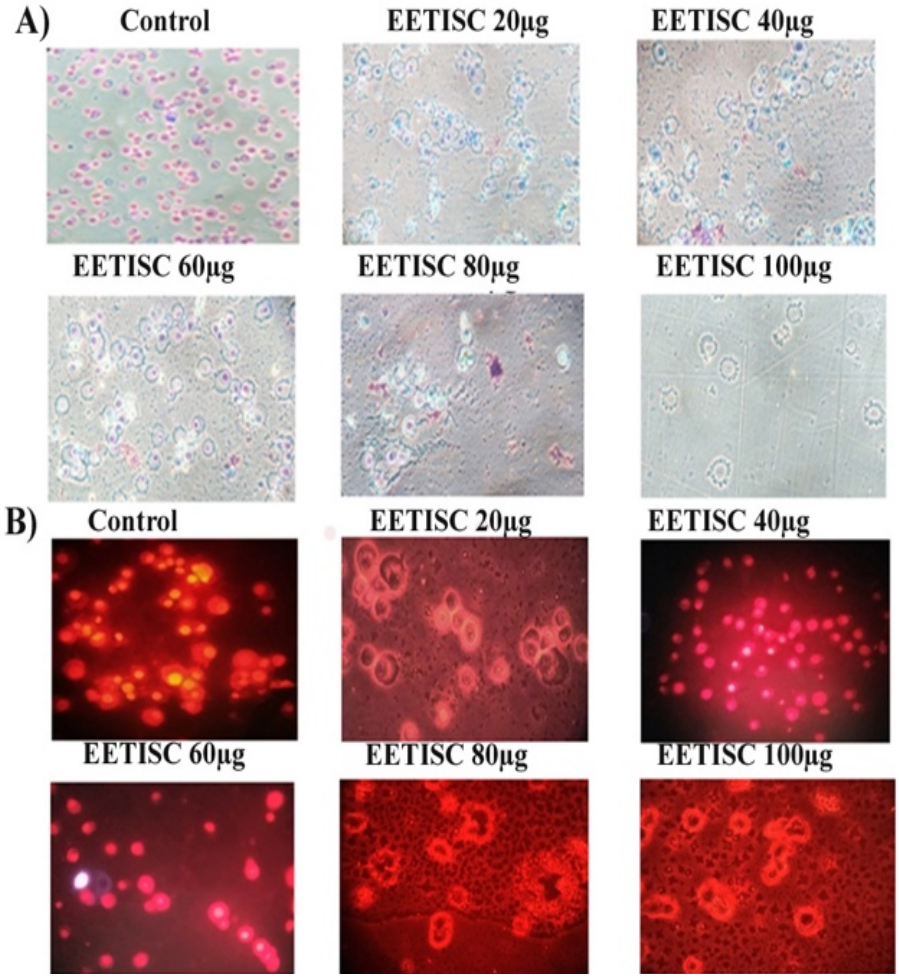

Fig.4 Anticancer activity of EETISC. **(A & B)** Dose dependent Effect of EETISC on Ehrlich ascites tumour (EAT) cell morphology: cells were treated with (0-100µg) of EETISC for 24h and photographed (phase contrast microscopy, 40×).

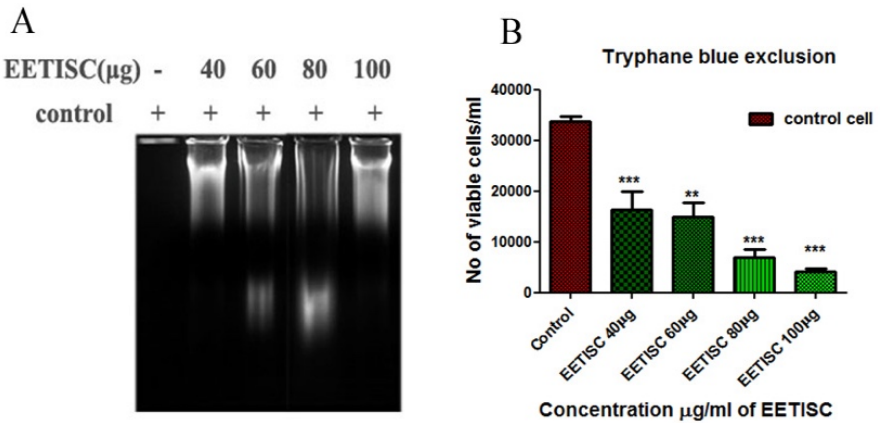

Fig.5(C) Effect of EETISC on DNA fragmentation of Ehrlich ascites tumour cells. DNA runs on 1% agarose gel electrophoresis. Lane1: DNA from control EAT cells, Lane2: DNA from EAT cells treated with 40µg of EETISC, Lane3: DNA from EAT cells treated with 60µg of EETISC, Lane4: DNA from EAT cells treated with 80µg of EETISC, Lane5: DNA from EAT cells treated with 100µg of EETISC. **D)** Cytotoxicity of EAT cells as assessed by Trypan blue dye exclusion assay: Cells ($3x10^4$) were treated with EETISC (40-100µg) along with respective positive and negative controls for 45min. The cells were harvested, washed with PBS and counted after adding trypan blue dye. The viable cells were counted, and the percentage viability was calculated. Data are presented as the mean ± SEM of three independent experiments; **P<0.01.

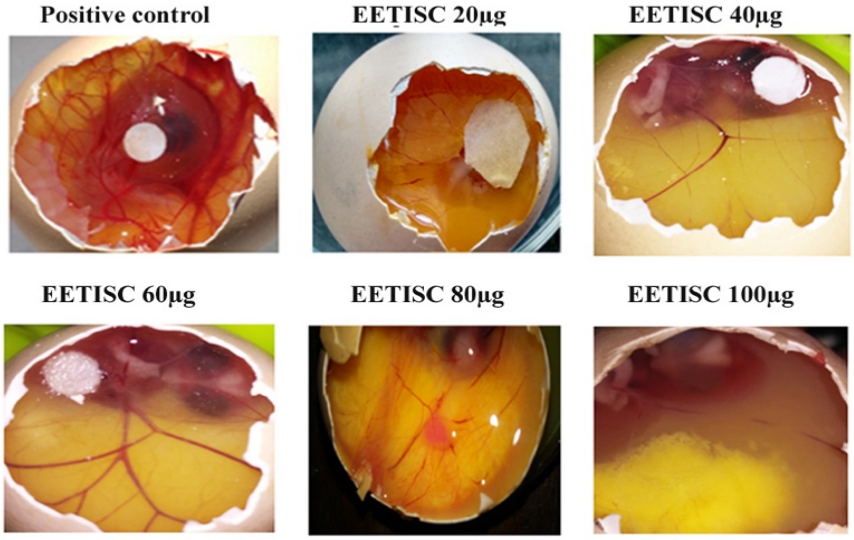

Fig.6 Dose dependent antiangiogenic activity of EETISC. **(A)** Chik embryo + VEGF (50 ng/ml posstive conrol) **(B)** Chik embryo + water (Negative control), **(C)** Chik embryo + VEGF+40 µg of EETISC, **(D)** VEGF+60 µg of EETISC, **(E)** Chik embryo + VEGF+80 µg of EETISC, **(F)** Chik embryo + VEGF+100 µg of EETISC.

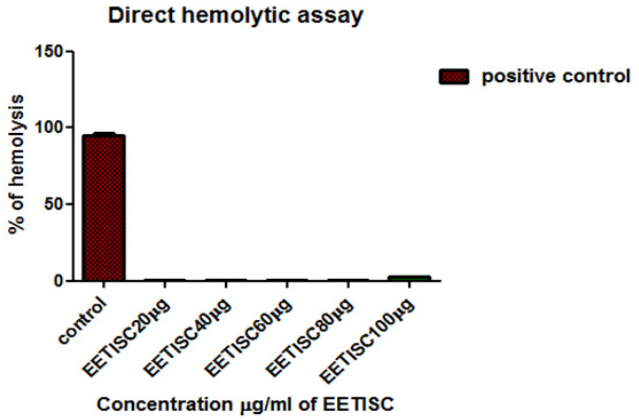

Fig.7 Effect of EETISC on RBC membrane and mice blood vessels **(A)** RBC cells treated with water (positive control) and RBC cells treated with EETISC (0-100µg)

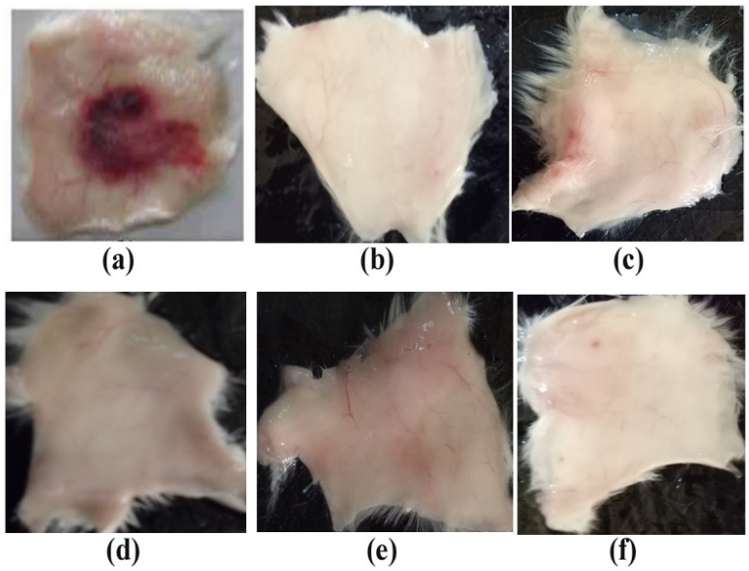

Fig.8 Dose-dependent haemorrhagic activity of EETISC. **(a)** Saline, **(b)** positive control 2 MDH venom**, (c)** 50µg**, (d)** 100µg, **(e)** 200µg of EETISC were injected independently into mice in a total volume of 50µl intradermal**.**

RESULTS

EETISC extract is rich in phytoconstituent

To identify the type of phytochemicals present in the EETISC extract, the sample was subjected to RP-HPLC- Photo Diode Array detector at 330nm using c18 column for 1h. The chromatogram of revealed two minor peaks and one major peak (Fig 1A). Suggesting the presence of bioactive components in the extract. While, the FTIR data of EETISC showed characteristic absorption bands at 3385 cm -1 and 1056 cm -1 (C-O) for a hydroxyl (-OH) group 2929 cm -1 (for C-H stretching), 1384 cm -1 (for C-H bending), 1707 cm -1, for an carbonyl group (C=O) and at 1622 cm -1 for C=C group (Fig 1 B). Suggesting the major functional group present in the EETISC are C-O, -OH, C-H,C=O and C=C.

EETISC exhibits strong anticoagulant activity

EETISC extract found to exhibit anticoagulant effect that was analyzed using both *in-vitro* (plasma recalcification time) and in-vivo (bleeding time) experiments. Interestingly, EETISC enhanced the clotting time of citrated human plasma from the control 225s and reached the extreme of 425s at the concentrations of 40µg and remained unaltered even at the increased dose up-to 20µg. (Fig. 2A) represents the dose-dependent anticoagulant activity of EETISC. The anticoagulant activity triggered by the EETISC was also authenticated by using in-*vivo* mouse tail bleeding assay. The intravenous injection of EETISC significantly prolonged the bleeding time in a dose-dependent fashion and the recorded bleeding time was more than 600s ($P<0.01$) at the concentration of 100mg against the PBS-treated control of 160.8s (Fig. 2B).

Furthermore, to identify the EETISC triggered anticoagulation could be due to its participation in intrinsic/extrinsic pathway. PT (Prothrombin Time) and APTT (Activated Partial Thromboplastin Time) assay was carried out. Interestingly, EETISC specifically prolonged the clotting time of only APTT, but it did not alter the clotting time of PT, suggesting its participation in intrinsic pathway of the blood coagulation cascade (Table 1).

EETISC is an effective scavenger of DPPH and superoxide radicals

EETISC showed DPPH radical scavenging activity in a dose dependent manner. The observed DPPH radical scavenging efficiency of EETISC extract at the concentration of 100µg/ml was found to be 58 % (Fig 3A) that could be compared with the positive control vitamin C a well-known antioxidant. The calculated IC50 value for EETISC and Vitamin C was found to be 25±1.02 µg/ml and IC50 of 0.80µg/mL respectively.

EAT cells treated with EETISC extracts showed significant decrease in SOD activity against the control cells (Fig 3B). EETISC stronly inihited the SOD activity and the maximum inhibition was found to be 80% at 80µg/ml. The extract was found to be an effective scavenger of superoxide radical generated by photo reduction of riboflavin. Inhibition of SOD causes accumulation of cellular O_2 and leads to free-radical-mediated damage to mitochondrial membranes, the release of cytochrome *c* from mitochondria and apoptosis of the cancer cells.

EETISC induces pro-apoptotic activity in EAT cells

EETISC found cause potent pro-apoptotic activity in the EAT tumour model. The cytotoxic effect of the EETISC was determined *in vitro* using EAT cells stained with Giemsa staining. The number of viable cells present in the cell suspension that was treated with EETISC extract was counted by differentiating the live cells from the dead ones. It is to state that a dose dependent decrease in viable cells was noticed in EETISC extract treated cells at the concentration of 100µg. The cytotoxic effect trigger by EETISC on EAT cells were further validated using dual staining by acridine orange and ethidium bromide under a fluorescent microscope. Interestingly, there was no significant morphological changes were observed in the control cells as most of them appeared green colour with intact nuclei (Fig 4A). However, the EAT cells treated with EETISC showed the early and late stages of apoptosis, manifested by the shrunken and crescent-shaped orange nuclei, membrane blebbing and apoptotic bodies containing fragmented nuclei (Fig 4B). The percentage of apoptotic cells was determined by counting the number of apoptotic cells under the microscope in ten random fields in

comparison to the vehicle treated control. The percentage of apoptotic induction triggered by EETISC was found to be 79% and 66% respectively, suggesting its anticancer potential (Fig 5B). EETISC triggered apoptosis was further authenticated using DNA fragmentation assay using agarose gel electrophoresis (Fig 5A). EETISC found to fragment the DNA in a dose dependent manner. EETISC at the concentration of 100µg generated the DNA fragmentation or lost chromosomal integrity in EAT cells. However, cells that were not treated with EETISC did not show any change in chromosomal integrity.

EETISC exhibits anti-angiogenic potential

EETISC strongly exhibited antiangiogenic potential by supressing the angiogenesis triggered by vascular endothelial growth factor (VEGF) in a dose dependent manner (Fig 6). When chick embryo was teated with VEGF with 50ng/ml, significant neovascularization was noticed. The neovascularized chick embryo was treated with various concentration of EETISC (0-100µg) and monitered for 11days. There was a significant decrease in the neovascularization was noticed at the concentration of 100µg.

Nontoxic properties of EETISC

EETISC was non-toxic to RBC as it did not lyse the RBC membrane that was compared with the positive control Triton X-100 (0.1%). In addition, it did not exhibit hemorrhage and edema in the experimental mice up to the concentration of 100mg, whereas positive control Daboia russelli venom induced hemorrhage and edema in experimental mice (Fig.7 & 8).

DISCUSSION

Belatedly, the tamarind seeds have been acknowledged much attention due to their immense therapeutic applications in managing several pathophysiological disorders such as, diabetes, snakebites, chronic diarrhoea, dysentery, jaundice, eye diseases, and ulcers [13, 14, 20]. In view of this, the current study reports on the anticoagulant, antioxidant and anticancer potential of Ethanolic Extract of *Tamarindus Indica* Seed Coat extract (EETISC). The chromatogram obtained from the EETISC on RPHPLC suggesting the presence of flavonoids. Flavonoids are important group of plant secondary metabolites found to contain antiviral, anticancer, anti-inflammatory and antiallergic activities [6, 13, 14, 20, 26]. The compounds such as Isorhamnetin, Kaempferol, Myricetin, Quercetin, Apigenin, Luteolin, Baicalein, Chrysin Eriodictyol, Hesperetin, and Naringenin were well characterized from various parts of the plants [20, 26]. FTIR (Fourier-transform infrared spectroscopy.) data showed that the presence of carbonyl group, hydroxyl group, ketone group is the functional groups in the extract that could be compared with the other known flavonoids [23, 27, 39, 40]. EETISC showed strong anticoagulant effect, specifically altered the clotting time of APTT but not PT suggesting that the anticoagulation induced by EETISC could be due to the participation in the intrinsic pathway of blood coagulation cascade. Haemostasis is a physiological phenomenon that involved in the arrest of bleeding due to injury [10, 18, 19]. It encompasses the participation of several coagulation factors those operates through the intrinsic and extrinsic pathways culminate in common pathway end up in the formation of fibrin clot [10-11]. In addition, activation of platelets also major contributing factors in forming a plug at the site of injury. Hence, hemostasis is tightly regulated pathway [28]. While, Environmental and genetic factors are directly/indirectly involved in the thrombosis. Thus, thrombosis is the pathological phenomenon involved in the formation of unusual clot in the arteries and veins. Thus, it increases cerebro and cardiovascular complications [1, 6-8, 10]. Mortality and morbidity rate has been tremendously increasing due to the secondary complications associated with the currently available antithrombotic agents. Thus, it could be better therapeutic candidate in treating thrombosis. The seed extract of Bitter gourd, Jackfruit, *Pisum sativum, Macrotyloma uniflorum* and flax seeds found to show anticoagulant effect [11, 12, 14,29,41] Reactive Oxygen Species (ROS) damages the biological systems and they are the major culprit in developing cardiovascular complications and cancer [1-4]. EETISC showed strong DPPH scavenging ability. DPPH a stable free radical having purple colour convert into yellow colour upon receiving an electron from an antioxidant. EETISC have the potential to scavenge the free radicals may be serve as a potent inhibitor of free radicals generated in response to oxidative stress. Superoxide dismutase (SOD) is a crucial enzyme accountable for the purging of superoxide radicals and is believed to be an important anti-oxidant in aerobic cells [3-5]. In view of the fact that, SOD is an important enzyme that play a pivotal role in the elimination of superoxide generated in the first metabolic step [7]. SOD Deficiency or its inhibition could be results in the accumulation of superoxide radicals in cells and cause their death [20, 21]. Therefore, SOD inhibition by natural compounds could afford a novel way to slaughter the cancer cells. EETISC significantly inhibited the SOD activity in EAT cells and SOD activity in normal cell is in control condition. Thus, it could be better agent to kill the cancer cells through a free radical mediated mechanism.

Furthermore, EETISC also showed pro-apoptotic potential on EAT cells as it was positive to Geimsa, Acridine orange- EtBr staining. An apoptosis is a phenomenon in which cell undergo programmed death that facilitates an organism to specifically remove unwanted/unhealthy cells in a controlled mechanism without harming a normal cell [23, 33]. Amelioration of apoptosis during the cancer progression is the crucial events, which encourage the cell proliferation in an abnormal way. Therefore, induction of apoptosis to the malignant cells paves the way to better development of the cancer treatment and prevention strategies. EETISC found to cause membrane blebbing, cell-shrinkage, chromosome condensation; nuclear fragmentation and aggregation of apoptotic bodies. Membrane blebbing and cell shrinkage are considered to be the key morphological alterations of early apoptotic cells. Moreover, EETISC caused the DNA fragmentation further strengthens its anticancer efficiency. Fragmentation of DNA, aggregation of apoptotic bodies are the final products of apoptotic event. Angiogenesis is the continuous process of growth of the blood vessels from the existing vasculature in both health and disease [27-30]. Thus, it is an essential event that helps capillaries in the tissues for the exchange of nutrients and metabolites. New growth in the vascular network play a crucial role in cell proliferation and metastatic progression depends on the influx supply of oxygen, nutrients and the removal of waste products [9, 22, 25, 34, 35]. Several anti-antigenic agents have been reported from the various research groups. Despite their beneficial efficiency the antiangiogenic effect trigger by them have been failed to increase the survival rate [24, 31, 36,]. Thus, the drug formulation that provides cumulative effect could be better therapeutic agent in managing the deadly diseases like cancer. EETISC inhibited VEGF induced angiogenesis in chick embryo [37, 38]. Thus, EETISC could be a better therapeutic agent to treat cancer as well. Above all, EETISC was devoid of toxicity as it did not cause edema, kept intact RBC membrane and blood vessels.

CONCLUSION

In conclusion, EETISC showed strong anticoagulant activity specifically interfering in intrinsic pathway of blood coagulation. Antioxidant property was proved in DPPH scavenging assay, further confirmed in, SOD activity. Anticancer effect of EETISC was confirmed in EAT cells apoptosis through DNA fragmentation and in additional EETISC revealed strong antiangiogenic potential. Hence from overall effects of EETISC proved to be a good candidate in the treatment regime of stress induced cardiovascular, cancer and its related complications.

ACKNOWLEDGMENTS

D.S and J.K thank the UGC, Government of India, New Delhi and Vision Group on Science and Technology, Government of Karnataka, Bangalore for financial assistance.

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Cite this article as:

Jayanna Kengaiah *et al*. Antioxidant, anti-cancer and anticoagulant properties of ethanolic extract of *Tamarindus indica* seed coat. Int. Res. J. Pharm. 2019;10(6):58-67 http://dx.doi.org/10.7897/2230-8407.1006204

Source of support: Department of Science and Technology Government of India New Delhi and Vision Group of Science and Technology Bangalore, India, Conflict of interest: None Declared