Journal of Ethnopharmacology 137 (2011) 893–901

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Gum resin of *Boswellia serrata* inhibited human monocytic (THP-1) cell activation and platelet aggregation

Praveen K. Kokkiripati[a], Lepakshi Md. Bhakshu[a,1], Swathi Marri[a,1], K. Padmasree[b], Anupama T. Row[c], Agepati S. Raghavendra[a], Sarada D. Tetali[a,*]

[a] Department of Plant Sciences, University of Hyderabad, Hyderabad 500046, India
[b] Department of Biotechnology, University of Hyderabad, Hyderabad 500046, India
[c] University Health Centre, University of Hyderabad, 500046, India

ARTICLE INFO

Article history:
Received 26 January 2011
Received in revised form 31 May 2011
Accepted 2 July 2011
Available online 8 July 2011

Keywords:
Anticoagulant
Antioxidant
Antiplatelet
Boswellia serrata gum resin
Monocytes
Śallakī

ABSTRACT

Ethnopharmacological relevance: Stem bark gum resin extract of *Boswellia serrata* is traditionally used in India for its hemostatic, antiinflammatory and cardiovascular health effects and it is named as Śallakī in Ayurvedic medicine.
Aim of the study: This study was conducted to evaluate the antioxidative and antithrombotic properties of stem bark gum resin extracts of *Boswellia serrata* (BS).
Materials and methods: The inhibitory activity of the BSWE and BSAE on $FeCl_3$ induced lipid peroxidation (*in vitro*) in rat liver and heart homogenates was measured spectrophotometrically. Their effect on H_2O_2 induced reactive oxygen species (ROS) generation in human monocytic (THP-1) cells was investigated by tracking intensity of a cell permeable fluorescent dye, H_2DCFDA and subjecting the cell samples to confocal microscopy. Further, the effect of BSAE and BSWE on ADP-induced platelet aggregation was assessed using a multimode detection plate reader, plasma coagulation times using an automated blood coagulation analyzer and on human blood clotting factors Xa and XIa using chromogenic substrate. Phytomarker analysis of the water (BSWE) and hydroalcoholic (BSAE) extracts of BS-gum resin was done through HPLC using a standard compound AKβBA.
Results: BSAE and BSWE inhibited, to varied extents, the lipid peroxidation in liver (80%) and heart (50%) tissue homogenates of male Wistar rats. Further, BSAE (30 μg dwt/mL) and BSWE (300 μg dwt/mL) attenuated ≥60% of H_2O_2 mediated ROS generation in THP-1 cells. In case of standard compounds, ascorbate (20 μg dwt/mL) and butylated hydroxytoluene (BHT) (10 μg dwt/mL) completely scavenged ROS in the cells. BSAE and BSWE at 3 mg dwt/mL completely inhibited ADP induced platelet aggregation and activities were comparable to 20 μg/mL of heparin. The extracts also showed very high activity in prolonging coagulation time periods. Both types of extracts extended prothrombin time (PT) from ~13 to >60 s and activated partial thromboplastin time (APTT) from ~32 s to >90 s. BSAE inhibited clotting factors Xa and XIa remarkably at 6 μg of dwt where as BSWE did not show much effect on FXa and showed 30% inhibition on FXIa at 120 μg. 10 μg of heparin was required to inhibit about 30% activity of the above factors. HPLC analyses suggested that BSAE and BSWE had AKβBA of 9% (w/w) and 7.8% (w/w) respectively.
Conclusion: Present study demonstrated antioxidant and antithrombotic anticoagulant activities of water and hydroalcoholic extracts of *Boswellia serrata*'s gum resin. We suggest that BS-gum resin as a good source for lead/therapeutic compounds possessing antioxidant, antiplatelet and anticoagulant activities.

Abbreviations: AKβBA, 3-acetyl-11-keto-β-boswellic acid; APTT, partial thromboplastin time; BHT, butylated hydroxytoluene; BSAE, hydroalcoholic extract of *Boswellia serrata*'s gum resin; BSWE, water extract of *Boswellia serrata*'s gum resin; H_2DCFDA, 2′,7′-dichlorofluorescin diacetate; PPP, platelet poor plasma; PRP, platelet rich plasma; PT, prothrombin time; ROS, reactive oxygen species; THP-1, human acute monocytic leukemia cell line.

* Corresponding author at: Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India. Tel.: +91 40 2313 4512; fax: +91 40 2301 0120.
E-mail addresses: sdtsl@uohyd.ernet.in, saradakanakagiri@gmail.com (S.D. Tetali).
[1] These authors contributed equally to the work.

0378-8741/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.jep.2011.07.004

1. Introduction

Extracts of *Boswellia serrata*'s stem bark gum resin (Śallakī or Salai guggal or Frankincense) are traditionally used in India (Ayurvedic/ethnomedicine) to treat various types of blood disorders, inflammatory health ailments, pain and cardiac debility (Paranjpe, 2001). There are several scientific reports on pharmacological activities of BS-gum resin. Gummy exudates of *Boswellia serrata*'s stem bark or its major constituents, boswellic acids have anti-inflammatory (Ammon, 2006; Shen and Lou, 2008), anticancerous (Aman et al., 2009) and anti-ulcerous (Singh et al., 2008) activities. Molecular targets for *Boswellia* extract in the inflammation pathway are identified and these include leukotrienes (inflammatory chemical messengers), 5-lipoxygenase, human leukocyte elastase, toposiomerase I and II, as well as IκB kinases (Poeckel and Werz, 2006).Clinical trials with *Boswellia serrata*'s gum resin extracts indicated its non-toxic nature (Arieh et al., 2010). A patented polyherbal formulation BHUx, containing gum resin of *Boswellia serrata* as one of the herbal components is formulated for treating atherosclerosis (Tripathi, 2009), a cardiovascular disease (CVD). Lately, CVD is recognized to develop as a result of complexed interactions between the processes of inflammation, oxidative stress and thrombosis (Ross, 1999; Boos and Lip, 2006). The objective of the present study is to investigate, if BS-gum resin extract can interrupt these complexed interactions and thus offer cardioprotective effect. To the best of our knowledge, there are no reports on antithrombotic properties of *Boswellia serrata*'s gum resin. Inhibition of platelet function represents a promising way for the prevention of thrombosis. Drugs with anticoagulant and antithrombotic effects, e.g. heparin, are among the primary drugs of choice, for the prevention of thromboembolic disorders. However, alternative drugs for heparin are in high demand due to the long-term side effects of heparin. Therefore, the objective of the present study is to evaluate antioxidant and antithrombotic activities using *in vitro* and cell based assays and to provide scientific basis for the traditional use of *Boswellia serrata*'s gum resin.

2. Materials and methods

2.1. Chemicals

Ascorbic acid, butylated hydroxytoulene (BHT) ferric chloride, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrezolium bromide (MTT), sodium acetate trihydrate, 2,4,6-tripyridyl-s-triazine (TPTZ) were from Hi-media, India. 3-Acetyl-11-keto-β-boswellic acid (AKβBA), 2,7-dichlorofluorescin diacetate (H_2DCFDA), ursolic acid, 2-thiobarbituric acid (TBA), and trypan blue were purchased from Sigma–Aldrich (Germany). RPMI 1640 medium, L-glutamine, and fetal bovine serum (FBS) were purchased from Invitrogen. Hydrogen peroxide (H_2O_2) obtained from Merck. Heparin purchased from Samarth Life Sciences (Mumbai). Human blood clotting factors Xa, XIa purchased from American Diagnostic Inc., Stanford. All other reagents used were of analytical grade.

2.2. Plant material and animal tissues

The voucher specimen (# 428) was identified by Dr. K. Madhava Chetty and deposited at Sri. Venkateshwara University Herbarium, Tirupati. Male Wistar rats were housed in ventilated cages and fed with pellet diet and water. Liver and heart tissues were kindly provided by Prof. P. Prakash Babu, Department of Biotechnology, University of Hyderabad.

2.3. Preparation of medicinal plant extracts.

Hydroalcoholic extract (BSAE) of gum resin was prepared by soaking dried gum powder in 80% ethanol followed by continuous stirring at 40 °C for 5–6 h. Water extract (BSWE) was prepared by soaking gum powder in water for overnight at room temperature. The extracts were centrifuged at 10,000 rpm for 10 min and subjected to various studies. BSAE or BSWE, obtained from 1 g of gum resin, was subjected to complete dryness under vacuum. Dry weight of the extract was determined to calculate the yield.

2.4. Phytochemical analysis of plant extracts

Preliminary phytochemical screening of the extracts showed positive reaction for triterpenoids, polyphenols and flavonoids. Using standard compound, i.e. one of the major boswellic acids, 3-acetyl-11-keto-β-boswellic acid (AKβBA) of 95% purity (Sigma), quantity of AKβBA in BSAE and BSWE has been quantitated by subjecting the extracts to HPLC analyses. The analysis was outsourced to Pharmatrain, Kukatpally, Hyderabad, methodology in brief is as follows. The instrument used for this analysis was Waters HPLC 2487 dual λ absorbance detector and 2695 separation module. The separation was performed on C_{18} 100 Å (250 mm × 4.6 mm, make: Waters) reverse phase analytical column. Compound was eluted using a mobile phase consisted of water (A) and acetonitrile (B), used in 35A and 65B in an isocratic mode at a flow-rate of 0.6 mL/min for 9 min. Absorption spectra and retention times were recorded at 210 with a UV detector connected to the HPLC system.

2.5. Cell culture

THP-1 human monocytes were purchased from National Centre for Cell Science (NCCS Pune, India) and cultured in RPMI 1640 medium (10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate) supplemented with 2 mM L-glutamine and 10% fetal bovine serum. Cells were maintained in 5% CO_2 and 95% air at 37 °C during growth and treatments.

Treatments with BSAE, BSWE or standard compounds were conducted at a cell density of ~0.5×10^6 cells/mL for 16–18 h. Vehicle (alcohol or water) concentration was limited to <1% of the cell culture volume. Cell viability was monitored after all the treatments using trypan blue exclusion and cell count was performed using an inverted microscope (Leica DMR) with the aid of a haemocytometer. MTT cell viability assay was performed according to manufacturer's protocol. Briefly, THP-1 cells were seeded in 24-well plates (2×10^5 cell per well). Cells were pre-treated with and without plant extracts or BHT for 24 h or with ascorbate for 1 h. After respective time periods of incubation, cells were washed twice with medium in order to remove trace amounts of extracts or standard compounds. Similarly, wells with media without cells were processed. MTT (5 mg/mL) was added to all the wells with or without cells and incubated for 3 h. The converted MTT dye was solubilised with 0.04 N HCl in isopropanol. Absorbance of the dye was measured at a wavelength of 570 nm with background subtraction at 690 nm. Media without cells with respective concentrations of extracts or standard compounds were taken as blanks.

2.6. Lipid peroxidation assay

2.6.1. Liver tissue homogenate lipid peroxidation

The peroxide formation was monitored according to the method of Gaurav et al. (2007) by measuring the colour of thiobarbituric acid reactive substances (TBARS) formed at the end of the reaction. The reaction mixture contained rat liver homogenate, 1 mM ferric chloride and various concentrations of BSAE/BSWE. Lipid peroxidation was initiated by adding 100 μl of 1 mM fer-

ric chloride and incubated for 30 min at 37 °C. The reaction was terminated by addition of 2 mL of ice cold HCl (0.25 N) containing 15% trichloroacetic acid (TCA), 0.38% TBA to the reaction mixture followed by heating at 80 °C for 60 min. The samples were then cooled and centrifuged at 5000 × *g* for 15 min, and the absorbance of supernatants was measured at 532 nm against the blank. Identical experiments were performed to determine the normal and induced lipid peroxidation. The protective effects of different extracts against lipid peroxidation were calculated as follows: % Inhibition = (control − sample/control) × 100. Ascorbic acid was used as a reference compound.

2.6.2. Heart tissue homogenate lipid peroxidation

This assay was performed according to the method reported by Meera et al. (2009). The reaction volume composed of 50 μL heart homogenate, 10 mM ferric chloride, and various concentrations of BSAE/BSWE. Buffer blank was prepared w/o ferric chloride. All the tubes were incubated at 37 °C for 1 h. After incubation, 500 μL of 70% ethanol was added to all the tubes to arrest the reaction. TBA (1%, 1 mL) was added to all the tubes followed by boiling in water bath for 20 min. After cooling to room temperature the tubes were centrifuged to clear the solution and the supernatants collected. To the supernatants, 50 μL of acetone was added. TBARS were measured at 532 nm using a spectrophotometer. An assay medium corresponding to 100% oxidation was considered by adding tissue homogenate, ferric chloride, without BSAE/BSWE. Ascorbic acid was used as a reference compound.

2.7. Measurement of ROS generated by H_2O_2 in THP-1 monocyte cell line using H_2DCFDA

Intracellular ROS were measured by using cell permeable fluorescent dye, H_2DCFDA according to Evgeniy et al. (2010). THP-1 cells were seeded in 24-well plates and pre-treated with or without different concentrations of BSAE/BSWE for overnight at 37 °C in a humidified atmosphere containing 5% CO_2. After incubation, wells were loaded with 5 μM H_2DCFDA and incubated for 30 min at 37 °C. Then the cells were washed twice with the growth medium to ensure the removal of unbound dye as well as plant extract in the medium containing the cells. After washing, cells were exposed to 10 μM H_2O_2 for 10 min. Images were obtained by subjecting the cells to confocal laser-scanning electron microscopy using excitation and emission wavelengths at 488 nm and 525 nm respectively. The quantitation of fluorescence intensities of the cell samples was done by spectrofluorimetry.

2.8. Assay of ROS scavenging potential in THP-1 cells

ROS scavenging assays were done in THP-1 cell lysates after the treatments done as detailed in Section 2.5. Cells were washed twice prior to exposure with H_2O_2 to avoid direct interaction between plant extracts and H_2O_2. After two washes, cells were exposed to H_2O_2 and then sonicated, thus obtained cell lysates were centrifuged at 10,000 rpm for 2 min. Supernatants were subjected to the following assays.

2.8.1. Catalase assay

Catalase (CAT) enzyme assay was done according to the method of Prasenjit et al. (2007). 7.5 mM H_2O_2 was added to cell-lysates and the decrease in absorbance at 240 nm was monitored at 37 °C using a multimode microplate reader for about 5 min and absorbance was recorded at an interval of 10 s. CAT activity was expressed in fold change which reduced 1 μmol of H_2O_2 per min at 25 °C.

2.8.2. Ferric reducing/antioxidant power (FRAP) assay

The FRAP assay was done according to the method of Prasenjit et al. (2007), with cell lysates by measuring the changes in absorbance at 593 nm. 1.5 mL of freshly prepared and prewarmed (37 °C) FRAP reagent (300 mM acetate buffer, pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ in the ratio of 10:1:1) was added to THP-1 cell lysates and incubated at 37 °C for 10 min. Blue colored Fe^{II}-tripyridyltriazine compound formed from the colorless oxidized Fe^{III} form by the action of electron donating antioxidants was assayed. The absorbance of the sample was read against reagent blank (1.5 mL FRAP reagent + 50 μL distilled water) at 593 nm.

2.9. Antiplatelet aggregation assay

Platelet aggregation activity was determined according to Mary et al. (2003). 10 mL of blood was obtained from normal healthy volunteers. Platelet rich plasma (PRP) was collected after centrifugation of blood at 200 × *g* for 5 min. 1.5 mL of acid citrate dextrose (ACD) was used as anticoagulant for every 8.5 mL of blood. Platelet poor plasma (PPP) collected by centrifugation (1500 × *g* for 5 min) was kept as reference. PRP with or without different concentrations of BSAE or BSWE or heparin were added to microwell plate. Aggregation of platelets in PRP was initiated by adding ADP to a final concentration of 20 μM. Absorbance of the samples was recorded at 600 nm using multimode detection plate reader preset at 37 °C for a time period of 12 min at an interval of 2 min. Commercial heparin (20 μg/mL) was used as reference compound.

2.10. Assay plasma clotting times

Prothrombin time (PT) and activated partial thromboplastin time (APTT) assays belong to the class of diagnostic tests were done to assess the function of coagulation system. APTT measures contact activation (intrinsic) pathway and PT measures tissue factor (extrinsic) pathway and initiated by release of tissue factor. To assay the effect of BSAE/BSWE on extrinsic and intrinsic pathways of coagulation, PT and APTT time periods of normal human plasma were compared in the presence of BSAE/BSWE. These reactions were carried out using kit UNIPLASTIN obtained from Tulip Diagnostics (Goa, India) by using the instrument MC100 single channel coagulation analyzer. Samples were outsourced commercially to analyse at Regional Diagnostic Centre, Hyderabad. The protocol followed was briefly as follows: 0.9 mL of blood sample was transferred into a 0.109 M trisodium citrate (1:9, v/v) and then centrifuged at 1800 × *g* for 10 min to obtain plasma. 100 μL of the plasma were mixed with different concentrations of BSAE/BSWE and the coagulation was started by addition of $CaCl_2$, 100 μL of thromboplastin and thrombin added to the incubated plasma for PT and APTT assays individually.

2.11. Clotting factor assays

Human clotting factors were assayed according to Robert et al. (2010). Incubations were performed in 96-well plates. The final concentration of the reactants included 70 ng of factor Xa or 100 ng of factor XIa and different concentrations of test sample in 100 μL of 50 mM Tris, pH 8.3 containing 5 mM calcium chloride and 0.2 mM sodium chloride. Factor Xa/XIa was added last to initiate the reaction. After 60-s of incubation at room temperature, 0.8 mM of chromogenic peptide substrate (CH3OCO-D-CHA-Gly-Arg-pNA-AcOH) for the above factor was added, and the absorbance at 405 nm was recorded for 5 min. Heparin was used as positive drug control.

2.12. Statistical analysis

All data obtained were analyzed by one way analysis of variance (ANOVA) test using Statistical Package for the Life Sciences (SPLS version 11). All results were expressed as mean ± standard deviation of mean (S.D.). $p < 0.001$ was considered to be statistically highly significant.

3. Results

3.1. Plant extracts

Hydroalcoholic extracts yielded 4.8% (w/w) and water extracts yielded 5.2% (w/w) based on their starting material of dried gum resin. The phytochemical screening of gum resin showed positive reaction for polyphenols, flavonoids and triterpenoids. BSAE contained 0.647 ± 0.09 UAE mg/mg dwt; 22.5 ± 1.6 GAE μg/mg dwt and 2.29 ± 1.4 QE μg/mg dwt. Whereas BSWE contained 0.271 ± 0.026 UAE mg/mg dwt, 79.2 ± 2.8 GAE μg/mg dwt and 6.35 ± 3 QE μg/mg dwt. In summary hydroalcoholic extracts had higher amounts of total triterpenoids and water extracts had higher amounts of TPC and flavonoids.

3.2. Quantititation of AKβBA

Under the experimental conditions, retention time of standard compound AKβBA was 4.784 min (Fig. 1B). HPLC analyses with BSAE and BSWE showed peaks corresponding to standard AKβBA with retention times of 4.623 and 4.616 respectively (Fig. 1C). Calculations with corresponding peak areas determined that AKβBA content of BSAE to 9% (w/w) and BSWE to 7.8% (w/w) of their extracts' dwt.

To test the linearity of the compound, we used standard solutions of AKβBA in the range from 10 μg/mL to 120 μg/mL. 20 μL of these standard solutions were injected for each assay, which correspond to 200–2400 ng. Least amount of detection and quantification under the experimental conditions was 2.69 (± 0.17) μg/mL and 9.08 (± 0.58) μg/mL respectively. Each sample was measured in triplicate (Fig. 1A).

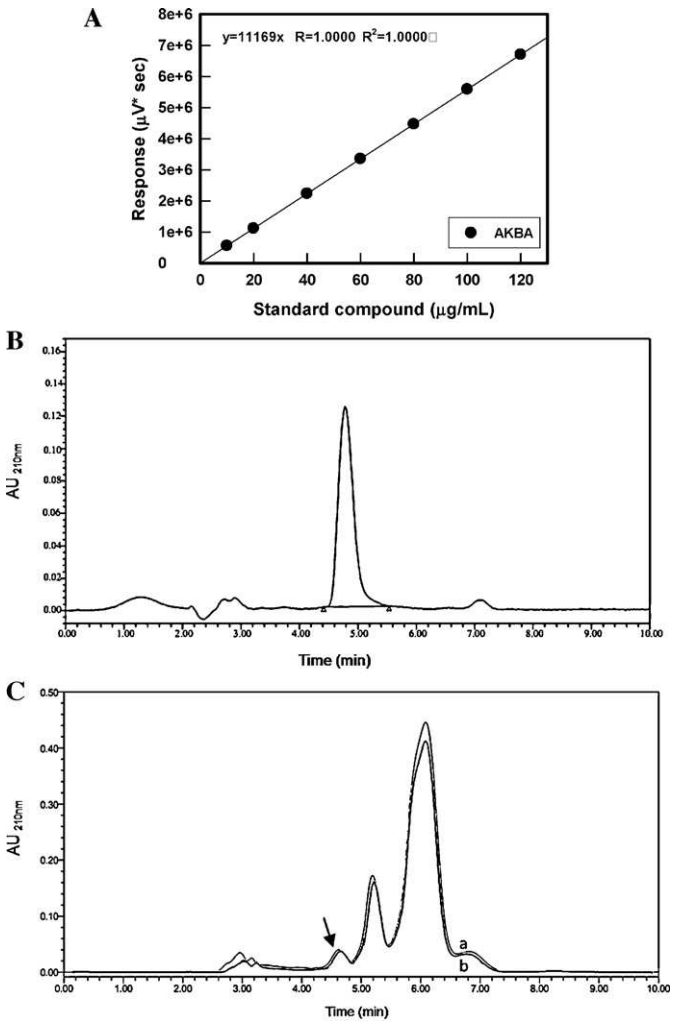

Fig. 1. HPLC analysis for quantification of AKβBA in plant extracts. (A) Regression plot with varied concentrations of AKβBA ranging from 20 to 120 μg/mL. LOD and LOQ of AKβBA are 2.69 ± 0.17 μg/mL and 9.08 ± 0.58 μg/mL. (B) An example chromatogram of standard compound AKβBA showing a peak with the retention time period of 4.784 min. (C) Example chromatograms of BSAE (a) and BSWE (b), with marked peak, corresponding to standard AKβBA with retention times of 4.623 min and 4.616 min respectively. Peak areas were used to determine the AKβBA content in the extracts.

3.3. Lipid peroxidation inhibition activity of BSAE/BSWE

BSWE inhibited $FeCl_3$ induced peroxidation in liver homogenates by 70% where as BSAE showed maximum inhibition of only 40% (Fig. 2A). In case of heart homogenate, both types of extracts inhibited maximum of 50% lipid peroxidation (Fig. 2B). The concentration of the BSWE needed for 50% inhibition in liver homogenate was 34 μg dwt/mL. In case of heart homogenate, IC_{50} of BSAE and BSWE were 129 μg dwt/mL and 88 μg dwt/mL respectively. Extracts showed lower effect in inhibiting peroxidation in heart tissue homogenates from 200 μg dwt/mL onwards. IC_{50} values for standard compound ascorbate were 38 μg/mL and 153 μg/mL for lipid peroxidation in liver and heart tissues respectively. Ascorbate showed maximum inhibition of 80% lipid peroxidation in liver tissue but only 55% inhibition with heart tissue. Thus our results indicated that water extracts of BS-gum resin were more potential than standard compound ascorbate in suppressing lipid peroxidation of both rat's liver and heart homogenates.

3.4. Cell viability

Dosage of plant extracts or standard compounds for THP-1 cell treatments were determined based on cell viability experiments (Fig. 3). At the used concentrations of plant extracts or standard compounds for the present study, no cell death was found, >95% cells were alive (Fig. 3). In case of standard compounds, there was no cell death found after treatments with ascorbate up to 50 μg/mL for 1 h time period (Fig. 3A) with BHT up to 10 μg/mL for 24 h time period (Fig. 3B). Cell death was not found after treatments with BSAE up to 40 μg dwt/mL (Fig. 3C) and up to 400 μg dwt/mL of BSWE (Fig. 3D). Therefore, we restricted our dosage of standard compounds and plant extracts to the maximum of above cited concentrations.

3.5. Attenuation of H_2O_2 induced ROS by BSAE/BSWE in THP-1 Cells

We evaluated the potential of BS-extracts in scavenging intra cellular ROS in human monocytic cell line using a cell permeable ROS sensitive fluorescent marker H_2DCFDA. THP-1 cells treated with 10 μM H_2O_2 for 10 min showed much brighter fluorescence compared to cells not treated with H_2O_2 (Fig. 4A). THP-1 cells treated with 10 μM H_2O_2 for 10 min showed 4.5 folds greater fluorescence compared to cells not treated with H_2O_2 (Fig. 4B). Pretreatment of cells with BSAE (30–40 μg dwt/mL) or BSWE (300–400 μg dwt/mL) markedly attenuated H_2O_2-dependent flu-

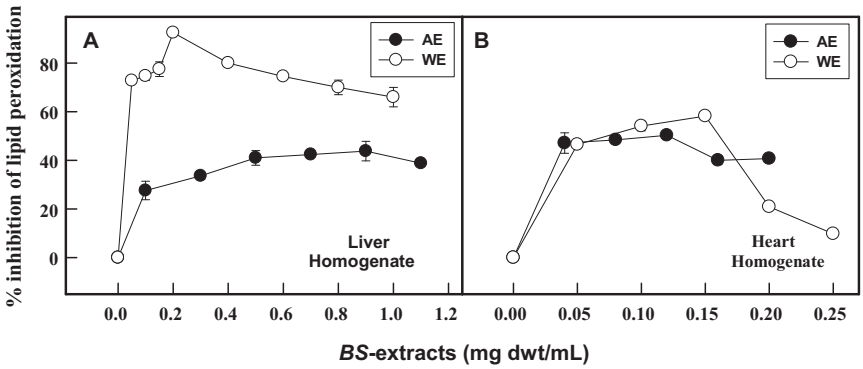

Fig. 2. Effect of BS-gum resin on ferric chloride induced lipid peroxidation on (A) liver and (B) heart tissue homogenates of rat. Data presented are mean ± S.D., $n = 6$. S.D. not seen where S.D. values are within the symbol of data point.

orescence increase with statistical significance of p value <0.001. IC_{50} of BSAE (0.011 mg dwt/mL) is much lower than IC_{50} of BSWE (0.08 mg dwt/mL), which reflects that alcoholic extract was highly potential in scavenging intra cellular ROS. Washing H_2DCFDA dyed cells twice with medium prior to the exposure of cells to H_2O_2 ensured not only the removal of excess fluorescent dye, but also removal of BS-extracts from the medium, otherwise there is a possibility of extracts scavenging H_2O_2 extracellularly in the medium. Positive drug controls, ascorbic acid at 20 μg/mL and BHT at 10 μg/mL completely scavenged intracellular ROS (Fig. 4B).

3.6. Effect of BSAE/BSWE on scavenging mechanisms of cellular ROS

To elucidate the mechanism offered by plant extracts in scavenging intracellular ROS, effect of BSAE/BSWE treatments (concentrations, from Fig. 4B, at which the extracts were effective in scavenging intracellular ROS), cells were treated similarly and subjected to cellular catalase enzyme and total reducing power assays.

On exposure of cells to H_2O_2 for a short period, catalase activity was increased by 40% (Fig. 5A) in THP-1 cells. Whereas, catalase activity was increased by two fold in the cells treated with BSWE at 300 μg dwt/mL, with a statistical significance of p value <0.001. BSAE treated cells showed an increase in the enzyme activity by only 40%. Ascorbate (20 μg/mL) increased catalase activity by two folds but BHT (10 μg/mL) inhibited the enzyme by 50% (Fig. 5A).

Total reducing power of the THP-1 cells was rapidly declined by 70% upon treatment with H_2O_2 (Fig. 5B). Plant extracts or standard compound treated cells maintained cellular reducing power even after treatment with H_2O_2 (Fig. 5B). BSAE at 30 μg dwt/mL sustained cellular reducing power by 80%, BSWE at 300 μg dwt/mL by 70%, standard compounds, ascorbate (20 μg/mL) by 60% and BHT

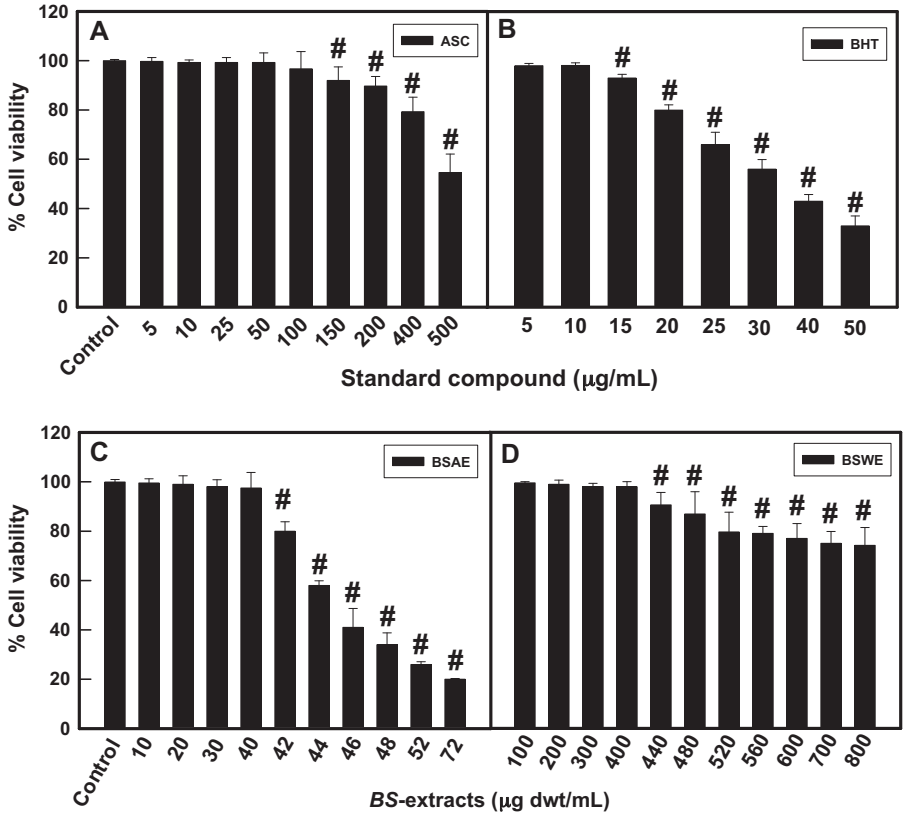

Fig. 3. Cell viability was checked by MTT assay after treatment with (A) ascorbate for 1 h, (B) BHT for 24 h, (C) BSAE for 24 h, (D) BSWE for 24 h, at 37 °C and 5% CO_2. Media without cells but with respective concentrations of standard compounds or plant extracts were used as appropriate blanks. Data presented are mean ± S.D., $n = 6$.

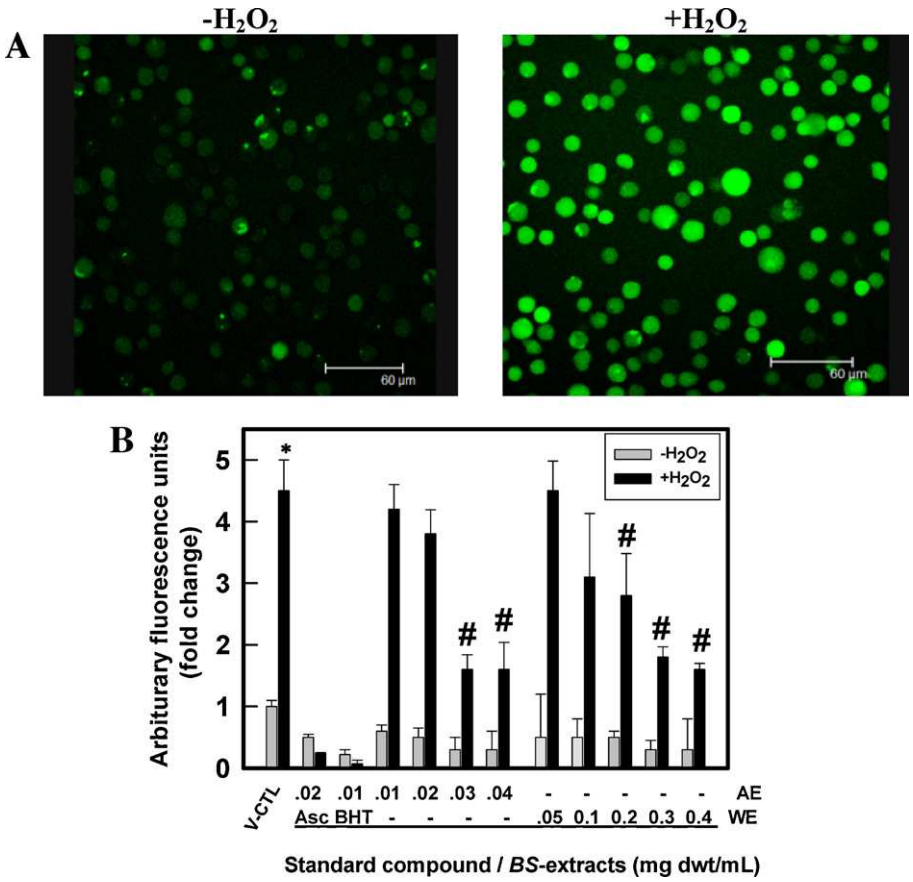

Fig. 4. Effect of BS-gum resin extracts on H_2O_2 induced ROS generation in THP-1 cells. (A) Confocal images: left panel – control cells – H_2O_2; right panel – cells + H_2O_2. ROS species were detected by cell permeable fluorescent dye, H_2DCFDA, and cells were observed with a fluorescence microscope. (B) Fluorescence intensity of H_2DCFDA taken by the cells treated as above was measured by spectrofluorimetry at excitation wavelength of 488 nm and emission wavelength of 525 nm. One-way ANOVA test was performed between the experimental groups represent mean ± S.D. ($n = 4$), *statistical significance of $p < 0.001$ within the control groups i.e. cells + H_2O_2 *vs* cells – H_2O_2, #statistical significance of $p < 0.001$ for groups of cells treated with H_2O_2 in the presence of BS-extracts *vs* in the absence of BS-extracts.

(10 μg/mL) by 100% that of H_2O_2 untreated cells. These results suggest that the selected plant extracts help the cells in maintaining their total reducing power under oxidative stress conditions and thus help in scavenging excess ROS.

3.7. Antiplatelet aggregation and anticoagulant activity of BSAE/BSWE

Addition of 20 μM ADP to human platelets caused the decrease in absorbance units indicating the aggregation of platelets (Fig. 6). In the present study heparin was used as positive drug control, which inhibited ADP-induced platelet aggregation as shown in Fig. 6. BSAE and BSWE markedly inhibited the platelet aggregation in a concentration dependent manner (Fig. 6). Both types of extracts BSAE and BSWE (3 mg dwt/mL) showed high antiplatelet aggregatory activity with p value <0.001 (Fig. 6). Activity of the extracts was comparable to heparin. Thus our results demonstrated antiplatelet aggregatory effect of BS gum resin.

Blood coagulation system, not only initiated by complex coagulation pathways but also involves the interactions between platelets and plasma factors. In this regard, APTT is used to evaluate intrinsic clotting index and PT for extrinsic index, these tests used to identify the coagulation risk factors.

The concentration-dependent effects of BSAE and BSWE on the PT and APTT clot times of human plasma are shown in Fig. 7. Both BSAE and BSWE showed very potent activity in prolongation of clot times. BSAE and BSWE prolonged APTT time period by 2.6 fold and PT time period by 4 folds. Thus our data on prolongation of both PT and APTT clotting times by both types of BS extracts clearly demonstrate anticoagulant property of BS-gum resin.

In order to know target sites of BSAE or BSWE in clotting cascade pathway, we tested the effect of extracts on human clotting factors Xa and XIa. BSAE inhibited both the factors significantly ($p < 0.001$). BSAE at 6 and 8 μg dwt/mL inhibited 70% of FXa (Fig. 8A) and 55% of FXIa (Fig. 8B). BSWE did not show inhibitory effect on FXa and showed about 25% inhibition on FXIa. Heparin showed about 35% on both the factors (Fig. 8A and B).

4. Discussion

The interest in natural plant products is on a steep rise due to their increased use of traditional medicine to treat chronic metabolic diseases (Mukherjee et al., 2010). *Boswellia serrata*'s gum resin is one such plant used in Indian Ayurvedic and folk medicine to treat blood disorders, curtail inflammatory diseases like rheumatoid arthritis and to promote cardiac health (Clarisse et al., 2008; Arieh et al., 2010). Present study is designed to investigate the mechanism by which BS-gum resin offers cardiac health. The link between the oxidative stress, inflammation and thrombosis leading to cardiovascular disease is well established (Ross, 1999; Libby, 2002; Boos and Lip, 2006). Therefore, antioxidant and antithrombotic properties of BS-gum resin have been investigated.

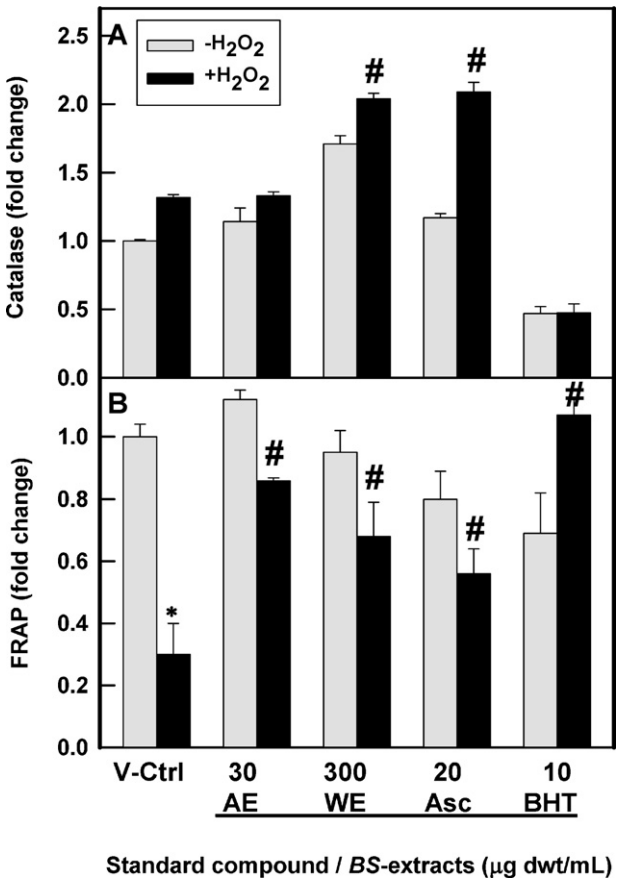

Fig. 5. (A) Effect of BS-extracts on catalase enzyme activity in THP-1 cells exposed to H_2O_2. Ascorbic acid and BHT serve as positive drug control. (B) FRAP assay performed with cells pretreated with BS-gum resin and induced with H_2O_2. Data represented mean ± S.D., $n = 4$; *statistical significance of $p < 0.001$ within the control groups i.e. cells + H_2O_2 *vs* cells − H_2O_2, #statistical significance of $p < 0.001$ for groups of cells treated with H_2O_2 in the presence of BS-extracts *vs* in the absence of BS-extracts.

4.1. Antioxidant activity of BS-gum resin

The enrichment of BS-gum resin with triterpenoids (Assimopoulou et al., 2005; Bushra et al., 2007; Magesh et al., 2008) implies their antioxidant activity based on its chemical composition but direct line of evidences for such antioxidant activity was not available. Investigating antioxidant activity of the BS-gum resin during our experiments, BSAE and BSWE showed anti-lipid peroxidation activity when checked with liver and heart

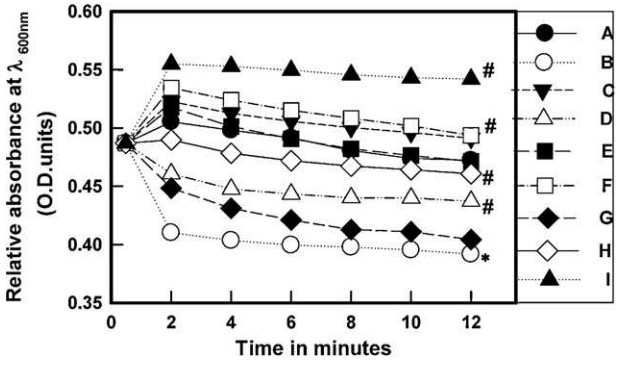

Fig. 6. Effect of BS-extracts on ADP induced aggregation of human platelets. (A) PRP; (B) PRP + ADP (20 μM); (C) PRP + Hep (20 μg/mL) + ADP; (D) PRP + BSAE (1 mg dwt/mL) + ADP; (E) PRP + BSAE (2 mg dwt/mL) + ADP; (F) PRP + BSAE (3 mg dwt/mL) + ADP; (G) PRP + BSWE (1 mg dwt/mL) + ADP; (H) PRP + BSWE (2 mg dwt/mL) + ADP; (I) PRP + BSWE (3 mg dwt/mL) + ADP. Data presented are mean ± S.D., $n = 3$. S.D. not seen where S.D. values are within the symbol of data point. #$p < 0.001$ between platelets treated with BS extracts and ADP *vs* treated with ADP alone, *$p < 0.001$ indicates comparison between PRP + ADP *vs* PRP alone.

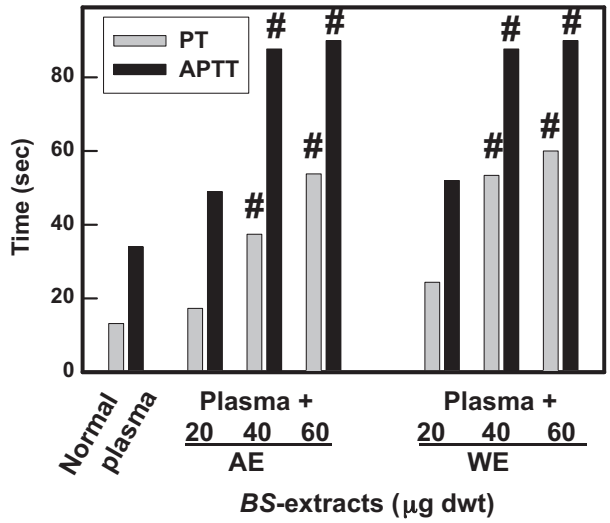

Fig. 7. Effects of BS-gum resin on clotting PT and APTT time periods. Data presented are mean ± S.D., $n = 3$. S.D not seen where S.D. values are within the symbol of data point. Statistically significant values are indicated by # with $p < 0.001$.

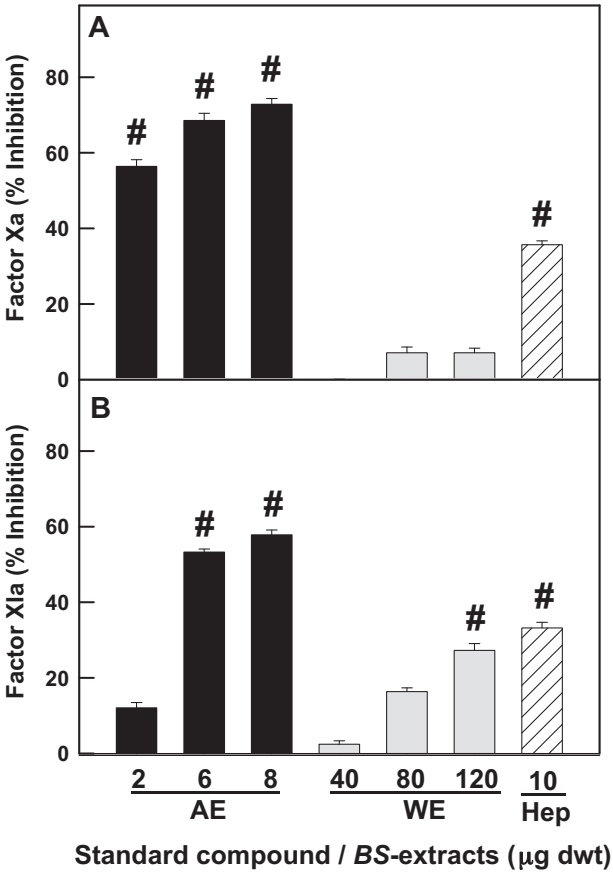

Fig. 8. % Inhibitory effect of BS-extracts or heparin on (A) factor Xa and (B) factor XIa. Clotting assays were conducted by monitoring decrease in the absorbance of chromogenic substrate using microplate reader. Values in the bar graphs represent mean ± S.D. ($n = 4$), #$p < 0.001$ highly significant compared with control.

homogenates. These extracts were also capable of scavenging ROS in human monocytic (THP-1) cells (Fig. 4B). Since activated monocytes promote inflammatory cascade of events (Ross, 1999) leading to atherosclerosis. The cellular ROS scavenging activity offered by BSAE and BSWE may help in attenuating the above outlined cascade of events.

BSAE did not show significant effect on catalase where as BSWE significantly increased cellular catalase activity in Thp-1 cells (Fig. 5A). BSAE and BSWE helped Thp-1 cells in sustaining their cellular reducing power to great extent, even on exposure to H_2O_2 (Fig. 5B). Therefore, we think that BSAE and BSWE scavenge excess ROS generated by oxidative stress by modulating either cellular reducing power or catalase enzyme activity.

The reasons for higher potential of BSAE (IC_{50}: 0.011 mg dwt/mL), that of BSWE (IC_{50}: 0.08 mg dwt/mL) in terms of ROS scavenging, cannot be explained based on the analyzed phytomarker, AKβBA. BSAE and BSWE contained AKβBA of 9% (w/w) and 7.8% (w/W) respectively. Detailed chemical composition analyses of these extracts may help in knowing other active metabolites.

4.2. Antithrombotic potential of BS-extracts

To further elucidate antiatherogenic potential of BS-gum resin, antiplatelet aggregation and anticoagulant activities of the extracts were examined. Platelets of the blood play an important role in the process of hemostasis. Platelet activation is essential to perform many of their functions, however, activated platelets also has tendency to stick to each other and form aggregates leading to thrombosis and clot formation in the vessel and thus contributes to cardiovascular disease and stroke (Ross, 1999; Boos and Lip, 2006). Inhibition of platelet aggregation and enhancing coagulation time can help to a great extent in the management of atherosclerosis (Ross, 1999). Alternative drugs to heparin are of great interest in view of its limitations and allergic problems (Henry et al., 2009). Therefore, efforts on identifying factor Xa inhibitors (Pinto Donald et al., 2010) and screening herbal resources possessing significant antithrombotic activity with minimal side effects are highly essential (Winston, 1999; Kim et al., 2010). To the best of our knowledge, there are no scientific studies reported on antithrombotic effect of BS-gum resin. In our present study, both BSAE and BSWE inhibited platelet aggregation (Fig. 6). We used ADP to induce platelet aggregation since it is an important endogenous aggregating agent involved in thrombus formation (Park et al., 2004). BSAE and BSWE at 3 mg dwt/mL showed stronger effect in inhibiting platelet aggregation and are as potential as positive drug control, heparin.

Both BSAE and BSWE also enhanced PT and APTT coagulation time periods (Fig. 7). Blood coagulation is not only the result of a complex process initiated by the intrinsic system or the extrinsic system and or/a common pathway, but also a highly regulated process involving interaction between platelets, plasma coagulation factors, and the vessel wall. Anticoagulant drugs are screened using PT and APTT tests (Arif et al., 2007). BSAE at 57 μg dwt and BSWE at 40 μg dwt exhibited maximum of 2.65 fold increase in clotting time period of APTT. The same concentrations increased clotting time periods of PT by four fold. The above concentrations of BSAE significantly inhibited FXa and FXIa and this could be the possible mechanism by which it enhanced the clotting time of both PT and APTT. Where as BSWE did not inhibit FXa but inhibited FXIa by 25%. Mechanism of enhancing PT time period by BSWE needs to be elucidated. Detailed analysis of these extracts on various factors and enzymes involved in clotting cascade may reveal the mechanism of action.

5. Concluding remarks

Conclusively, our results indicated that both water and hydroalcoholic extracts of *Boswellia serrata*'s gum resin contain high amounts of AKβBA and other boswellic acids, also contain significant levels of phenolic compounds, which may be responsible for its exhibited high antioxidant and antithrombotic activity. BSAE and BSWE showed cellular ROS scavenging, antiplatelet aggregation and anticoagulation activities. With all these shown wide spectrum of activities of phytomedicine, BS-gum resin can be considered as an effective antiatherogenic resource for preventing coronary artery diseases and may serve as a good source for isolating lead compounds of antiplatelet and anticoagulant therapeutics.

Acknowledgements

We are grateful to Prof. P. Prakash Babu, Department of Biotechnology for providing rat liver and heart tissue samples and Dr. Madhava Chetty, Department of Botany, S.V.U. Tirupathi for providing plant material. We are thankful to Ms. Nalini Manthapuram, CIL for her assistance in collecting confocal images. We are also thankful to Pharma Train, Hyderabad for their timely help on HPLC analyses. This part of the work is supported by DBT (BT/PR/10972/GBD/27/123/2008) and UGC (37/532/2010 SR) research projects of Sarada D. Tetali. Dr. D.S. Kothari F.4-2/2006(BSR)/13-252/2009(BSR) dated 15/02/2010 of Dr. L. Md. Bhakshu. We are thankful to DBT-CREBB, DBT-FIST and UGC-SAP for supporting infra structural facilities of Department of Plant Sciences and School of Life Sciences.

References

Aman, U., Balu, G., 2009. Pharmacological activities of *Boswellia serrata* Roxb—mini review. Ethnobotanical Leaflets 13, 766–774.
Ammon, H.P.T., 2006. Boswellic acid in chronic inflammatory diseases. Planta Medica 72, 1100–1166.
Arieh, M., Raphael, M., 2010. *Boswellia* resin: from religious ceremonies to medical uses: a review of *in-vitro*, *in-vivo* and clinical trials. Journal of Pharmacy and Pharmacology 61, 1281–1293.
Arif, H.K., Ayalew, T., Rajiv, K.P., 2007. How to interpret and pursue an abnormal prothrombin time activated partial thromboplastin time, and bleeding time in adults. Mayo Clinic Proceedings 82, 864–873.
Assimopoulou, A.N., Zlatanos, S.N., Papageorgiou, V.P., 2005. Antioxidant activity of natural resins and bioactive triterpenes in oil substrates. Food Chemistry 92, 721–727.
Bushra, S., Farooq, A., Roman, P., 2007. Antioxidant activity of phenolic components present in barks of *Azadirachta indica*, *Terminalia arjuna*, *Acacia nilotica*, and *Eugenia jambolana* Lam. Trees Food chemistry 104, 1106–1114.
Boos, C.J., Lip, G.Y.H., 2006. Blood clotting inflammation and thrombosis in cardiovascular events: perspectives. Frontiers in Bioscience 11, 328–336.
Clarisse, C., Ludivine, B., Eric, B., Corinne, C., Daniel, S.A., Felicitas, G., Berhold, B., Tatiana, S., Thomas, S., Mustapha, R., 2008. Antiinflammatory and antiatherogenic effects of the NF-kB inhibitor acetyl-11-keto-β-boswellic acid in LPS-challenged ApoE−/− mice. Arteriosclerosis, Thrombosis, and Vascular Biology 28, 272–277.
Evgeniy, E., Sergei, K., 2010. Identification of ROS using oxidized DCFDA and flow-cytometry. Advanced Protocols in Oxidative Stress II, Methods in Molecular Biology, 594.
Gaurav, V.H., Chandragauda, R.P., Mahesh, R.P., 2007. Protective effect of *Kalanchoe pinnata* pers (Crassulaceae) on gentamicin-induced nephrotoxicity in rats. Indian Journal of Pharmacology 39, 201–205.
Henry, B.L., Jay, N.T., Erika, J.M., Donald, F.B., Umesh, R.D., 2009. Characterization of the plasma and blood anticoagulant potential of structurally and mechanistically novel oligomers of 4-hydroxycinnamic acids. Blood Coagulation and Fibrinolysis 20, 27–34.
Kim, S.Y., Koo, Y.K., Koo, J.Y., Ngoc, T.M., Kang, S.S., Bae, K.H., Kim, Y.S., Yun-Choi, H.S., 2010. Platelet anti-aggregation activities of compounds from *Cinnamomum cassia*. Journal of Medicinal Food 13, 1069–1074.
Libby, P., 2002. Inflammation in atherosclerosis. Nature 420, 868–874.
Meera, R., Devi, P., Kameswari, B., Madhumitha, B., Merlin, N.J., 2009. Antioxidant and hepatoprotective activities of *Ocimum basilicum* Linn. and *Trigonella foenum-graecum* Linn. against H_2O_2 and CCl_4 induced hepatotoxicity in goat liver. Indian Journal of Experimental Biology 47, 584–590.
Magesh, V., Raman, D., Pudupalayam, K.T., 2008. Genotoxicity studies of dry extract of *Boswellia serrata*. Tropical Journal of Pharmaceutical Research 7, 1129–1135.

Mary, K., Achuthan, C.R., Babu, B.H., Padikkala, J., 2003. *In vitro* antioxidant and antithrombotic activity of *Hemidesmus indicus* (L) R.Br. Journal of Ethnopharmacology 87, 187–191.

Mukherjee, P.K., Venkatesh, P., Venkatesh, M., Ponnusankar, S., Khan, Y., 2010. Strategies for revitalization of traditional medicine. Chinese Herbal Medicines 2, 1–15.

Pinto Donald, J.P., Smallheer Joanne, M., Cheney Daniel, L., Knabb Robert, M., Wexler Ruth, R., 2010. Factor Xa inhibitors: next generation antithrombotic agents. Journal of Medicinal Chemistry 53, 6243–6274.

Prasenjit, M., Mahua, S., Prasantha, P., Parames, C.S., 2007. Arjunolic acid, a triterpenoid saponin, ameliorates arsenic induced cyto-toxicity in hepatocytes. Chemico-Biological Interactions 170, 187–200.

Paranjpe, P., 2001. Śallakī – *Boswellia serrata*. In: Indian Medicinal Plants – Forgotten Healers: A Guide to Ayurvedic Herbal Medicine. Chaukhamba Sanskrit Pratishthan Publishers, Delhi, pp. 233–234.

Park, W.H., Ahn, J.C., Kim, H., Lee, Y.C., Kim, C.H., 2004. Effects of Korean herbal formulation Silsosangami, consisting of seven medical herbs and its seven herbs on endotoxin induced experimental thrombosis in rats. Phytotherapy Research 18, 224–229.

Poeckel, D., Werz, O., 2006. Boswellic acids: biological actions and molecular targets. Current Medicinal Chemistry 13, 3359–3369.

Robert, S., Baccelli, C., Devel, P., Michel Donge, J., Quetin-Leclercq, J., 2010. Effects of leaf extracts from *Croton zambesicus* Muell. Arg. on hemostasis. Journal of Ethanopharmacology 128, 641–648.

Ross, R., 1999. Atheroscelerosis—an inflammatory disease. New England Journal of Medicine 340, 115–126.

Shen, T., Lou, H., 2008. Bioactive constituents of myrrh and frankincense, two simultaneously prescribed gum resins in Chinese traditional medicine. Chemistry and Biodiversity 5, 540–553.

Singh, S., Khajuria, A., Taneja, S.C., Khajuria, R.K., Singh, J., Johri, R.K., Qazi, G.N., 2008. The gastric ulcer protective effect of boswellic acids, a leukotriene inhibitor from *Boswellia serrata*, in rats. Phytomedicine 15, 408–415.

Tripathi, Y.B., 2009. BHUx: a patented polyherbal formulation to prevent hyperlipidemia and atherosclerosis. Recent patents on inflammation allergy and drug. Discovery 3, 49–57.

Winston, J.C., 1999. Health-promoting properties of common herbs. American Journal of Clinical Nutrition 70, 491S–499S.