Full Length Research Paper

Cardiovascular benefits of an organic extract of *Tulbaghia violacea:* Its anticoagulant and anti-platelet properties

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Garlic (*Allium sativum* L.) is used as a traditional medicine throughout the world for the treatment of a variety of diseases. Dietary garlic has been recognized for its beneficial health effects. In particular, garlic consumption has been correlated with: (i) reduction of risk factors for cardiovascular diseases and cancer; (ii) stimulation of immune function; (iii) enhanced detoxification of foreign compounds; (iv) hepatoprotection; (v) antimicrobial effect, (vi) antioxidant effect, and most importantly, (vii) its hypoglycemic and anticoagulant properties. Due to these beneficial properties, garlic and its closely related genera, which includes *Tulbaghia violacea*, may be useful in the therapy of cardiovascular disease. Platelets were exposed to various extracts of *T. violacea* to determine their effects on platelet aggregation, adhesion and protein secretion in both *in vitro* and *ex vivo* models. It was noted that the organic bulb extract had a higher inhibition on platelet aggregation and adhesion than the positive control, aspirin. It reduced clotting times in the prothrombin time test (PT), but prolonged the clotting time in the activated partial thromboplastin time (APTT) assay in the *ex vivo* model displaying its antithrombotic ability. The bulb organic extract increased the D-dimer and fibrinogen-C concentrations in the *in vitro* model, but had no effect on the D-dimer concentrations and lowered the fibrinogen-C results in the *ex vivo* model. This study has demonstrated that an organic extract of *T. violacea* has a beneficial effect similar to that of garlic on the cardiovascular system.

Key words: Anti-platelet, anticoagulation, antithrombic, fibrinogen-C, *Tulbaghia violacea*.

INTRODUCTION

Hemostasis is a fundamental defence mechanism of all vertebrates, and involves complex processes which require multiple interdependent interactions between platelets, endothelial cells, white cells and plasma proteins (Gentry, 2004). Blood platelet activation and aggregation is linked to a number of cardiovascular disease (CVDs), which are linked to abnormal excessive activation of platelets, which can be prevented by several anti-platelet drugs, namely acetylsalicylic acid (aspirin) (Vane and Botting, 2003). Medicinal plants have become important for pharmacological research and drug development, where the plant constituents are used directly as therapeutic agents, as templates for the synthesis of drugs or as models for pharmacologically active compounds (Levetin and McMahon, 1999).

Both garlic (*Allium sativum* L.) and onion (*Allium cepa* L.) have been used since ancient times for the treatment of many diseases. *Tulbaghia violacea* Harv. (Alliaceae) which is closely related to garlic is a small bulbous herb indigenous to KwaZulu-Natal, Gauteng and the Eastern Cape region in South Africa. The evergreen leaves of *T. violacea* exhibit a garlic-like smell when bruised and has been used in some cultures as a substitute for garlic and chives (Maoela, 2005). It is used by traditional healers to treat a number of ailments, including but not limited to hypertension, diabetes, fever, colds, asthma, tuber-culosis, and gastrointestinal ailments (Burton, 1990).

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In previous studies by Low Ah Kee et al. (2008) and Bungu et al. (2008), it was demonstrated that *T. violacea* displayed antithrombotic activity, which was dependent on the extraction method and seasonal variations. Further studies on *T. violacea* have shown that it reduces systemic arterial blood pressure in the Dahl rat due to a decrease in renal AT1 receptor gene expression (Duncan et al., 1999; Mackraj et al., 2008). Recent studies have focused on the effects of a *T. violacea* extract to reduce hypertension (Raji et al., 2012). The present study was undertaken firstly, to identify which part of the *T. violacea* plant displayed the best anticoagulant and antiplatelet activity, secondly if an aqueous or organic extraction method would be most suited and thirdly, to determine how the coagulation pathway and platelet aggregation were affected using both an *in vitro* and *ex vivo* rat model.

MATERIALS AND METHODS

Ethical clearance

Ethical clearance was obtained in accordance with the guidelines set by the Animal (409-SCI-BCM-003) and Human (N 01/11/03/07) Ethical Committees of Nelson Mandela Metropolitan University (NMMU). Human plasma was collected from healthy donors free of medication for 2 weeks for the *in vitro* model. Male Wistar rats were used for the ex *vivo* model.

Plant collection

T. violacea was collected from the NMMU Herbarium and identified (voucher no. 20083) by the curator of the Herbarium at the NMMU, Kirsten Ellis. All debris and soil were removed before dissecting the plant material into its various components, namely rhizomes, leaves and bulbs.

Organic and aqueous *T. violacea* extraction

The plant components were dried in an oven at 40°C for 48 h and ground to fine powder. 10 g of the dried powder was mixed with 50 ml methanol and extracted overnight at 4°C. The crude *T. violacea* extracts were centrifuged at 1500× *g* for 10 min at 4°C. The supernatant was then filtered using a Whatman No. 1 filter to remove all residual plant debris. The filtrate solvent (methanol) was removed in a rotavapor evaporator at 40°C, and the residues kept at 4°C. The subsequent residue was mixed with 10 ml deionised distilled water, frozen at -80°C and freeze dried. Aqueous extraction was performed using the method described earlier, but replacing methanol with saline (0.15 M NaCl). These extracts were centrifuged at 4500× *g* for 20 min and the pellet was discarded. Acetone was added to the supernatant in an 80% v/v ratio to precipitate any protein. The resulting solution was centrifuged at 4500× *g* for 20 mins at 4°C. The supernatant was discarded and the pellet re-dissolved in 10 ml distilled water, frozen at -80°C and freeze dried. All freeze-dried extracts were stored in the dark at 4°C. The extract was dissolved as a stock in 100% dimethyl-sulfoxide (DMSO) and used at a final concentration of 0.1% as required (Serra et al.*,* 2005; Bungu et al.*,* 2008*)*. Six extracts were prepared, that is, an organic rhizome (RO), leaf (LO) and bulb (BO) extracts as well as aqueous rhizome (RA), leaf (LA) and bulb (BA) extracts.

HPLC fingerprinting

HPLC fingerprints were completed via RP-HPLC using a C18 Grace Vydac analytical column, (4.6 × 250 mm, 5 µm) and an ion-pairing Kinetex C18 pentafluorophenyl (PFP) column (Phenomenex, 10 × 0.46 cm, 2 µm). Various ratios of HPLC grade acetonitrile and water were used to optimise the HPLC fingerprints for the organic *T. violacea* extract, with the selected conditions shown in Figure 1.

Coagulation studies

Measurements of the activated partial thromboplastin time (APTT), prothrombin time (PT) and fibrinogen-C and D-Dimer levels were performed using a CL analyser (Instrumental Laboratory Corporation, Beckman) and an ACL-assay reagent kit (Instrumental Laboratory Corporation, Beckman). For the *ex vivo* rat model, the assays (PT, APTT, D-Dimer and Fibrinogen-C) were performed by replacing the calibration plasma with rat plasma.

Platelet aggregation and adhesion studies

Isolation of platelets

Blood was obtained via venipuncture using vacutainer tubes containing 3.8% sodium citrate (1:9) from various healthy adult volunteers, free of medication. Platelets were isolated by differential centrifugation of the blood (20 min at 200× *g*). Platelet-rich plasma (PRP) was separated and centrifuged for 20 min at 1000× *g* to sediment platelets. The resulting pellet was gently resuspended in Ca^{2+}/Mg^{2+}- free modified Tyrode's buffer (140 mM NaCl, 10 mM glucose and 15 mM Tris/HCl, pH 7.4). Platelet-poor plasma (PPP) was prepared by centrifugation of a fraction of the PRP at 1500× *g* for an additional 10 min. The platelet suspension was kept at 4°C and utilized within 1 h of harvesting (Bellavite et al., 1993).

Platelet adhesion and aggregation

Adhesion of the activated platelets to the extracellular matrix (ECM) coated plates was performed according to the method of Eriksson and Whiss (2005). The isolated platelets obtained were incubated in the absence and presence of the extracts at a concentration range of 0.25 to 1 mg/ml for 1 h at 37°C. A volume of 1 ml of platelets (30×10^6 platelets/ml) was then activated using thrombin (Sigma) (0.25 U/ml, 30 min at room temperature). The platelet suspension was added to a 3 cm diameter ECM cell culture plate (Nunc) and incubated for 1 h at 37°C without shaking to allow the platelets to adhere. The plate was microscopically examined (400× magnification using a Zeiss microscope). The same method was used to determine the effect of *T. violacea* extracts on platelet aggregation; however, ECM plates were replaced by culture plates free of any coating to allow platelet suspensions to aggregate unhindered (Eriksson and Whiss, 2005).

Protein secretion assay

Isolated platelet suspensions (1 ml of 30×10^6 platelets/ml) were incubated in the absence and presence of the extracts at a range of 0.25 to 1 mg/ml for 1 h at 37°C activated with thrombin (0.25 U/ml, 30 min at room temperature) and centrifuged (20 min at 1100× *g*). The level of proteins released to the supernatant by the activated platelets was determined by the Bicinchoninic acid (BCA) assay and a standard curve. The protein levels were expressed as mg/ml of platelet suspension. These were compared to the untreated control (CON) and the percentage inhibition of platelet protein
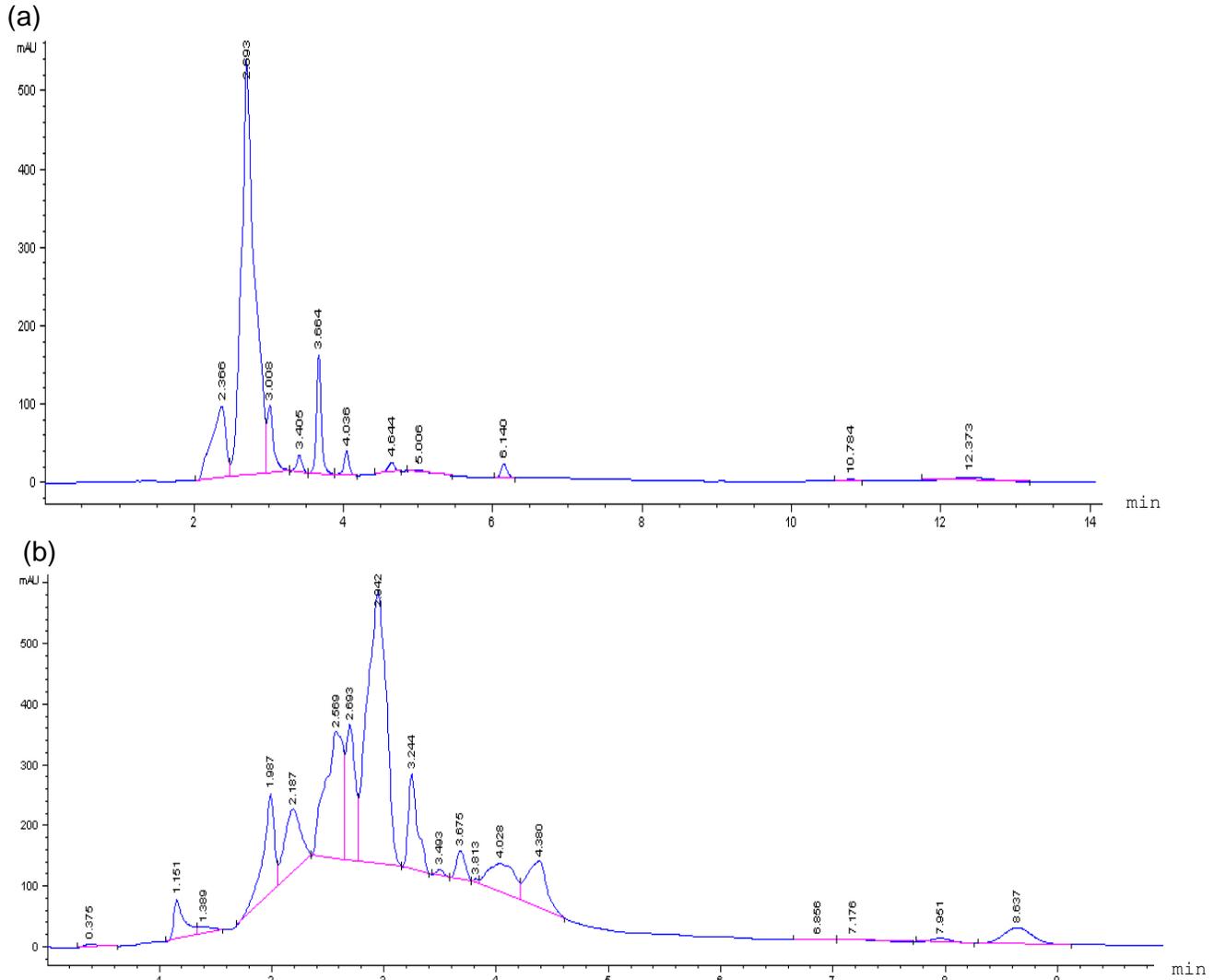
Figure 1. HPLC fingerprints using an isocratic gradient: a) C18 RP-HPLC (80:20 acetonitrile:water; flowrate of 1 ml/min; 4.6 × 250 mm) and (b) PFP-HPLC (95:5 acetonitrile: water; flowrate of 0.25 ml/min; 10 × 0.46 cm) using an organic *T. violacea* bulb extract (1 mg/ml), using a 10 µl injection loop.

secretion was determined (Erikson and Whiss, 2005).

Ex vivo rat model of platelet aggregation studies using flow cytometry

Male Wistar rats, 6 weeks old were randomly divided into three experimental groups of 6 rats per group: vehicle control (CON) (1% Tween 80 dissolved in saline), positive control (PC) (50mg/kg aspirin in 1% Tween 80 in saline) and experimental (50 mg/kg BO extract in 1% Tween 80 in saline). The rats were fed on rat chow (Epol, SA) and allowed to acclimatise for two weeks to facility conditions (19 to 25°C and a 12 h light/dark cycle). Water was available *ad libitum.* Rats were treated for 7 days with the aforementioned treatments. Rats were anesthetized with pentabarbitol (1 ml). Blood was collected from the heart using a syringe with 3.8% sodium citrate (1:9) (Gardi et al*.,* 2009) and used for coagulation and platelet studies. The method used to test platelet aggregation was adapted and optimised from Michaelson et

al. (1991, 1996, 2004). A GPIIb antibody was used to quantify the amount of platelet aggregation by flow cytometry.

Statistical analysis

A minimum of three experiments were performed, with each experiment conducted in triplicate. Data was expressed as a mean ± SD. The significance of these results was further analysed using a two tailed student's test ($P \leq 0.05$).

RESULTS

HPLC fingerprints of the organic *T. violacea* extract

Figure 1 illustrates HPLC fingerprints of the BO extract, using both RP-HPLC and ion-pairing chromatography.

Optimisation of the fingerprints was first completed using a C18 RP-HPLC method (Figure 1a) as this is most commonly used in most laboratories. However, variation of the mobile phase conditions failed to optimise separation as noted in Figure 1a, conversely, ion-pairing chromatography using PFP-HPLC (Figure 1b), led to identification of numerous peaks since the extract contained a mixture of polar and non-polar components.

Effect of *T. violacea* extracts on clotting factors *in vitro*

The concentration range of the various *T. violacea* extracts tested had no significant effect on the clotting times obtained using the APTT and PT tests. In the D-dimer test (Figure 2a), 10 and 20 µg/ml of the BO extract brought about a significant Increase of 1.4-fold in the D-Dimer concentration compared to the control. In the fibrinogen-C test (Figure 2b), 20 µg/ml BO extract produced a 1.25-fold increase in fibrinogen concentration compared to the control. A significant decrease of 0.8-fold (p < 0.05) in fibrinogen concentrations was observed using the LA and BA extracts at the lowest concentration (5 µg/ml). The BO extracts displayed concentration dependent results for D-Dimer and fibrinogen-C assays.

Effect of *T. violacea* extracts on platelet aggregation *in vitro*

All the organic extracts of *T. violacea* (BO, LO and RO) were found to inhibit thrombin-induced platelet aggregation. The BO extract at 1 mg/ml displayed 90% platelet inhibition, while the LO and RO extracts inhibited platelet aggregation by 69 and 50%, respectively. It was noted that the aqueous extracts (BA, LA and RA) at 1 mg/ml displayed between 20 to 49% inhibition of platelet aggregation. The BO extract inhibited both platelet aggregation and adhesion (Figure 3a and b). With an increase in concentration of the BO extract (0.25 to 1 mg/ml) there was a decrease in the ability of the platelets to aggregate and adhere to the ECM. BO extracts (1 mg/ml) inhibited platelet aggregation equivalent to that of the non-aggregated (or non-thrombin activated) platelets.

Protein secretion results obtained for the different treatments on the platelets, indicated that most of the extracts (aqueous and organic), at the various concentrations, significantly inhibited protein secretion relative to control conditions. The organic extracts showed a concentration dependent increase in platelet protein secretion inhibition with the BO having the highest inhibition ability (35 to 53%), compared to the RO (31 to 39%) and LO (38 to 43%). The BO extract inhibited protein secretion by 53% (1 mg/ml) (Figures 3c). The aqueous extracts inhibited protein secretion; with the insert 1mg/ml RA fraction (37 to 45%) having the highest

concentration dependent inhibition of protein secretion compared to the LA (39%) and BA (36%) fractions of similar concentration. Since concentration dependent results for the BO extract were also observed for platelet aggregation, adhesion and protein secretion inhibition, this extract was selected for further testing in an *ex vivo* rat model.

Effect of *T. violacea* extracts on platelet aggregation *ex vivo*

In the *ex vivo* rat model, it was observed that the BO extracts significantly decreased thrombin-induced platelet aggregation by 50% (P<0.01). Aspirin inhibited platelet aggregation by 30% which is consistent with its known properties as a platelet aggregation inhibitor at the concentration tested. These results indicate that the BO extract is 1.67-fold more efficient as a platelet aggregation inhibitor than aspirin (Figure 4).

Effect of *T. violacea* extracts on clotting factors *ex vivo*

It was observed that BO induced a 2.0-fold reduction in the PT clotting time, relative to both the PC and CON (P<0.01) (Figure 5). The result of aspirin (PC) was expected as it exerts its effect on the platelet surface and not on the clotting factors. In contrast to the *in vitro* results, the BO extract produced a significant 1.5-fold increase in APTT clotting time (relative to the CON) and a 1.2-fold increase (relative to the PC). There was a significant reduction in fibrinogen C (Figure 5b) in response to BO; however, no alteration of the D-dimer formation (Figure 5c) was noted.

DISCUSSION

The challenge associated with the use of all medicinal plant extracts used as traditional treatments, and this includes garlic preparations may differ widely in their biochemical composition. As a result, they may possess different pharmacological properties, depending upon the method of processing or preparation (Alder et al., 2003). This accounts for the accompanying differences in efficacy (Duke et al., 2003; McKenna et al., 2002; Kasuga et al., 2001; Munday et al., 1999). Therefore in our study, we have included routine RP-HPLC and PFP-HPLC chromatography of our extract as shown in Figure 1.

In clinical tests of blood coagulation, PT is used to evaluate the overall efficiency of the extrinsic clotting pathway; a prolonged PT indicates a deficiency in coagulation factors V, VII and X. On the other hand, APTT is a test of the intrinsic clotting activity; a prolonged APTT usually represents a deficiency in factors VIII, IX,
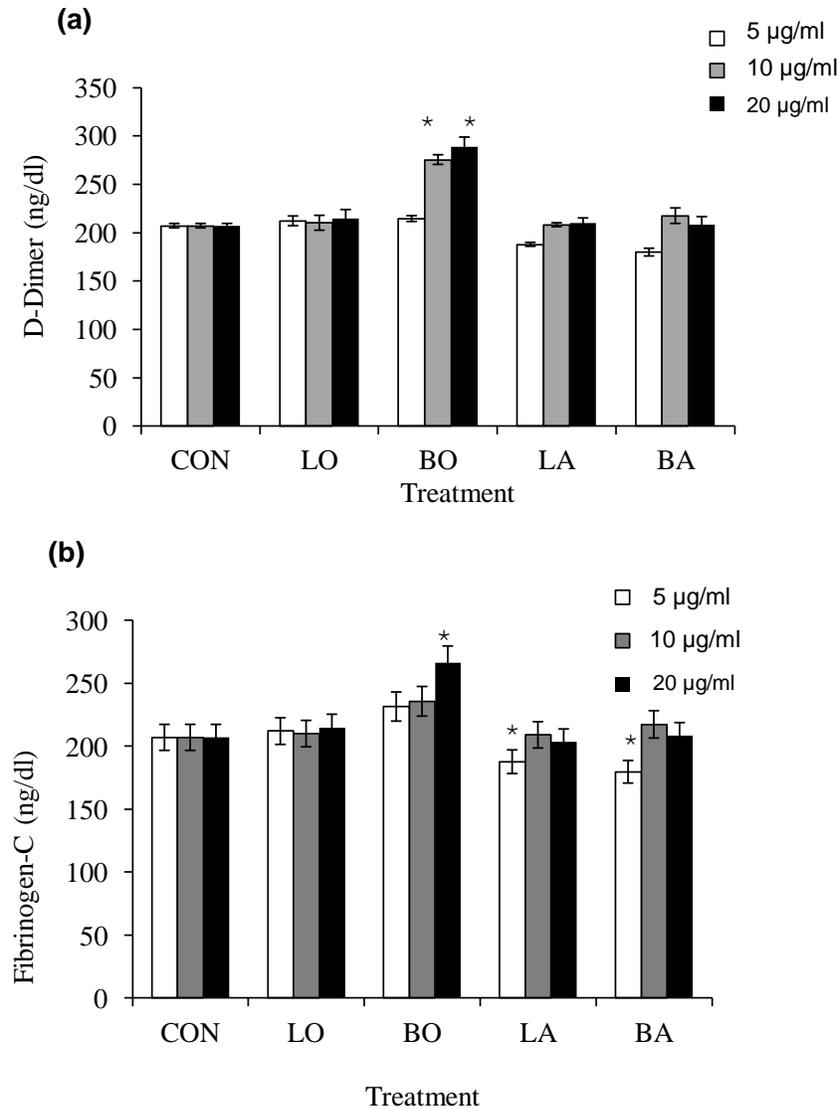
(a)

(b)

Figure 2. (a) The effect of *T. violacea* extracts on D-Dimer formation; (b) Fibrinogen-C formation *in vitro* (n = 5). *indicates p < 0.05 relative to the CON.

XI, XII and Von Willebrand's factor (Laffan and Bradshaw, 1995). In both the *in vitro* PT and APTT tests no significant effect on clotting time was observed for any of the extracts at the concentrations tested. Bungu et al. (2008) noted a prolongation of the clotting time of 28 to 36 s with aqueous extract infusions of *T. violacea* leaves (30 μg/ml) and bulb (40 μg/ml). However, these extract concentrations are higher than those tested in the present study. Bungu et al. (2008) also reported that the effectiveness of the antithrombotic activities of *T. violacea* extracts were seasonal for the aqueous extract; however, this was not noted for the organic extracts. These findings correlate with our results.

Our findings showed that in the *ex vivo* rat model, the BO extract resulted in a 2-fold decrease of the PT, relative to both the control and positive control.

Elevations in the normal clotting times can be observed for both the control and positive control in rats compared to humans (data not shown). Although a difference is observed between the *in vitro* and *ex vivo* models in the control of the APTT, it is well known that human plasma usually clots faster (16.5 ± 0.4 s) than rat plasma (33.8 ± 2.3 s) (García-Manzano et al*.,* 2001). Appropriate controls were used for each experiment and the variation in clotting times can therefore be attributed to inter-species differences. In the *ex vivo* rat model, the BO extract produced a 1.5-fold increase in the clotting time relative to the control, and a 1.2-fold increase relative to the positive control, for the APTT test. The results of APTT accounted for the platelet interaction.

In the *in vitro* model, the D-Dimer and fibrinogen-C test were performed using the various *T. violacea* extracts.
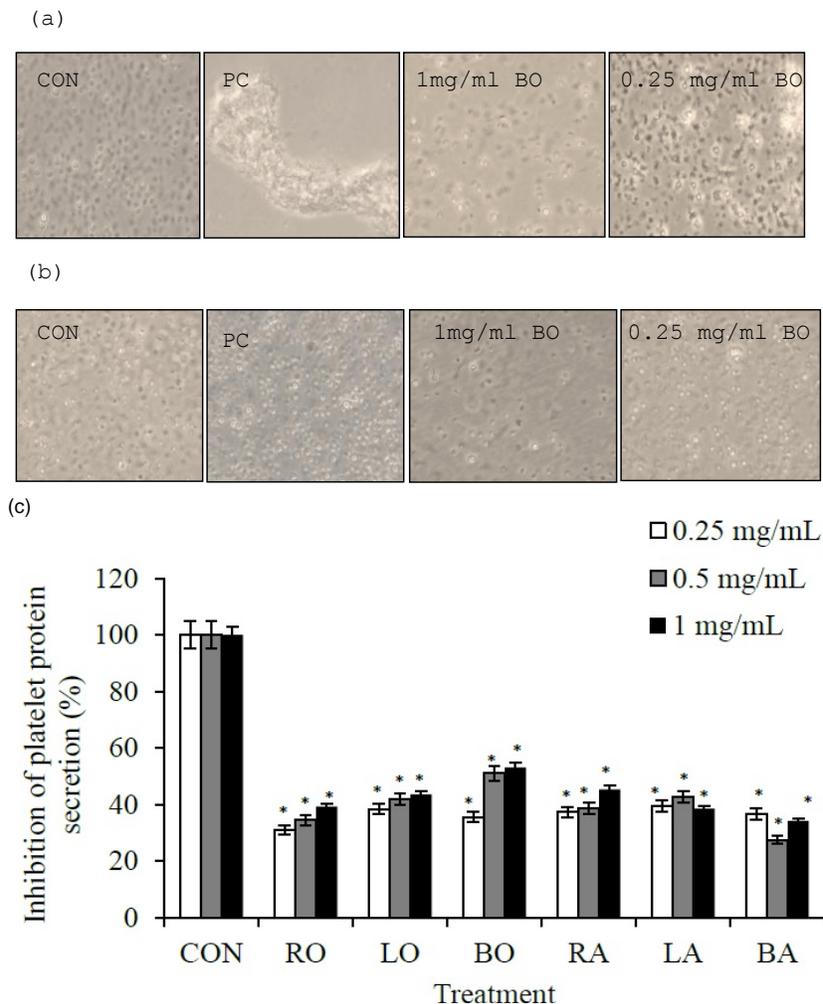
Figure 3. (a) An example of aggregated platelets, activated with thrombin (0.25 U/ml) evaluated microscopically. (i) Non-aggregated platelets, the CON platelets; (ii) activated platelets in the absence of any *Tulbaghia violacea* extracts, acting as a positive control of platelet aggregation (PC); (iii) activated platelets in the presence of 1 mg/ml of BO extract and (iv) 0.25 mg/ml BO extract. (b) An example of adherent platelets, activated with thrombin (0.25 U/ml) evaluated microscopically. (i) Non-adherent platelets, the CON platelets; (ii) activated platelets in the absence of any *Tulbaghia violacea* extracts, acting as a control of platelet adhesion (PC); (iii) Activated platelets in the presence of 1 mg/ml of BO extract and (iv) 0.25 mg/ml BO extract (400X magnification). (c) Effects of *T. violacea* extracts, at different concentrations, on the release of protein secretion from the thrombin-activated platelet (n = 3). * $P<0.01$ relevant to the control.

The BO was found to increase the amount of D-Dimer released and fibrinogen-C formation. A concentration dependent increase was found, with the highest concentration displaying a 1.4 and a 1.25 fold increase for D-Dimer and fibrinogen-C assays, respectively. These results indicate that the BO extracts may enhance fibrinolysis and support findings in other studies using garlic extracts showing that fibrinolysis is enhanced by garlic extracts, resulting in the dissolution of clots and thrombi (Rahman and Lowe, 2006). The D-Dimer and fibrinogen-C test provide an indication of the effect on fibrinolysis by the BO extracts in the *ex vivo* rat model. The BO extract produced a 2.6- and 3.0-fold decrease in fibrinogen-C levels, relative to the control and positive control, respectively. No effect of BO extract was noted for the D-Dimer assay. The fibrinogen concentration of the *in vitro* control was lower than that of the *ex vivo* control. This is due to the physiological range of fibrinogen in rats being higher than in humans. No effect was observed in the D-Dimer formation. This could be
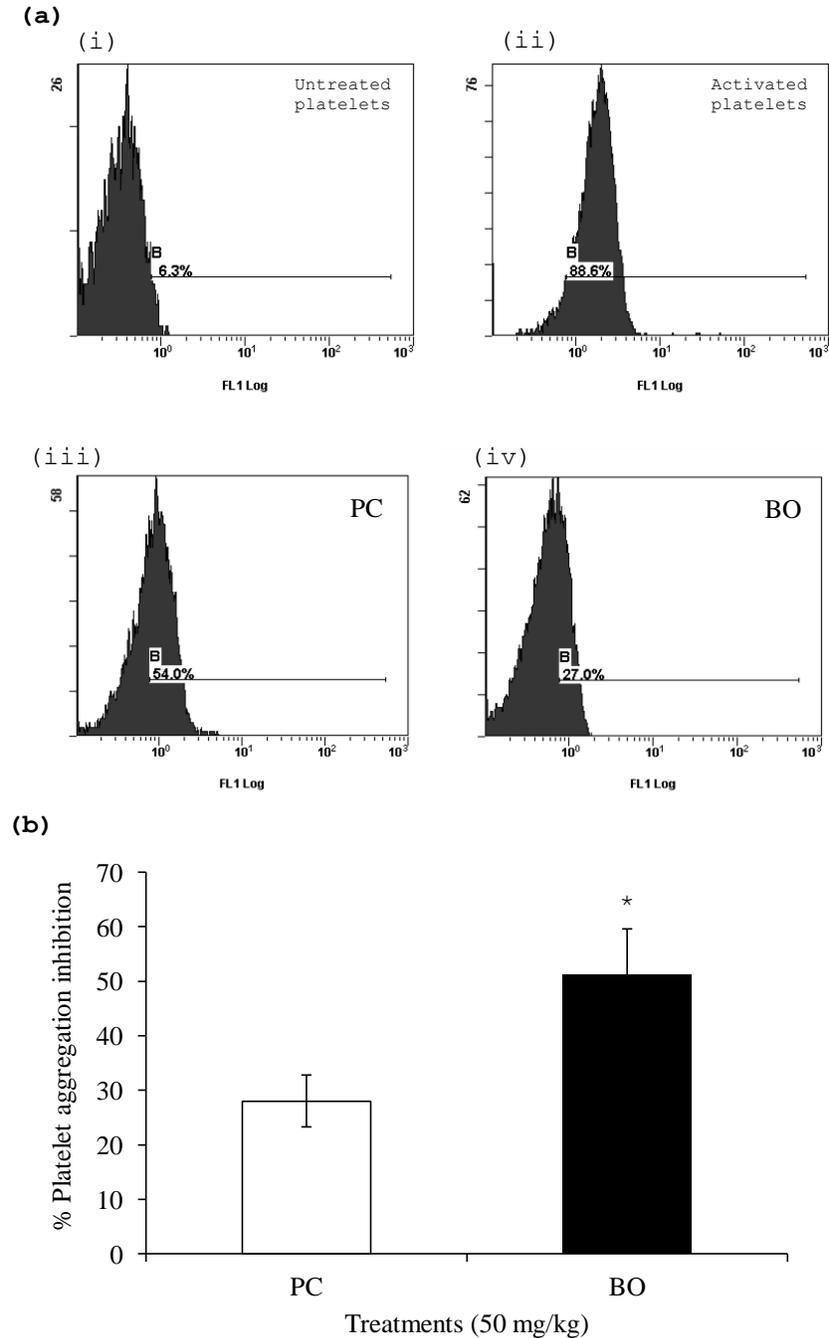
(a)

(b)

Figure 4. (a) Examples of flow cytometry histograms of rat platelet aggregation, and the percentage of aggregated platelets where (i) untreated CON where no platelet aggregation has been initiated (ii) activated platelets obtained from untreated rats (iii) platelets from aspirin treated rats (PC) activated with thrombin (iv) platelets from BO (50 mg/kg) treated rats activated with thrombin. (b) The effect of on platelet aggregation in an *ex vivo* rat model (n = 5) *indicates P<0.01 relative to the PC.

attributed to the low fibrinogen-C levels and thereby affecting the formation of D-Dimers.

Generally, saponins have a positive effect on the prevention of platelet aggregation, blood coagulation and fibrinolysis (Lanzotti, 2006). Based on the D-Dimer results, the BO extract had an effect on *in vitro* fibrinolysis; however, this was not observed for the concentrations tested in the *ex vivo* rat model.

The presence of saponins in *T. violacea*, especially in organic extracts, has been identified and could account
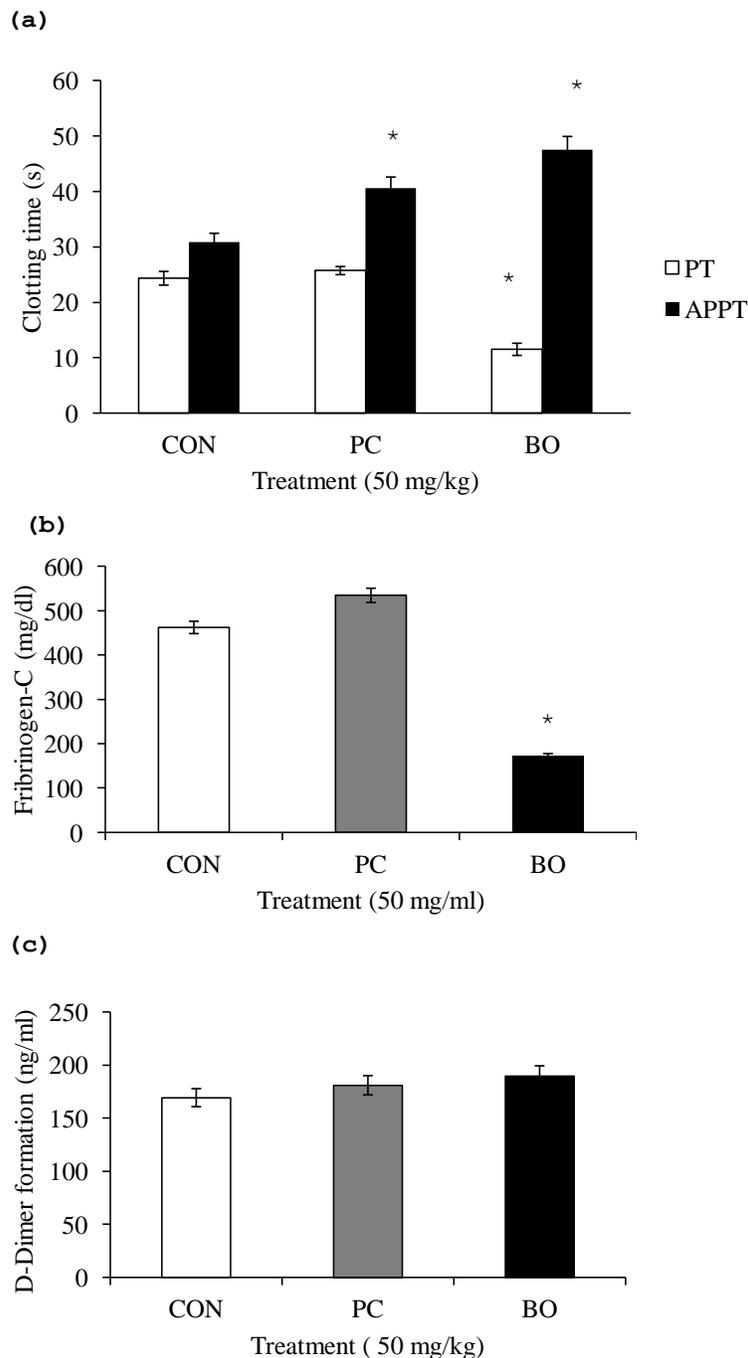
(a)

(b)

(c)

Figure 5. (a) The effect of the BO extracts on the clotting time using the APTT and PT test; (b) the effect of aspirin (PC) and extract (BO) on the Fibrinogen-C test; (c) the effect of aspirin (PC) and extracts (BO) on D-Dimer formation in an *ex vivo* model (n = 6) *p < 0.01 relative to the CON.

for this enhanced *in vitro* fibrinolytic ability (Burton, 1990, 1992).

The role of garlic in preventing CVDs has been extensively studied over the last decade (Brace, 2002; Rajaram, 2003; Borek, 2006). Several studies have indicated that preparations of garlic increase fibrinolytic activity but inhibit platelet aggregation, as well as lowering blood pressure and cholesterol in humans (Brace, 2002; Rajaram, 2003; Borek, 2006). It has been proposed that the mechanism of inhibition of platelet aggregation by garlic's

constituents is via inhibition of calcium mobilization and inhibition of several steps of the arachidonic acid pathway in platelets (Allison et al., 2006; Bordia et al., 1996).

In this study, all the extracts tested were able to inhibit protein secretion and platelet aggregation and adhesion, in a concentration dependent manner, with the organic extracts exhibiting the greatest degree of *in vitro* inhibition. The BO extract (1 mg/ml) displayed the highest degree of inhibition (53%) of all the extracts, with most of the extracts inhibiting protein secretion by more than 50%. These results were supported via microscopic evaluation of the platelet adhesion and aggregation induced by the extracts. The BO extract predominantly modulates its antithrombotic effect by preventing platelet aggregation, as evidenced by the *ex vivo* model. In the *ex vivo* rat model, it was observed that the BO extracts decreased thrombin-induced platelet aggregation by 50%. This was significant when compared to aspirin, a known platelet aggregation inhibitor, at the concentrations tested which inhibited platelet aggregation by 30%. These results associated with *T. violacea* are consistent with numerous *in vitro* studies conducted on garlic, showing a reduction in the parameters associated with CVD.

This work has demonstrated that an organic bulb extract of *T. violacea* has a beneficial effect on the cardiovascular system by decreasing fibrinogen levels, increasing the APTT time and through the modulation of platelet activation, via the GPIIb receptor present on platelets. These results provide the basis for future studies which should include determining the active component(s) of the organic bulb extract, responsible for the cardioprotective effect, using mass spectrometry and nuclear magnetic resonance. In addition, it needs to investigate whether *T. violacea* extracts also effect calcium mobilisation during platelet activation, as was found with garlic. Since *T. violacea* is commonly used traditionally in South Africa, this study provides insight into the cardiovascular benefits associated with an organic extract of the bulb of the plant material and provides a basis for future studies similar to those completed on garlic.

ACKNOWLEDGEMENTS

Abbreviations: AGE, Aged garlic extracts; **APTT,** activated partial thromboplastin time; **BA,** bulb aqueous; **BCA,** bicinchoninic acid; **BO,** bulb organic; **CON,** control; **COX,** cyclooxygenase; **CVD,** cardiovascular disease; **DAT,** diallyl trisulfide; **DMSO,** dimethyl-sulfoxide; **ECM,** extracellular matrix; **LA,** leaves aqueous; **LO,** leaves organic; **NMMU,** Nelson Mandela Metropolitan University; **PC,** positive control; PFP, pentafluorophenyl; **PPP,** platelet poor plasma; **PRP,** platelet rich plasma; **PT,** prothrombin time; **RA,** rhizome aqueous; **RO,** rhizome organic.

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