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Research Article

IN VITRO PLATELET AGGREGATION INHIBITORY EFFECT OF TRITERPENOID COMPOUND FROM THE LEAF OF *ELEPHANTOPUS SCABER* LINN

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Abstract

Elephantopus scaber Linn. (Compositae) has been widely used for the prevention and treatment of Bronchitis, Small pox, Diarrhoea, Brain-tronic. The different concentrations of Triterpenoid compound from this plant were investigated for the inhibitory effect on platelet aggregation invitro. The results indicated that the higher concentration (100µg/ml) Triterpenoid compound significantly inhibited thrombin induced platelet aggregation and the lower concentration (25 µg/ml) had little activity. The higher concentration Triterpenoid compound exhibited higher anti platelet activity than lower concentration with IC_{50} inhibition % ranging from 60% - 95%. The inhibitory mechanism of different concentration on analysis showed that the inhibition of extra cellular signal-regulated kinase (ERK 1/2) pathway may contribute to antiplatelet activity of Lupeol, Terpenoid compounds. These results indicating that the isolated triterpenoid compound has higher antiplatelet activity, therefore the consumption of *Elephantopus scaber* products may help to prevent or treat some cardiovascular disorders (i.e) thrombosis; but it must be used with caution by patients with bleeding disorders.

Key words: *Elephantopus scaber* Linn, Triterpenoid, Platelet aggregation, Antiplatelet activity, Lupeol

INTRODUCTION

Elephantopus scaber Linn. (Compositae) is a small perennial herb found in tropical conditions, almost throughout the world. Leaves are mostly in basal rosette and oblong-ovate to oblong-lance like, 10-25 cm in length and often very much notched on the margins. Flowers are Purple 8-10 mm long. Flowering heads borne in clusters at the end of the branches and usually enclosed by 3 leaf-like bracts which are ovate to oblong-ovate. The flowering heads many-crowded in each cluster. Fruits are achenes, ribbed. Tamil name is Aanaikalsuvati; In Indian traditional system of medicine, Siddha physician use the leaves for bronchitis, small pox, diarrhoea and braintronic. In the system of Chinese medicine this whole plant is used as a diuretic, antiviral and antibacterial agent as well as in the treatment of hepatitis, bronchitis, in cough associated with pneumonia [1]. In Taiwan folk Medicines these plant are used in the treatment of nephritis, edema, dampness, pain in the chest, fever and cough of pneumonia scabies and wound[2]. In Brazil traditional medicine the whole plant is used in the form of decoction to stimulate diuresis, reduce fever and to eliminate bladder stones[3]. The phytochemical of whole plant have been reported stigmasterol, germacranolide dilactone 11, 13 dihydrodeoxyelephantopin, elephantopin, epifriedelinol, epifriedelanol, scarbetopin[4].

Blood platelets are implicated in the haemostatic process and also in thrombus formation, which is one of the important contributors to pathogenesis of many thrombotic disorders, including hypertension, atherosclerosis and ischemic heart diseases[5, 6]. Thus, anti-platelet compounds have wide therapeutic potential for various circulatory diseases. Clinically, platelets play a pivotal role in initiating and sustaining the process of abrupt arterial occlusion. These studies have demonstrated that plaque rupture is not a necessary initiating component in platelet thrombus formation; consequently, shear forces appear to be the principal factor in initiating platelet activation. In vitro studies have confirmed that shear forces can directly activate platelets causing platelet aggregation and subsequent thrombosis[7].

MATERIALS AND METHODS

General chemicals

Anti-phospho p-44/42 MAP Kinase (Thr202/Tyr204) antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-mouse Ig, horseradish peroxidase linked whole antibody was from Amersham Biosciences (Buckinghamshire,UK). Thrombin and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA), and pentobarbital was obtained from Vetbutal (Polfa, Poland). All other reagents were of analytical grade.

Plant material

Leaves of *Elephantopus scaber* Linn. were collected from Gloris Biomed Research Centre Herbal garden, Chennai 600093, Tamil nadu, India.

Extraction and isolation of antioxidant compound

Dry leaves (3.0 kg) were soaked in Methanol for 24 h. The Methanol extract was fractioned by dry flash chromatography on silica gel using chloroform and methanol of increasing polarity, yielding 123X50 ml fractions which were reduced to triterpenoid after comparison by TLC.

Analysis of Isolated Compound

^{1}H NMR and ^{13}C NMR spectra were recorded at 399.952 and 100.577 MHz, respectively, on a Varian UNITY-400 spectrometer and with CDCl3 and $(CD_3)_2CO$ as solvent. The resonances of residual $CHCl_3$ and C_3H_6O at δ_H 7.25 and 2.04 and signals of $CDCl_3$ and $(CD_3)_2CO$ δc 77.0 and 206.0, respectively were used as internal reference for ^{1}H and ^{13}C spectra. Mass spectra were obtained using a VG 1250 or a Kratos MS- 80 RFA instrument at 70 eV. The IR spectra were recorded on a Bio-Rad FTS-7. Optical rotations were determined using a Perkin-Elmer polarimeter model 241 set on the sodium D line.

Animals

Male Wistar rats, weighing approximately 250–300 g, were supplied by Tamilnadu Veterinary & Animal Sciences University, Chennai-600 007, Tamil Nadu, Chennai. The animals had free access to food pellets (C.P. feed number 082) and tap water. They were kept in a room with controlled temperature (23±2 °C), humidity (50%), and light cycle (12-h light/12-h dark), and one animal per hanging cage. An acclimatization period of 7 days was allowed before experimentation.

Preparation of washed platelets

Rats were anesthetized by dose of sodium pentobarbital (50 mg/kg). Blood samples were taken from the heart and transferred into plastic tubes containing anticoagulant acid – citrate – dextrose (ACD) (75mM trisodium citrate, 38 mM citric acid and 138 mM glucose) in a volume ratio 6:1. The blood was centrifuged at 400Xg for 10 min to obtain Platelet rich plasma. The plasma was collected and again centrifuged at 800xg for 10 min. The platelets were washed with Tyrode buffer (136 mM NaCl, 2.7 mM KCl, 10 mM HEPES, 12 mM $NaHCO_3$, o.34 mM Na_2HPO_4, 1mM $MgCl_2$, 5.5mM Glucose) Containing 0.35% albumin and 10% ACD, pH 6.5. Washed platelets were then centrifuged at 800 xg for 10 min and resuspended in Tyrode buffer containing 0.1% albumin pH 7.4. Platelet number was counted by a coulter counter and adjusted to 3×108 cells/ml.

Assay of Platelet aggregation in vitro

Washed platelets were incubated with a range of tested compound on vehicle at 37°C for 15, 30 and 60 min. Aggregation experiment was done using lumi-aggregometer. To the Platelets in a silicone treated glass cuvet, aggregation was indeed using an agonist, thrombin with continuous stirring at 1000rpm, to a final volume of 250 µl. The reaction was then permitted to proceed for 4 min. The aggregation was expressed as the percentage of aggregation of the control values. In support antiplatelet experiments, the aggregation was performed using the threshold concentration of thrombin (0.1 U/ml). Threshold is defined as the lowest concentration of an agonist that evokes irreversible aggregation, with amplitude between 65% and 85% of the potential maximum deflection in light transmission.

Analysis of ERK phosphorylation in platelets

Washed rat platelets (3×108/ml) were incubated with different concentration of Triterpenoid 12.5, 25, 50 and 100 µg/ml or vehicle control (DMSO) for 1 h at 37 °C then thrombin (0.1 U/ml) under stirring for the 0.5, 1, 2, or 3 min. The platelets were dissolved in 2X Laemmli sample buffer (62.5 mM Tris–HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue) containing 5% 2-mercaptoethanol and then heated at 95 °C for 5 min. Proteins were separated in 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were washed with TBST (20 mM Tris-buffer saline 0.1 % Tween 20) for 5 min and then incubated in a blocking buffer (5% nonfat dry milk in TBST) for 1 h with agitation at room temperature. The membranes were incubated overnight with anti-phospho p-44/42 MAP Kinase (Thr202/Tyr204) antibody (Cell Signaling Technology; Beverly MA, USA) at 4 °C with gentle agitation. In the next day, blots were then incubated with anti-mouse Ig, horseradish peroxidase linked whole antibody (Amersham Biosciences, Buckinghamshire,UK) for 2 h at room temperature.

Statistical analysis

The means and standard errors of means were calculated for all experiment groups. The data were subjected to the Student's t-test and one-way analysis of variance followed by Duncan's multiple range tests to determine whether means were significantly different from each other or the control group.

RESULTS

Isolation of the in vitro platelet aggregation compound by Column chromatography from the leaves of *Elephantopus scaber*

The extraction of dry leaf of *Elephantopus scaber* with Methanol gave a crude extract which was chromatographed on a silica gel column (Flash chromatography) eluted with chloroform and methanol (9:1) mixtures of increasing polarity. Medium polar fractions yielded compound ES1 20mg. The Purity of isolated compound tested by Pre-coated TLC plates (60 F_2 54 Merck) and developed with a solvent system of hexane, chloroform and methanol in the ratio of 1:0.5:0.1 was efficient. The developed plate was viewed under UV 240nm and 360nm.

Identification and analysis of in vitro platelet aggregation compound from the leaf of *Elephantopus scaber*

Fraction eluted with 9:1 Chloroform: Methanol was purified repeatedly to field a while solid. The molecular formula was formulated by HR-EIMS at M/2 426-3855 ($C_{30}H_{500}$). While solid exhibited molecularation peak at M/2 426 and EI mass spectrum ion peaks at M/2 411 $[M\text{-}CH_3]^+$, 218 $[M\text{-}C_{14}H_{28}]$+, 207$[M\text{-}C_{16}H_{27}\}$+, which form pentacyclic triterpenes with an isopropenyl moiety structure when compared with Waller[9].

The IR spectrum showed absorption bands at 3400 cm-1 and 1640 cm-1 which are hydroxyl and olefinic functions.

In ^{1}H-NMR spectrum seven tertiary methyl's at δ 0.77, 0.79, 0.84, 0.97, 0.98, 1.04 and 1.69 appeared as singlets where as signals appeared at δ 1.69 which should allylic coupling (δ = 1.3112) with H-29. A pair of multiplets at δ 4.56 and 4.69 (1H each) showed the presence of terminal isopropenyl moiety. This revealed that the purified white solid belongs to the lupane class of triterpenoid.

The ^{13}C- NMR assignments of various carbons were substantiated by this experiment, which revealed the presence 15.2 ppm due to methyl protons and the remaining aliphatic carbons appeared between 22.7 to 45.1 ppm. Based on the both ^{1}H-NMR and ^{13}C- NMR characterization data the structure of the anti-platelet aggregation compound might suggest Triterpenoid [10, 11].

Anti-Platelet activity of different concentration of Triterpenoid compound from leaves of *E.Scaber*

Isolated compound of Triterpenoid lupeol from the leaf of *E. scaber* on platelet aggregation are exposed in (Fig 1). It was observed that different concentration of isolated Triterpenoid lupeol compound from the leaf of *E. scaber* showed platelet aggregation activity to range of (15.00 ± 9.28%, 35.56±18.421%, 57.25±18.22%, 85.23±13.23% and for 12.5, 25, 50 and 100µg/ml respectively). Higher concentration of Lupeol strongly inhibited platelet aggregation indeed by thrombin than the lower concentration with maximum inhibitory activity of 85.23% at 100 µg/ml.

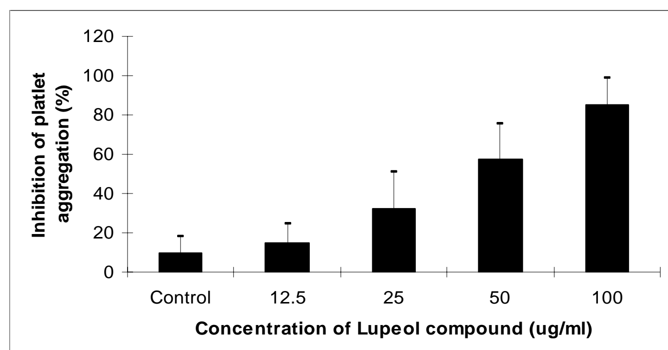

Fig. 1: Effect of different concentration of Triterpenoid compound from *E.Scaber* leaves on ERK Phosphorylation in platelets.

Thrombin induce the activation of extracellular signal regulated kinase (ERK 1/2)[8] and this activation has been involved in platelet aggregation. The effect of different concentration of triterpenoid compound of *E. Scaber* leaves showed that ERK_2 was not phosphorylated in resting platelets (Fig 2).But ERK_2 phosphorylation occurred in thrombin activation starting at 30s after thrombin activation and reached maximum at 2 min.

On investigation on effect of pre incubated isolated triterpenoid compounds on ERK phosphorylation in thrombin – activated platelet aggregation, the higher concentrated terpenoid compound (100 µg/ml) showed maximum inhibitory effect on ERK_2 than the lower concentration Triterpenoid compound.

The inhibitory effect of 100 µg/ml concentrated triterpenoid compound on ERK_2 phosphorylation was prolonged more than the lower concentration and was still observed from 1 to 3 min. This result was in similar to inhibition of platelet aggregation assay.

Washed rat platelets were pre incubated with different concentration of Triterpenoid (12.5 to 100µg/ml) or vehicle control (DMSO) for 60 min at 37°C and then stimulated with thrombin (0.1 U/ml) in the presence of 1 mM CaCl2 for 3mins with stirring.

Platelet lysates were analyzed by SDS- PAGE followed by western-blotting using antibody especially recognizing phosphorylated ERK1/2. Results are representative of 3 experiments.

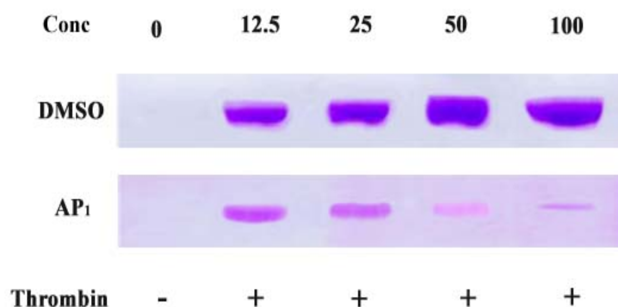

Fig. 2: Effect of the Triterpenoid Lupeol compound on the phosphorylation of ERK2.

DISCUSSION

Platelet aggregation causes major disease in Humans. The medical plant possesses variety of phytochemicals which help human from escaping from various diseases.

E. Scaber has been widely used in traditional system of medicine for the treatment of small pox, brachiates, diarrhoea, antiviral antibacterial diseases. In the present study we investigate on platelet aggregation inhibitory effect of Triterpenoid compounds isolated from *E. Scaber.*

The lower concentration (25 µg/ml) of Triterpenoid compound was found to be weak inhibitor and higher concentration (100 µg/ml) of Triterpenoid compound to be strong inhibitor this shows the antiplatelet activity of Triterpenoid compounds of *E. Scaber* leaves. Similar results was shown in Coumarin derivative compounds from roots of Angelica genuflexa[12] Stilbene derivatives from Rheum undu latum[13] also indicated that flavanoid and flavanoid-glucosides from saphare japonica have antiplatelet activity.

The IC_{50} value of Triterpenoid compounds of *E. Scaber* suggested that the highest concentration (100 µg/ml) strongly inhibited platelet aggregation (25 µg/ml) by only 70%.

In platelets ERK have been activated after stimulation by thrombin [8] also high Ca^{2+} level activate platelet[14]. The release of Ca^{2+} activates the ERK_2 for signaling in Platelets[15]. The higher concentration Triterpenoid of *E.Scaber* leaves act as antagonist to platelet aggregation by blocking calcium channel blocking. These results concluded that the platelets aggregation leading to thrombus formation play critical role in cardiovascular diseases[16]. The active triterpenoid compounds isolated from *E. Scaber* showed inhibitory action on platelet aggregation invitro and therefore can be used to treat at present in cardio vascular diseases and also suggest that antiplatelet aggregation inhibitory compound can be isolated and can be utilized as drugs for cardiovascular diseases.

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