

Full Length Research Paper

Bioactivity of the lanostane-type of triterpenoid from *Protorhus longifolia* stem bark

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This work evaluated the anticoagulant and anti-inflammatory activity of a lanosteryl triterpene (3 β -hydroxy-28-norlanosta-9,24-dien-21-oic acid) isolated from *Protorhus longifolia* stem bark. Tail bleeding time assay was used to investigate the *ex vivo* anticoagulant activity. The effect of the triterpene on the thermally induced aggregation of malate dehydrogenase (MDH) and citrate synthase (CS) was studied. The anti-inflammatory activity of the triterpene was investigated using the cotton pellet-induced granuloma model in rats. Granuloma formation was measured following 7 days of oral administration of the experimental rats (two groups) with the triterpene at 50 and 250 mg/kg body weight (b.w). The compound (50 mg/kg) significantly ($p < 0.05$) increased bleeding time in rats by up to 7 min as compared to 2.5 min observed in the normal control group. It also improved the activity of Hsp70 on MDH and CS aggregation suppression. The reduction of the granuloma formation by up to 40.3% was observed. It is apparent that the triterpene has potential to inhibit the aggregation of proteins.

Key words: Triterpene, anti-inflammatory activity, *Protorhus longifolia*, protein aggregation.

INTRODUCTION

Thromboembolic disorders are the leading cause of stroke, myocardial infarction, and pulmonary embolism. These disorders contribute to over 20% of annual deaths globally (Cohen et al., 2011). There is an established link between blood coagulation and inflammation (Verhamme and Hoylaerts, 2009; Chu, 2011). This linkage is observed under both pathophysiological and physiological conditions. Upon endothelial injury, inflammation and blood coagulation become

autocatalytic. Inflammation suppresses the natural anticoagulant system, which favors the expression of prothrombogenic molecules (Verhamme and Hoylaerts, 2009). Furthermore, inflammation has also been linked with most chronic illnesses including diabetes and cardiovascular diseases (Aggarwal et al., 2006). Anticoagulants and anti-inflammatory compounds are thus, important in the management of thromboembolic disorders (Hirsh et al., 2007). The reported (Khanapure et

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al., 2007; Mavrakanas et al., 2011) undesirable side-effects of the current anticoagulant and anti-inflammatory agents have stimulated the search for new generation of more effective agents from natural sources.

Medicinal plants are reservoirs of bioactive compounds vital to human health. Their extracts provide a useful source of biologically active compounds which can be used as novel structural templates or be directly developed as active agents (Kee et al., 2008). Natural products identified from traditional medicinal plants have become very important in the development of therapeutic agents. Triterpenes are a group of biologically active plant secondary metabolites predominantly found on plant surfaces such as stem bark and leaves (Jäger et al., 2009). These compounds have been reported to exhibit potentially significant pharmacological effects such as anti-platelet aggregation (Mosa et al., 2010a), anti-hyperglycaemic (Ghosh et al., 2011), anti-hyperlipidemic (Liu et al., 2007), and anti-inflammatory activities (Yadav et al., 2010). Plant derived triterpenes have thus become new targets for drug development.

Protorhus longifolia (Benrh.) Engl. (Anacardiaceae) is an evergreen tall (about 15 m) tree indigenous to Southern Africa. In the genus *Protorhus*, *P. longifolia* is the only species that is found in Southern Africa (Archer, 2000). The bark of this plant is traditionally used to cure various ailments. Among its many known traditional uses is its use to "thin" blood. Anti-platelet aggregation activity of the crude extracts as well as the lanosteryl triterpenes from of *P. longifolia* stem bark have been reported (Mosa et al., 2011a, b).

As part of our on-going evaluation of some bioactivity of the lanostane-type of triterpenoid (3 β -hydroxylanosta-9,24-dien-21-oic acid) from *P. longifolia*, we report, in this work, the *ex vivo* anticoagulant and anti-inflammatory activity of this triterpene.

MATERIALS AND METHODS

Reagents and chemicals

Unless otherwise stated, the reagents and chemicals were purchased from Sigma-Aldrich (St Louis, USA).

Plant

Plant material (fresh stem barks) of *Protorhus longifolia* was collected from Hlabisa, KwaZulu-Natal, South Africa. The plant (voucher specimen number RA01UZ) was authenticated by Dr. N.R. Ntuli, Department of Botany, Zululand University. The plant material was air-dried, powdered and stored at 4°C until processing.

Extraction and isolation

The method of extraction and isolation of the triterpene from *P.*

longifolia stem bark have been previously described (Mosa et al., 2011a; Mosa et al., 2014). Briefly, the plant material was first defatted with *n*-hexane and then extracted with chloroform. The triterpene was isolated from the chloroform extract (13 g) over column chromatography (24 × 700 mm; Silica gel 60; 70-230 mesh ASTM; Merck, Darmstadt, Germany), eluted with *n*-hexane-ethyl acetate solvent system (9:1 to 3:7, gradient). The collected fractions (20 ml) were analysed with thin layer chromatography (TLC aluminium sheets, F₂₅₄). Structure of the compound (RA5, 0.72 g) was confirmed using spectroscopic techniques. Melting point was determined using Stuart SMP 11 melting point apparatus (Shalom Instruments supplies, Durban, South Africa).

Malate dehydrogenase (MDH) and citrate synthase (CS) aggregation suppression assay

The assay was conducted by evaluating the ability of the triterpene to suppress MDH and CS thermally induced aggregation. The heat stability of the protein and the triterpene was assessed following the method described by Ramya et al. (2006) with some modifications. Heat shock protein 70 (Hsp70, Human recombinant) and the triterpene (100 μ l each) were separately prepared in the assay buffer (100 mM NaCl, 20 mM Tris, pH 7.4) at a final concentration of 1.3 μ M. Turbid metric changes at 48°C were then followed at 340 nm for 45 min with Biotek plate reader (ELx 808 UI, Biotek Instrument Supplies) equipped with Gen5 software. The effect of the triterpene to prevent thermally induced aggregation of MDH and CS at 48°C was conducted as described for heat stability assessment (Shonhai et al., 2008). MDH and CS (each at 1.3 μ M) were separately incubated with the Hsp70 (1.3 μ M) in the presence and absence of the triterpene at 1.3 and 5.2 μ M. The thermally-induced aggregation of MDH and CS at 48°C was then separately followed by reading turbidity changes at 340 nm for 45 min. For control experiments, aggregation of MDH alone, aggregation of MDH in the presence of 1.3 μ M bovine serum albumin (BSA), and aggregation of MDH in the presence of Hsp70, was separately assessed. Similar controls were used for CS and the experiments were replicated three times.

Animals

Approval (UZREC 171110-030 Dept 2012/23) for use of animals and experimental protocols was issued by the Institutional (Zululand University) Research Animal Ethics Committee. Adult Sprague-Dawley rats (7 to 9 weeks old, 220 $\pm$ 20 g) of either sex were obtained from the Department of Biochemistry and Microbiology animal house, Zululand University. The rats were maintained under standard conditions (12 h light-dark cycle at 23 $\pm$ 2°C); they had free access to enough drinking water and standard pellet rat feed.

Anticoagulant activity (*ex vivo*)

Tail bleeding time assay

Tail bleeding time assay described by Gadi et al. (2009) was adapted with some modification to evaluate the anticoagulant activity of the triterpene. The adult Sprague-Dawley rats (220 $\pm$ 20 g) were randomly divided into four groups of five rats per group. Two groups of the rats were administered (orally) with different concentrations (50 and 250 mg/kg body weight- b.w) of the triterpene 2 h before experiment. Positive and negative control

groups were administered with acetylsalicylic acid (ASA) (30 mg/kg b.w) and 2% Tween 20, respectively. The rats were slightly anaesthetized. Bleeding time was measured by cutting off the rat's tail tip (5 mm) and blood was blotted on a filter paper at 30 s interval until no blood was observed on the filter paper. The period between the tail amputation and the cessation of bleeding was taken as the bleeding time in minutes.

Platelet preparation

After the bleeding time has been determined, blood samples from the rats in the respective groups were collected to obtain platelets. The animals (rats) were killed by a knock on the head and this was followed by immediate collection of the blood from abdominal aorta. The blood collected was separately mixed with an acid-dextrose-anticoagulant (0.085 M trisodium citrate, 0.065 M citric acid, 2% dextrose). The platelets were obtained through successive centrifugations followed by washing of the blood (Tomita et al., 1983). The obtained platelets were further suspended in a buffer (resuspending buffer, pH 7.4).

Ex vivo anti-platelet aggregation

The *ex vivo* anti-platelet aggregation activity of the triterpene was determined on thrombin-induced platelet aggregation (Mekhfi et al., 2004). The platelets (200 μ l) from the different groups were separately pre-incubated in a 96-well micro plate for 5 min at 37°C before addition of thrombin (20 μ l, 5 U/ml). The reaction was monitored by reading absorbance at 415 nm (Biotek plate reader, ELx 808 UI) for 20 min at 30 s interval. The experiment was replicated three times and the mean slope (A) $\pm$ SD was reported. Percentage (%) inhibition of platelet aggregation was calculated from the given formula:

$$\% \text{ Inhibition} = [(A_0 - A_1)/A_0 \times 100]$$

Where, A_0 represent the mean slope of control and A_1 represent the mean slope of the test drug.

Cotton pellet-induced granuloma

The anti-inflammatory activity of the triterpene was investigated using cotton pellet-induced granuloma model (Penn and Ashford, 1963). The rats (220 $\pm$ 20 g) were divided (randomly) into four groups of five rats per group. The animals were orally pre-administered with the drugs 30 min before interscapular implantation (under slight anaesthesia) of pre-weighed sterile cotton pellets (20 mg). The experimental groups (I and II) were administered with the triterpene at 50 and 250 mg/kg b.w, respectively, dissolved in 2% Tween 20. The positive and negative control groups (III and IV) were administered with 10 mg/kg b.w of indomethacin and 2% Tween 20, respectively. The rats were orally administered with the drugs for seven consecutive days. On the eighth day, the rats were slightly anaesthetised and the cotton pellets were carefully removed without the extraneous tissue. The wet pellets were weighed and dried at 37°C for 24 h. The increment in weight of the dry pellets was considered as a measure of granuloma formation. The anti-proliferative activity of the triterpene was compared with that of the control. Percentage inhibition of granuloma formation was calculated from the formula:

$$\% \text{ Inhibition} = (W_c - W_t/W_c) \times 100$$

Where, W_c represents the pellet weight from the animals in the control group and W_t represents the pellet weight from the animals in the drug-treated group.

Biochemical estimation

The method previously described by Nagar et al. (2011) was adapted with modifications to prepare tissue homogenate. The granuloma tissue was homogenized (10 ml/g) in cold Tris-HCl buffer (0.1 M, pH 7.8, containing ethylene diaminetetraacetic acid - EDTA). This was followed by centrifugation at 10,000 $\times$ g for 15 min at 4°C. The supernatant was collected and kept on ice. The supernatant was used for estimation of protein content, catalase and superoxide dismutase (SOD) activities.

Protein content

The Bradford assay was used to determine protein content of the homogenate. Coomassie Brilliant Blue G-250 (5 ml) was added to 100 μ l of the diluted (1:10) homogenate. The mixture was incubated for 5 min. BSA at concentrations ranging from 5 to 100 μ g/100 μ l normal saline, was used as a standard. The Biotek plate reader was used to read absorbance at 595 nm. Protein concentration was determined from a calibration curve.

Catalase activity

Catalase activity was estimated by measuring H_2O_2 decomposition (Aebi, 1983). The reaction mixture consisted of the homogenate (20 μ l) and 2 μ l of phosphate buffer (0.1 M, pH 7.2). The reaction was then initiated by adding 250 μ l of 30 mM H_2O_2 . The change in absorbance was monitored at 240 nm with UV-Vis spectrophotometer for 3 min at 30 s interval. The results were expressed as H_2O_2 decomposed/minute/mg protein, using 43.6 as the molar extinction coefficient of H_2O_2 .

Superoxide dismutase activity

The nitroblue tetrazolium (NBT) reaction method (Glannopolittis and Ries, 1977) was followed to estimate the superoxide dismutase activity. The reaction mixture contained 100 μ l of homogenate, 0.4 ml of 0.01% NBT, 1 ml of 0.05 M sodium carbonate, and 0.2 ml of 1 mM EDTA. Initial absorbance (zero minute) was read at 630 nm and this was followed by addition of 0.4 ml of 2.4 mM hydroxylamine hydrochloride to initiate the reaction. The reaction mixture was then incubated at 25°C for 5 min. The NBT reduction was measured at 630 nm (Biotek plate reader, ELx 808 UI) and one enzymatic unit of SOD was taken as the amount of protein (in the form of enzyme) present in 100 μ l of homogenate. The enzymatic activity of SOD was expressed as unit/mg protein.

Data analysis

The results were presented as mean $\pm$ SEM. One way analysis of variance (ANOVA) followed by Tukey multiple comparison test, were used to assess statistical differences. Where the *p* value is less than 0.05 the results were taken to be statistically significant.

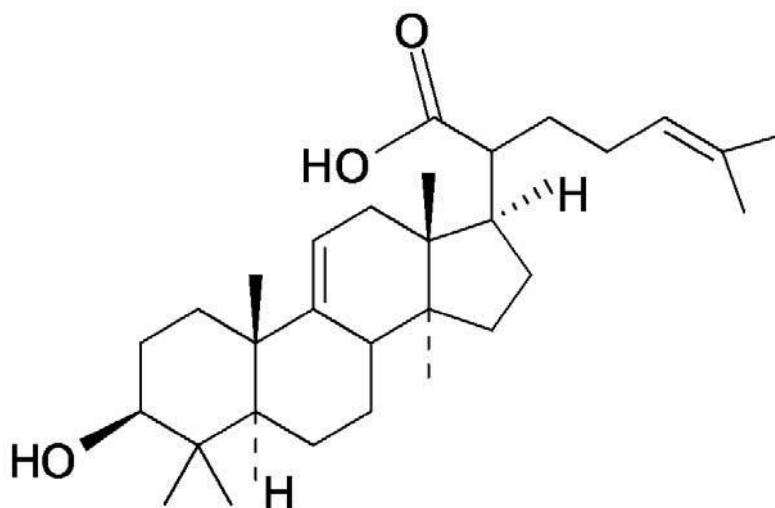


Figure 1. Chemical structure of 3 β -hydroxy lanosta-9,24-dien-21-oic acid (RA5).

RESULTS AND DISCUSSION

The lanosteryl triterpene, 3 β -hydroxy lanosta-9,24-dien-21-oic acid (RA5, Figure 1) has previously been isolated and characterized from *P. longifolia* stem bark (Mosa et al., 2011a; Mosa et al., 2014). Even though inappropriate protein aggregation is normally prevented by complex cellular mechanisms, however under certain circumstances aggregation of proteins occurs. Misfolding and inappropriate aggregation of proteins is commonly associated with a number of neurodegenerative diseases (Lendel et al., 2009). Proteins are known to aggregate in response to heat stress. The thermal aggregation of MDH and CS, and the effect of Hsp70 on their aggregation are well documented (Shonhai et al., 2008). Hsps are ubiquitous proteins found in the cells of all living organisms. These proteins function as intra-cellular chaperones for other proteins, preventing protein misfolding and unwanted protein aggregation (Shonhai et al., 2008). Hsps are involved in many disease processes, including cardiovascular diseases (Whitley et al., 1999). Therefore, there is a growing interest in discovery of pharmacologically active drugs that up regulate expression of Hsps as a potential therapeutic effect in human disease.

Since blood coagulation and inflammation are both mediated by activation and aggregation of proteins, it was appropriate to evaluate the effect of RA5 (triterpene) on the MDH/CS/ Hsp70 system. Figures 2 and 3 indicate that, like the Hsp70, the triterpene suppressed the thermally induced aggregation of MDH and CS. The triterpene (1.3 and 5.2 μ M) also improved the ability of

Hsp70 to suppress aggregation of the proteins. The effect of the triterpene was concentration dependent. The ability of the compound to improve the activity of Hsp70 suggests that the triterpene could partly be exerting its therapeutic properties through inhibition of protein aggregation.

The *in vitro* anticoagulant and/or anti-platelet aggregation activity of some plant-derived triterpenoids have been reported (Habila et al., 2011; Lee et al., 2012). The ability of RA5 to inhibit the aggregation of platelets induced by thrombin and other clotting agents has also been reported (Mosa et al., 2011a). The *ex vivo* anticoagulant activity of the triterpene was assessed using tail bleeding time assay. It is apparent that the triterpene significantly ($p < 0.05$) prolonged the tail bleeding time (Table 1) and thus exhibits anticoagulant properties. The anti-platelet aggregation activity of the triterpene was however, less than that of ASA - a standard anti-platelet agent. The lower activity relative to the standard drug could be due to the bioavailability of the compound. Furthermore, previous studies (Mekhfi et al., 2008; Gadi et al., 2009) have demonstrated that there is not always a direct correlation between inhibition of platelet aggregation and the bleeding time. Also, the possibility that ASA could have permanently inhibited activation and aggregation of the platelets could not be ruled out in contributing to the higher anti-platelet aggregation activity in the positive control.

In our previous study (Mosa et al., 2011a) we reported the anti-inflammatory activity of RA5 using the carrageenan-induced paw edema model which is used for acute inflammation. In this study, the anti-proliferative

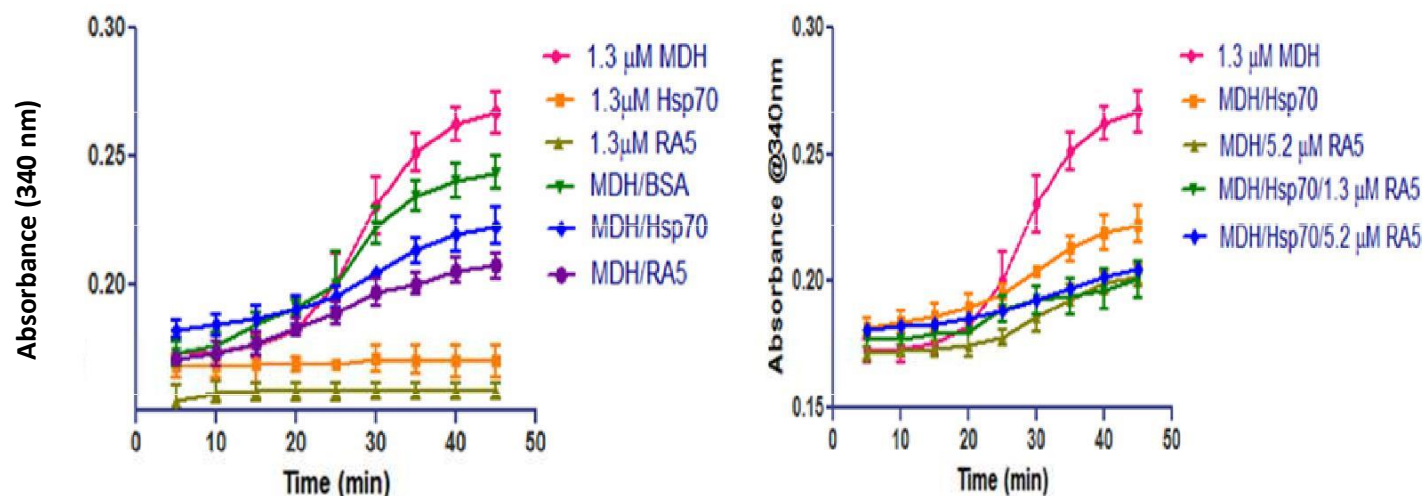


Figure 2. Effect of RA5 (1.3 and 5.2 μ M) on thermally induced aggregation of MDH at 48°C. The aggregation of MDH (1.3 μ M) was induced in the absence and presence of the triterpene at different concentrations. For control experiments, aggregation of MDH alone, MDH aggregation in the presence BSA, and MDH aggregation in the presence of Hsp70 were separately assessed. Data were expressed as Mean $\pm$ SEM.

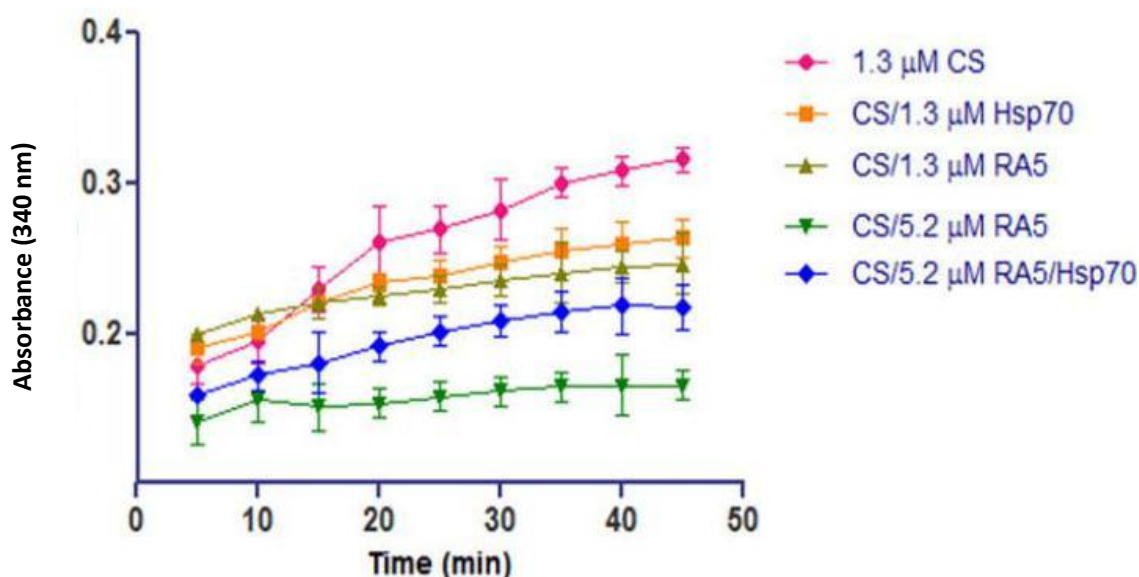


Figure 3. Effect of RA5 on thermally induced aggregation of CS at 48°C. CS (1.3 μ M) was exposed to heat stress in the absence and presence of the triterpene (1.3 and 5.2 μ M). Data were expressed as Mean $\pm$ SEM

activity of the triterpene was investigated using the cotton pellet-induced granuloma model. Chronic inflammation is characterized by infiltration of mononuclear cells, fibroblast proliferation and increased connective tissue formation (Beni et al., 2011). Cotton pellet-induced granuloma model is commonly used as a suitable *in vivo* test for chronic inflammation. The weight of wet and dry

cotton pellets correlates with the amount of formed transudate material and granuloma tissue, respectively. The triterpene (50 and 250 mg/kg b.w) significantly ($p < 0.05$) decreased transudates and granuloma formation in the rats (Table 2). The inhibitory effect of the compound compared favorably with that of the indomethacin (10 mg/kg), a known non-steroidal anti-inflammatory drug

Table 1. Effect of the RA5 on tail bleeding time and inhibition of platelet aggregation.

Parameter	Control	RA5		ASA
Dose	2% Tween 20	50 mg/kg	250 mg/kg	30 mg/kg
Bleeding time (min)	2.50	7.00 ± 1.04*	6.00 ± 0.79*	4.50 ± 0.46*
Antiplatelet aggregation (%)	0.00	4.53	9.32	19.3

Values are expressed as mean ± SEM, n = 5. *p < 0.05 compared to control

Table 2. Effect of RA5 on transudative and granuloma formation.

Group	Dose mg/kg	Granuloma wet weight (mg)	Granuloma dry weight (mg)	Transudative weight (mg)	Granuloma inhibition (%)
Control	-	338 ± 4.05	96.6 ± 0.51	241.4 ± 4.33	-
Indomethacin	10	168 ± 2.43*	65.9 ± 2.43*	102.1 ± 2.33*	31.8
RA5:	50	222 ± 2.90*	67.9 ± 0.76*	154.1 ± 1.42*, †	29.7
	250	152 ± 2.15*, †	57.7 ± 0.81*, †	94.3 ± 3.18*, †	40.3

Values are expressed as mean ± SEM, n = 5. * p < 0.05 compared to control, † p < 0.05 compared to 50 mg/kg.

Table 3. Effect of RA5 on protein content, catalase and SOD activity on the cotton pellet-induced inflammation in rats.

Group	Dose (mg/kg)	Protein content (mg/ml)	Catalase activity (μmol H ₂ O ₂ decomposed/min/mg protein)	SOD activity (units/mg protein)
Control	-	0.68 ± 0.11	96.7 ± 2.88	0.75 ± 0.42
Indomethacin	10	0.56 ± 0.19	119.1 ± 1.01	1.26 ± 0.75
RA5	50	0.48 ± 0.94	242.5 ± 0.83*, †	1.04 ± 0.09
	250	0.52 ± 0.07	256.4 ± 1.22*, †	1.08 ± 0.26

Values are expressed as mean ± SD, n = 5. * p < 0.05 compared to control, † p < 0.05 compared to indomethacin

(NSAID). The anti-proliferative activity of NSAIDs is characterized by decrease in granuloma tissue formation, collagen fibre generation, and suppression of mucopolysaccharides (Verma et al., 2010). The results from this study suggest the NSAIDs character of the triterpene from *P. longifolia* which indicates its potential to prevent chronic inflammation. The anti-inflammatory effect of other triterpenes has been previously reported (Yadav et al., 2010).

Inflammation is associated with excess production of superoxide radicals (Chang et al., 2010) which intensify inflammation by stimulating secretion of inflammatory mediators from macrophages (Porfire et al., 2009). Endogenous antioxidant enzymes such as SOD and catalase help to destroy the free radicals. Table 3 shows that the triterpene did not only reduce the protein content in the granulation tissue but also significantly increased

the activity of catalase (p < 0.05) and SOD as compared to the control group. These results partly explain a relatively higher (40.3%) inhibitory activity of the triterpene (250 mg/kg) observed on the granuloma formation. It is apparent that the ability of the compound to increase activity of SOD and catalase is vital in the compound's anti-inflammatory activity.

Conclusion

Platelets play a central role in linking inflammation and blood coagulation. Activated platelets release pro-inflammatory mediators and cytokines (Blair and Flaumenhaft, 2009). Consequently, inflammation suppresses the natural anticoagulant system, which favors the expression of prothrombogenic molecules

(Verhamme and Hoylaerts, 2009). Anti-inflammatory compounds may significantly prevent inappropriate blood coagulation and therefore alleviate thromboembolic disorders. This study demonstrated that the triterpene (3 β -hydroxylanosta-9,24-dien-21-oic acid) from stem bark of *P. longifolia* possesses anticoagulant and anti-inflammatory activity. The observed activities of the triterpene could be attributed to its ability to inhibit protein aggregation. Further work is required to evaluate the effect of this compound on the Hsp70 expression.

Conflict of Interest

The authors have not declared any conflict of interest.

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