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Antithrombotic potential of esculin 7, 3′, 4′, 5′, 6′-*O*-pentasulfate (EPS) for its role in thrombus reduction using rat thrombosis model

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ABSTRACT

Currently available anticoagulants for prevention and treatment of thrombosis have several limitations, thus, small organic scaffolds that can dissolve clots *in vivo* in a dose dependent manner with lesser side effects are highly desirable. Here we report the synthesis of esculin pentasulfate (EPS) and assessment of its *in vitro*, *in vivo* and *ex vivo* anticoagulant and antithrombotic potential. Assessment of *in vitro* clotting times showed prolonged activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) in the presence of EPS. EPS also showed remarkable reduction in thrombus formation when administered in occlusion induced thrombotic rats at a low dose (2.5 mg/kg). Further, assessment of clot rate with plasma isolated from EPS treated rats confirmed its anticoagulation potential. EPS at varying concentrations showed no significant cytotoxic effect on HEK293 cell line. Further, molecular docking analysis of EPS with known anticoagulant proteins [(antithrombin (ATIII) and heparin cofactor II (HCF II)] that require heparin revealed good binding affinity (−7.9 kcal/mol) with ATIII but not with HCF II. ATIII when incubated with EPS showed increased fluorescence intensity, with no change in secondary structure. Overall, our results clearly show the *in vivo* modulation of thrombus formation using a modified natural scaffold EPS.

1. Introduction

Under physiological conditions the anticoagulant, procoagulant, and fibrinolytic pathways together regulate hemostasis to avoid inadvertent clotting of blood due to endothelial injury, hyper-coagulability or hindrance in blood flow [1]. Until very recently, pharmacologic prophylaxis of venous thromboembolism was based on three types of anticoagulants, *i.e.* warfarin, unfractionated heparin, and low-molecular-weight heparins. These antithrombotic agents are multi-targeted, *i.e.* act on a number of coagulation factors like factor Xa (FXa), thrombin (IIa); new antithrombotic drugs selectively target one specific coagulation factor [2–10]. Heparin based anticoagulants have numerous limitations like frequent monitoring, dose response, drug-drug and diet-drug interactions, narrow therapeutic range along with purple toe syndrome, eclampsia, risk of haemorrhage and genetic polymorphisms [5,6,8,12]. Thus these major clinical drawbacks and other therapeutic shortcomings of these conventional anticoagulants indicate the need for better anticoagulant agents with better efficacy, improved safety, cost effectiveness and oral administration [2,3,5,9,11–17].

Attempts have been made previously to design synthetic sulfated molecules with an aim to mimic glycosoaminoglycans (GAGs) for possible modulation of GAG activated anticoagulant enzymes [18]. The currently employed antithrombotic therapy relies upon the derivatives of conventional anticoagulant heparin. The latter belongs to the group of GAGs including heparan sulfate, chondroitin sulfate, keratin sulfate, dermatan sulfate. Sulfate group (s) play critical role in interacting with target protein. GAGs are 50–500 residue long polymeric chain of hexamine-hexuronic acid. GAGs translate into numerous biological responses such as modulation of coagulation, angiogenesis, inflammation, cell growth and signaling, cell host defense and morphogenesis [18,19]. Small sulfated non-saccharide molecules possess anionic, aromatic, hydrophobic and hydrogen binding atmosphere which forces interactions with protein(s) resulting in specificity of action relative to parent GAGs. Moreover, the small sulfated non-saccharide can be easily synthesized in one step [20]. Further, in light of the available literature, the choice for sulfation of biological macromolecules has essentially emerged as a significant modification to improve anticoagulant property [18–22]. In many natural and synthetic anticoagulants it has been well established that sulfate moieties are involved in molecular interaction with serine protease inhibitors such as antithrombin and heparin Cofactor II [2,13,14,20, 23–26]. Many naturally occurring polyphenols like

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flavonoids, xanthones and phenolic acids are known to possess sulfate moieties and exhibit anticoagulant activity. Based on the evidences of antithrombotic effect of compounds with sulfated moieties, interest has increased to synthesize small scaffolds based polysulfated molecules with anticoagulant potential, It has also been reported that sulfated flavonoids activate antithrombin for accelerated inhibition of factor Xa [13,20,23,27,28]. Indeed, our lab previously reported the anticoagulant and antiplatelet effect of trehalose octasulfate [29]. These organic scaffolds are water soluble and easily metabolised, however, regardless of their novel features, activation achieved against heparin binding enzymes are rare and minimal [20,29]. More importantly the efficacy of sulfated compound for *in vivo* clot dissolution has not been tested and hence their targets and potential remains to be explored. Further, an *in vivo* thrombosis model may serve as a better predictor of anticoagulant and antithrombotic potential of any new compound [30–32].

Esculin, 6, 7-dihydroxycoumarin-6-*O*-β-glucoyranoside, is a coumarin derivative that occurs naturally in various plants including *Aesculus hippocastanum* and *Fraxinus rhynchophylla*. Esculin has been reported to possess multiple biological functions including antioxidant, antitumor, anti-inflammatory, antidiabetic, and antiapoptotic activities besides also having moderate *in vitro* anticoagulant effect [33–37]. Here, we aimed to do a comparative assessment of *in vitro* anticoagulant potential of sulfated esculin (EPS) and esculin (E), and assess if it translates into antithrombotic activity *in vivo* as well, in terms of clot dissolution.

We report the *in vitro* and *in vivo* anticoagulant property of EPS. The anticoagulant activity of EPS showed significant prolongation of APTT, PT and TT at micromolar (μM) range, indicating its promising role in delaying coagulation. Moreover, the *in vivo* antithrombotic effect of EPS at a low dose (2.5 mg/kg body weight of rat) demonstrated that intravenous administration of EPS significantly reduced thrombus formation in flow restriction induced rat model of venous thrombosis. Further, monitoring of clotting times in plasma samples isolated from rats infused with EPS prior to thrombus formation showed delayed APTT and PT in comparison to E (non-sulfated) in injected rats.

2. Materials and methods

Chemicals used were of analytical grade and procured from international standard commercial companies. E was purchased from MP-Biomedicals, USA. Triethylamine sulfur trioxide adduct and Dimethyl Acetamide (DMA) and solvents of HPLC were purchased from Sigma Aldrich, USA. Ammonium persulfate (APS), (TEMED), Bis-acrylamide were purchased from HiMedia Laboratories, India. Acrylamide was from Sisco Research Laboratories (SRL), sodium chloride; ethylene di amine tetra acetic acid (EDTA) and precoated aluminium sheets of Silica gel 60 F254 used for thin layer chromatography (TLC) was from Merck (India) Ltd. Syringe filters of pore size 0.45 μm and 0.22 μm were from Millipore Corporation. Whatman filter paper was from Whatman laboratories, England. Human plasma was procured from Rotary blood bank, Tughlakabad, New Delhi. Hi-Trap heparin columns were from GE Biosciences. Clotting time kits used for APTT (00597) and TT (00611) were from C.K. Prest, France, and PT kit (00667) was from Neoplastine C1 Plus. Dulbecco's modified Eagle's medium (DMEM), 0.25% trypsin, and 0.02% EDTA mixture were purchased from Hi Media (Mumbai, India). Fetal bovine serum (FBS) was obtained from Gibco (Grand Island, NY). The human embryonic kidney (HEK293) cell line was a gift from the National Centre for Cell Sciences (NCCS), Pune, India.

2.1. Synthesis of esculin pentasulfate (EPS)

Synthesis of EPS from a coumarin derivative E was achieved using previously reported method [29,38]. Adduct triethylamine sulfur trioxide was used for sulfation in dimethyl acetamide (DMA) as the solvent. To a solution of E (0.3583 mg, 10 mM) in DMA (15–20 ml) in a round bottom flask, 5.2 g triethylamine sulfur trioxide adduct (4–5 equivalent/OH, 32 mmol) was added and the suspension was stirred at 65 °C for overnight. The progress of the reaction was monitored by TLC After completion of the reaction, the reaction mixture was poured into 100 ml ice-cold acetone, maintaining the basic conditions by adding few drops of triethylamine and kept at 4 °C for 24 h. Further acetone was removed and crude oil was washed with acetone and diethyl ether. The retained oil was later dissolved in aqueous solution of 30% sodium acetate (5 ml). The suspension was added drop wise in ethanol to precipitate the sodium salt of sulfated derivative of E. The suspension was then stored overnight and semisolid product was lyophilized and stored in 4 °C for further structural characterization.

2.2. Analysis of EPS purity and structure

The purity of the synthesized EPS was assessed by TLC and high-pressure liquid chromatography (HPLC). EPS showed sharp single spot on TLC, distinct from its corresponding substrate, establishing the purity of EPS. In the HPLC analysis, the mobile phase consisted of ultra-pure milli Q water, acetonitrile and acetic acid in the ratio of 78:21:1 respectively. 20 μl sample was injected in a 20 μl loop and mobile phase was made to run isocratically for 35 min. Flow rate was maintained at 0.8 ml/min. UV detection was made at 220 nm. The major HPLC fractions were collected manually and were then lyophilized and characterized by spectral analysis. Melting point was measured on Buchi M-560 melting point instrument. Fourier transforms infrared spectroscopy (FTIR) of E and EPS were recorded on Agilent Cary 630 FTIR spectrometer to study the structural changes due to sulfation. To further characterize and confirm the structure, ^{1}H NMR spectroscopic measurements were performed on a Bruker AV-400 NMR spectrometer using tetramethylsilane (TMS) as internal reference. ^{1}NMR was carried out at 400 MHz using deuterated dimethyl sulfoxide-d_6 (DMSO-d_6) as the solvent for EPS.

Electro-spray ionization (ESI) mass spectra were recorded on Q star XL hybrid electrospray ionization high resolution mass spectrometer (Applied Biosystems). Electron ionization (EI) of EPS involves complex fragmentation as is the case with other flavonoids [29,39,40].

Structural characterization esculin 7, 3′, 4′, 5′, 6′-*O*-pentasulfate (EPS): Creamy white solid; Melting point: 210–230 °C; yield: 60%. FTIR (neat): 1722.76, 1627.34, 1506.09, 1398.44, 1045.50, 1015.37, 844.08, cm^{-1}; ^{1}H NMR (DMSO d_6) 7.86 (d,1H, Ar), 7.40(s,1H,Ar), 6.81 (s,1H,Ar), 6.24(d,1H,Ar), 5.04(s,1H,—CH), 4.78(d,1H,—CH), 4.62 (m,1H,—CH), 3.52(d,2H,—CH_2); ESI-MS (m/z): 677.89, 333.8, 453.8, 413.0, 703.

2.3. Cell proliferation assay

The cells were cultured and maintained as a monolayer in DMEM supplemented with 10% FBS and antibiotics (100 units per ml penicillin and 100 μg/ml streptomycin) at 37 °C under a humidified atmosphere of 5% CO_2in T-25 flasks. The cells were sub-cultured twice in a week.

Initially a cell count of approximately, 2×10^4 cells/well was seeded into flat bottom 96-well plates (150 μl/well) in triplicates, allowed to attach and grow. It was subsequently treated with varying concentrations of E and EPS ranging from 25 μg/ml to 400 μg/ml. After 48 h of treatment at 37 °C the medium was removed and cells were incubated with 20 μl of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml in PBS) in fresh medium for 4 h at 37 °C. Formazan crystals, formed by mitochondrial reduction of MTT [34], were solubilized in DMSO (150 μl/well) and quantification was performed by reading the absorbance at 540 nm after incubation period of 15 min on the iMark Microplate Reader (Bio-Rad). All analyses were performed in triplicates [41].

2.4. In vitro anticoagulant studies

Human blood was collected from healthy donors without any history of bleeding or thrombosis and who had not taken any medication